Македонски Фармацевтски билтен

Macedonian Pharmaceutical Bulletin

волумен **62** (додаток) 2016 / volume **62** (supplement) 2016

Македонско фармацевтско друштво, ул. Маршал Тито 13б/8, Скопје, Македонија
Macedonian Pharmaceutical Association, Marshal Tito 13b/8, Skopje Macedonia

It is our great pleasure to present this Supplement Issue on "*Macedonian Pharmaceutical Bulletin*" to the scientific and professional community. This supplement includes the short communications from the *Sixth Congress of Pharmacy in Macedonia with International participation*, as the largest gathering for the pharmacy profession held in the Republic of Macedonia. The main theme of the Congress was "Modern pharmacist - bridging science with practice".

A broad spectrum of topics within the pharmaceutical sciences and practice carefully selected for this special occasion in order to build up a highly interesting and comprehensive program were covered. The contributions submitted to the Congress included 6 plenary lectures, 84 section lectures, and more that 240 posters. This Congress, followed the excellent international tradition, was attended by close to 1000 domestic and foreign participants. We received 326 short paper submissions from more than 25 countries. These numbers show that our Congress is aiming for the highest scientific standards, and that it can be considered a well-established venue for researchers in the broad fields of Pharmaceutical sciences and practice.

We would like to thank all internationally prominent researchers for their contribution to reinforcing the overall quality of the Congress. They give the state of the art of the recent advances in the field of pharmacy research.

Sincere thanks to the hosts of the Sixth Congress of Pharmacy in Macedonia with International participation, Macedonian Pharmaceutical Association and Faculty of Pharmacy, Ss Cyril and Methodius University in Skopje for their vision and commitments.

We acknowledge the sponsoring companies: the platinium sponsor AD ALKALOID, Skopje, the golden sponsor PLIVA, the silver sponsor EUROFARM and the bronze sponsor SEPTIMA, for the permanent support to our efforts during the organization.

We would also like to thank our members of the Scientific Committee for their volunteer time and dedication to the critical peer review process and in the organization of the program. We also wish to thank all the members of the Organizing Committee, whose work and commitment was invaluable.

On behalf of the Advisory and Scientific Committees, we would like to especially thank the authors, whose work was the essential part of the congress and contributed to a very successful event. Besides the many academic staff and professionals who contributed to the success of the Congress, we are grateful to the students who participated with oral presentations and posters.

The pharmaceutical sciences continue to grow as dynamic scientific interdisciplinary fields. We believe that published short communications will be an excellent source of scientific material in the fast evolving fields in Pharmaceutical sciences and practice.

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Chair of the Organizing committee

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Your hosts
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The present issue *of Macedonian Pharmaceutical Bulletin* is a special issue of the 6th Congress of Pharmacy in Macedonia with international participation.

This issue of *Macedonian Pharmaceutical Bulletin* contains short papers accepted by the scientific committee for the presentation at the Congress.

The authors are fully responsible for the contents of their short papers.

All reviewers that were involved in the short papers revision process are sincerely acknowledged.

Opening lecture

Macedonian pharmaceutical bulletin, **62** (suppl) 7 - 8 (2016)
ISSN 1409 - 8695
UDC: 615.15
Short communication

The modern pharmacist: Is the future in the past?

Roberto Frontini

Centre for Patient Safety, University Hospital of Leipzig, Liebigstraße20, 04103 Leipzig, Germany

Background

The role of the pharmacist changed during the centuries. In the 20th century most of the pharmacists accepted a role as provider of medicines and the education was missing any clinical aspect. But looking back to the 18th century in Italy we discover that the role of the pharmacist was different and similar to what should be the modern profession. In the statute of the Ospedale Maggiore della SS Annunziata di Savigliano (1762**)** was indeed stated: "*The pharmacist must accompany the physician in all rounds...and more he will make daily additional rounds to judge the pain of the most worsening patients and their immediate needs..***"**.The pharmacist had an important clinical role and was a partner of the physician. Even more is part of the agreement between the Ospedale Maggiore della SS Trinità di Fossano and Gino Stefano Bertolo, speziale (pharmacist) (1741**):** **"***... he has to prepare the prescribed medicines... and bring them personally to the patients labelling every cup or decanter with the number of his or her bed and compassionately and gently inviting to take them either immediately or at least at time of the mass in the cathedral...*". The contract describes in fact the competencies of a modern pharmacist. He or she is the expert in preparing medicines and understands the formulation as an important issue. But his or her commitment does not end in the preparation. He or she has to go to the patient and promote adherence and best use of the medicine while using strategies for the reduction of medication errors.The challenge to speak about the future is not to re-invent the past.

The environment of the 18th century was indeed different to what we have nowadays and in this short article I would like to underline some of the challenges of a modern pharmacist both in the hospital as well as in the community setting. I think thatin the whole system the pharmacist should be the healthcare professional in the care team who seeks to ensure that pharmacologically active ingredients achieve the best possible benefit for the individual patient.

The competencies of a modern Pharmacist

The pharmacist needs first skills and competence in the formulation of a medicine. This does not necessarily mean in the era of industrial production that the medicine is compounded in the pharmacy, but the properties of the formulation may influence the correct use and the adherence as well. Only the pharmacist can understand this in deep. Sometime is also necessary to adapt medicine to the individual, especially in paediatric patients. Skills in compounding are therefore of primary importance and also the modern pharmacist should not forget the roots of our profession as the only manufacturer of medicines.

A further taskfor a pharmacist is to seek the balance between expected effects and drug related problems. It is essential to underline that the best outcome for patients is only achievable in a multidisciplinary care team (Zuling et al., 2013) and the pharmacist as part of this team should be alert when advising patients. He or she needs social competence in communication with the aim to understand any problem related to the medication and explain the appropriate use of drugs to the patient "*..in terms he can understand*" (EAHP, 2014). This social competence is also necessary in the team work. Physicians, nurses, pharmacists and other healthcare professionals have to abandon a hierarchy driven relationship and accept each other as a partner in achieving the best for the patient. As well the patient has to be part of any decision and has to articulate the desired outcome. Especially in oncology those expectations should be respected and the goal for the care team is not necessarily to prolonger life but to create quality of life.

The challenges of a modern Pharmacist

In the daily work the modern way of life and technology challenge the modern pharmacist. The daily increasing knowledge about drugs and their effect, complex medications, personalised medicines,IT technology, the overwhelming information through social media, the explosion of costs hampering healthcare systems, the globalisation of the supply chainare some examples.

One example of personalised medicine may underline those challenges: Enthusiastic researchers believed that we are "*on the leading edge of a true revolution in medicine"* (Collins, 2010) but critical authors say that "*they can also obscure more effective approaches to common complex disorders*" (Juengst et al., 2012). What is true? We do not know yet as data are contradictory. But an interesting paper on the adherence to the targeting INR in Warfarin medication may show the dilemma (Kimmel et al., 2013). Kimmel et al. (2013) explored two different approaches to control Warfarin medication: the first based on the genomic of the patients, the second based on clinical interventions. In average no difference was found between the two groups bringing to the conclusion that "g*enotype-guided dosing of warfarin did not improve anticoagulation control*". Interestingly in the subgroup of black patients the mean percentage of time in the therapeutic range was less in the genotype-guided group than in the clinically guided group. This example may underline that clinical interventions are still important even if our knowledge of genomics helps us to make better decisions. The modern pharmacist has to consider this while advising patients. In another paper (Wright et al., 2013) Wright concluded that "*Our ability to generate data now far outstrips our ability to interpret it*".

A second example of challenge for the modern pharmacist is the information technology of today. No doubt that internet and social media have completely changed our sources of information. The modern patient is informed and internet is after the physician the first source of information according to a study by Marrie (2013). There is some differences between young and old people (Couper et al., 2010) and persons with mental disease (e.g. depression) (Pohjanoksa-Mäntylä et al., 2011), the last using more and more the internet as an information source. But the quality of the internet information is not necessarily the best. Celebrities e.g. misuse their influence promoting products without scientific evidence (Hoffman and Tan, 2013) and for non-healthcare professionals is not easy to distinguish between spam and real scientific message. The challenge is for the modern pharmacist that patient sometime know more than the healthcare professional but they do not understand the background. The pharmacists as well as the physician must learn comprehensive, clear and for the patient understandable communication and educate him or her in the proper use of internet-information. This is no easy task as demonstrated by Watermeyerand Penn (2009). Healthcare professional's education should therefore include communication training as an essential part for the transfer of scientific data to the patient.

Conclusion

In conclusion between a medicine with its active ingredient and the outcome of the patient there are a lot of barriers like e.g. drug formulation, patient attitude, social context, medication errors, patient's genomic, biased information. The pharmacist has to help patients in overcoming such barriers but this is only possible if he or she is part of a cure team working in collaborative way without hierarchy barriers.

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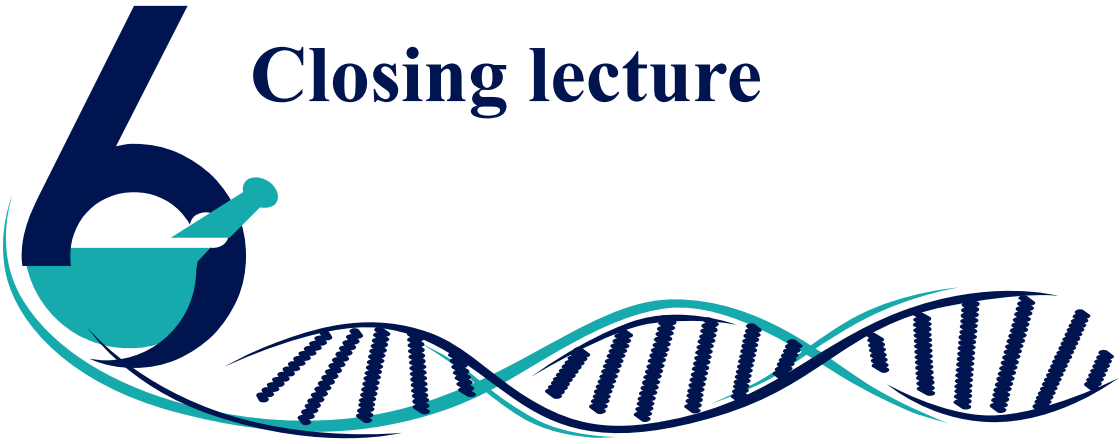
Closing lecture



Macedonian pharmaceutical bulletin, **62** (suppl) 11 - 12 (2016)
ISSN 1409 - 8695
UDC: 615.15:37.035
Short communication

Commitment to quality means commitment to change

Michael J. Rouse

Accreditation Council for Pharmacy Education, 135 South LaSalle Street, Suite 4100, Chicago, Illinois, 60603-4810, United States of America

Pharmacy practice and the education and training required to prepare pharmacists for practice have undergone dramatic change in the past several decades, and more change will come. Beyond ensuring that the right medicine is delivered to the right patient in the right dose, there is now a greater focus on pharmacist-delivered patient-centered care. The aims of such care are to ensure optimal therapeutic outcomes and improved quality of life, reduce adverse reactions to medication and the risk of patient harm, improve access, and reduce the overall cost of health care delivery. For pharmacists, the focus has shifted from the medication itself to the correct and optimal use of the medication by the patient.

With advances in technology, medical and pharmaceutical knowledge, medication use has a greater potential for good, but therapy is often more complex, and the risk of suboptimal outcomes or even patient harm through inappropriate selection and/or use of medication can be high. The need for a competent healthcare professional to advise on selection and manage the medication therapy is becoming increasingly clear, both at the individual patient level as well as at a societal level.

The foundational knowledge needed by pharmacists in the biomedical and pharmaceutical sciences has not diminished but additional knowledge is now needed in social, behavioral, administrative, and clinical sciences. But it does not stop there; in order to effectively manage the medication use process of patients and populations, pharmacists need to acquire new skills and develop the appropriate attitudes and values. Together, these reflect the competency profile of a contemporary pharmacist, which has been described by the International Pharmaceutical Federation across four domains: pharmaceutical public health, pharmaceutical care, organization and management, and professional/personal (FIP, 2012).

Schools of pharmacy have to undertake a major reform of their curriculum - both in terms of content and delivery – in order to prepare graduates with the needed competencies, to be "fit for purpose" and able to deliver the services required to meet societal needs. In addition, models for continuing education and continuing professional development have to change, because it is likely that the majority of pharmacists in practice today do not have all the competencies to deliver this model of care.

In addition to practice and education, a third sector plays a vital role when it comes to advancing the profession of pharmacy. This is the regulatory sector, which is by nature, however, more conservative due to its primary role to protect the public. For obvious reasons, professional regulations cannot be changing constantly, but change is certainly needed in this sector. With the roles of pharmacists evolving and expanding, regulation of the profession has also had to change. In addition, regulation has had to change – or in some cases be introduced for the first time – for the pharmacy support workforce, members of which play a vital role in supporting pharmacists to free them up to deliver pharmaceutical care, or "medication therapy management" services as now referred to in some countries.

It is evident that for improvements and advancement to come in the profession of pharmacy, change is required, but change will not come without commitment – commitment from practitioners, educators, regulators and all other key stakeholders who have a role to play. People can often recognize the change that they want to happen in their environment to improve their situation. It is, however, not always as easy for individuals to recognize and be committed to the change that must occur within themselves. We assume that all pharmacists want to succeed in their professional careers. At the end of their career when they look

* mrouse@acpe-accredit.org

back, they want to believe that they have made a difference …. left some "footprints." What are some of the factors that will determine whether or not pharmacists will, in fact, make a difference in the lives of patients, their professional colleagues, and others they meet and work with? What will determine if the "seeds" that pharmacists plant in the "field of pharmacy" will grow and produce a harvest?

Pharmacists work in different "fields" and likely have different ideas about what results they want to achieve … what impact they want to have personally and professionally, but are there some principles and values that are common – and important - regardless of where pharmacists live and work, and what they do?

Today, pharmacists acquire expert knowledge and skills, firstly through their pre-service education and training, and after that through continuing education and continuing professional development activities. How does the profession ensure that pharmacists also develop the right attitudes and values to be ethical, professional and caring pharmacists, and members of inter-professional teams, working together to achieve optimal medication therapy outcomes for patients? Can these attitudes and values be "taught" in a classroom? How else can they be cultivated?

Beyond having the right knowledge, skills, attitudes, and values, what else determines whether or not pharmacists will bring about the changes that are needed and have the desired impact; whether or not pharmacists make the difference in life that they aspire to make? That takes commitment, and "Commitment to Change" is the bridge that spans the "chasm" between learning and behavior change, between good intentions and real impact (Wakefield et al., 2003; Wakefield, 2004).

While some things must change in order to achieve improvement, certain values and attitudes must be preserved throughout the career of a pharmacist for him/her to be a successful professional, delivering quality care and other services. Important lessons learned and attitudes, values and habits that the author has found to be important in his pharmacy career include: have a desire to innovate; build the best team; learn to work with others who are not like you, take pride in what you do; keep searching for the things that are hard to find; keep climbing; draw on others' experience; find a mentor; be a mentor; take some chances; build strong networks; know your strengths and don't underestimate your contribution; find things to be passionate about; don't suffer from "paralysis by analysis" or always insist on perfection; there are no shortcuts to any place worth going; partner with people who share your vision; not all change leads to improvement… but all improvement requires change; and perhaps most importantly, sometimes "availability" is more important than "ability."

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Plenary session

Macedonian pharmaceutical bulletin, **62** (suppl) 15 - 16 (2016)
ISSN 1409 - 8695
UDC: 615.214(438)
Short communication

New psychoactive substances - analytical challenges and threats to the public health: European and Polish experience in the new drugs combating

Zbigniew Fijalek

Department of Bioanalysis and Drug Analysis, Faculty of Pharmacy,
Warsaw Medical University, Banacha 1, 02-097 Warsaw, Poland

Introduction to the problem and European NPS market

In recent years, the UE has seen the emergence of new drugs that have similar effects to drugs that are internationally controlled. These drugs can be collectively called New Psychoactive Substances (NPS). The key features are that NPS are psychoactive i.e. ones that stimulate, or depress the central nervous system, or cause a state of dependence; have a comparable level of potential harm to internationally controlled drugs; and are newly available, rather than newly invented. These substances may be the active pharmaceutical ingredients (APIs) used in authorised medicines (phenibut) or suspended/withdrawn medicines (sibutramine, DMAA) or potential medicines in the making (remimazolam). Methcathinone was originally used as an antidepressant in the former Soviet Union in the 1930s, but very quickly it became a recreational drug. Pyrovalerone and amfepramone have been used as anorectics, but they are currently obsolete. Bupropion is used as antidepressant and as an aid for those who wish to quit tobacco smoking. But NPS goes beyond APIs, in fact most of them come originally from the scientific and patent literature as a result of Pharma and academic institutes' research and development efforts. They have been designed to evade drug laws, are widely available and have the potential to pose serious risks to public health and safety and can even be fatal. The short-term harms of NPS can include paranoia, psychosis and seizures and their long-term harms are often unknown. NPS a new class of psychoactive substances, known as 'legal highs', 'herbal highs', 'designer drugs' or 'party pills' has emerged on the drug use market. They are frequently advertised as 'plant fertilizers/food', 'air fresheners', 'herbal incenses', 'spice', 'bath salts', 'research chemicals' (in Poland commonly known as 'dopalacze' - literally translated as afterburners).

Based on their chemical structures, designer drugs can be classified into amphetamine types, 2,5-dimethoxy amphetamines, 2,5-phenylamines, β-keto amphetamines (cathinones), phencyclidines, piperazines, pyrrolidinophenones, fentanyls, piperidines, tryptamine derivatives and synthetic cannabinoids. Based on the spectrum of exerted psychoactive effects, NPS can be classified into four basic categories: synthetic cannabinoids, stimulants, opioid-like compounds, and hallucinogenic/dissociative (psychodysleptic); however, they may have a combination of these effects due to their designed chemical structure.

From 2008 there has been a rapid increase in the number and range of new substances with greater ease of availability, with their open sale in offline retail outlets and through the global marketplace of the internet ('clearnet' and 'darkweb'). The Early Warning System (EWS) run by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) detected 86 novel NPS in 2013, 101 in 2014 and 100 in 2015.

Whilst generally there has been an increase in the number of novel NPS detected, it is important to note that the vast majority are permutations of groups of similar substances with similar effects (e.g. cathinones), or dissimilar substances that produce similar effects (e.g. synthetic cannabinoids), rather than new distinct types of drugs. Furthermore, it is likely that many of the substances identified are not in widespread or even limited use. It may be that the market, to some extent, regulates itself with less effective or more harmful NPS only being seen for very short periods of time or in a limited number of countries (Dar-

gan and Wood, 2013; Zawilska and Andrzejczak, 2015; Zawilska, 2015).

Polish new drugs market

New psychoactive substances were present on the Polish drug scene before 2008. At the beginning, there were occasional sale offers of psychoactive substances on the Polish Internet forums, which were in compliance with legal regulations at that time. In 2008, a website called 'dopalacze.com' was launched. It offered a new quality approach in terms of professional and marketing strategy. The website products were advertised as safe alternatives to illicit psychoactive substances. One advertising slogan of 'dopalacze.com' emphasised the harmless effects of its products: 'Life is too short to take unhealthy pills'. NPS were marketed as collectibles not intended for human consumption. They were described as legal in the European Union, controlled, regulated and safe. They were also marketed as the so-called 'party drugs'. The shops also stocked herbal concoctions, known for centuries in various cultures and used for a number of purposes including rituals e.g. *Salvia divinorum*. It came as a big surprise to NPS users that herbal concoctions contained synthetic cannabinoids, which were to a large extent, if not wholly, responsible for the psychoactive effects thereof. In mid-2008, following the opening of the first high street smart shop, trade in NPS entered the reality offline. As soon as by the end of 2008, 40 high street smart shops were operational in the centers of major Polish cities offering an increasingly wide range of psychoactive products. While describing the beginnings of the legal highs scene it is worth mentioning a first synthetic cannabinoid called JWH-180, which was identified in Poland by the Central Forensic Laboratory in February 2009. This substance gave rise to a massive supply of such-like substances on the Polish market. At the same time, other new psychoactive substances and products were arriving rapidly including synthetic cannabinoids, mephedrone and later on a whole range of cathinone-type substances. New smart shops started springing up exponentially and soon, by the end of 2010, 1 400 smart shops were up and running across the country. After the closure of high street smart shops by sanitary inspection, NPS kept being sold online (Jablonski and Malczewski, 2014).

Unfortunately in next years, some shops have been reopened and the situation regarding increased hospitalizations in July 2015 was similar to that from 2010, so therefore, it became necessary to develop a new orthogonal approach to solve the problem of identification of the new chemical structures appearing on the market by applying complementary techniques, i.e. LC-MS/MS-TOF, GS-MS/MS, LC-CAD and NMR. In December 2010 analysis of NPS samples collected by the sanitary inspection found seven main chemical groups; the highest incidences being MDPV (23%), and 16% for JWH-081 and RCS-4. From 2010 Polish National Medicines Institute have analyzed over 7000 various designer drugs and herbal highs products. About 160 psychoactive compounds were identified including: substituted cathinones, phenethylamines, synthetic cannabinoids, phenylpiperazines, tryptamines, pharmaceuticals and other. Poland still has one of the fastest growing market for new psychoactive substances in the EU with hundreds of hospitalizations resulting (approx. 1100 in 2013, 2000 in 2014 and 7000 in 2015).

Conclusions

Designer drugs present an ongoing challenge to chemists, toxicologists, and law enforcement agencies due to their dynamic and changing markets. To effectively combat the problems associated with designer drugs, a collective effort needs to be in place from forensic scientists, health professionals, and law enforcement authorities. It is important to stay at the forefront of drug detection techniques and strategies to allow speedy identification of new substances as they emerge. Metabolism and toxicity data need to be collected to facilitate diagnosis and treatment of designer drug intoxication. At the same time, drug scheduling authorities need to schedule these drugs promptly to enable laws to be applied to the production, distribution, and consumption of these illicit substances to safeguard society.

The global nature of the NPS phenomenon also presents opportunities for enhanced international collaboration, and the importance of data sharing within the European and international authorities. The Poland is currently an active participant in the European Early Warning System on NPS and this network has proved useful in the early identification of emerging threats in this area. Sustained collaboration in the future would not only continue to allow the rapid exchange of information between countries but also the potential for mutual benefit through collaboration in research initiatives or through the sharing of reference material and toxicological information.

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Macedonian pharmaceutical bulletin, **62** (suppl) 17 - 18 (2016)
ISSN 1409 - 8695
UDC: 615.015.4:620.3
Short communication

Green "cage" nanoparticles as efficient carriers for challenging drugs

Ruxandra Gref

Institut des Sciences Moléculaires d'Orsay, UMR CNRS 8214, Avenue Jean Perrin, Université Paris Saclay, 91400 Orsay, France

Background

Nanotechnology revolutionizes drug delivery by enabling achieving: i) targeted drug delivery; ii) transcytosis of drugs across biological barriers; iii) delivery of drugs to intracellular targets and iv) visualization of sites of drug delivery (theranostics). High loadings and surface engineering are important prerequisites for efficient drug nanocarriers.

Green "cage" nanocarriers

Will be presented a few examples of "cage" nanoparticles composed of cyclodextrins (CDs) (Gref et al., 2006) or made of highly porous Metal-Organic Frameworks (MOFs) (Horcajada et al., 2010). "Cage"nanoparticles were produced by solvent-free green procedures such as microwave assisted hydrothermal synthesis. The nanoparticles were fully characterized to determine their chemical composition, morphology, size distribution and specific surface.

Drug substances were encapsulated in the "cages" of these nanoparticles, thus ensuring optimal interactions with the matrices, leading to high loadings and controlled release properties.

Antibiotics, anticancer and antiviral drugs were loaded simply by soaking the nanoparticles in aqueous solutions of the drugs, reaching in most cases efficiencies close to 100%. Remarkably, drugs with different physico-chemical properties could be co-encapsulated in different interconnected cages inside the porous nanoparticles. Furthermore, to achieve stable, versatile coatings on highly porous MOF nanoparticles without altering their ability to entrap molecules of interest, the outer surface of the nanoparticles was functionalized with CDs in a biofriendly, non-covalent manner (Agostoni et al. 2015). Versatile coatings were obtained in aqueous media, within a few minutes. Ligands could be coupled to the shell, to ensure specific interaction with cancer cells.The coatings were remarkably stable in cell culture media, despite their non-covalent nature.

Conclusion

Drug loaded, surface modified "cage" nanoparticles were prepared using only biofriendly solvent free procedures. Each step of the fabrication procedure (systhesis, drug encapsulation, coating) was performed in aqueous medium, at room temperature, without the need of coupling reagents nor protective surfactants.

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Macedonian pharmaceutical bulletin, **62** (suppl) 19 - 20 (2016)
ISSN 1409 - 8695
UDC: 61-027.12(569.4)
Short communication

Data-driven innovation in health policy

Ran Balicer

Clalit Research Institute, Tel-Aviv &Public Health Department, Faculty of Health Sciences
Ben-Gurion University of the Negev, P.O.B. 653 Beer-Sheva 84105, Israel

We live in an era of ensuing prevalence rates of non-communicable disease, which pose an increasing risk to the financial sustainability of health systems worldwide. It has been suggested that a paradigm shift is required if we are to properly deal with these challenges, transforming our healthcare system to one which can bridge the silos of care provision in a patient-centered approach, move from reactive therapeutic to proactive preventive care, and abandon our paternalistic narrative to a participatory and engaging patient-physician relationship.

It has been realized in recent years that a key driving force and a pre-requisite for these changes is the availability and intelligent integrated use of data and information technology. Israel has benchmarked high in global NCD mortality and complications measures. Over the last two decades. These clinical improvements were supported by wide early adoption of Electronic Health Records throughout the country, all tiers of community care.

Clalit is Israel's largest healthcare organization which serves as insurer/payer and integrated care provider for over half of the Israeli population – over 4.3 million people. Clalit has been leading innovative interventions using clinical data to drive people-centered targeted and effective care models, for NCD prevention and control. In its strategic plans, Clalit aims to perform a paradigm shift to properly deal with these challenges, transforming the healthcare system to one which can bridge the silos of care provision in a patient-centered approach, move from reactive therapeutic to proactive preventive care, and abandon our paternalistic narrative to a participatory and engaging patient-physician relationship (Balicer et al., 2015; Leventer-Roberts et al., 2015; Feldman et al., 2014; Shadmi et al., 2015).

We at Clalit believe that a key driving force and a pre-requisite for these changes is the availability and intelligent integrated use of EHR-based clinical data, and have been practicing innovative utilization of this data for quite a few years with successful measurable outcomes. Many of the unique innovative interventions introduced by Clalit are data-driven, made possible by real-time data provided to physicians and nurses, in an actionable, decision-supporting format. Clalit has 100% (single software) Electronic Health Records coverage of ambulatory and hospital care, with an aggregated data warehouse that received feeds from both, on our 4.3 million members, for well over 1.5 decades. This data included detailed and full demographic (i.e. place of birth of person and parents), diagnoses (both EMRs and Claims), measures (i.e. as BMI, blood pressure), full laboratory test data, imaging data, patient reported such as smoking status and willingness to quit smoking, cost (pricelist and real-life monthly cost per patient), both prescription and dispensing medication data, and administrative health services consumption data. These data are augmented in Clalit by the largest ongoing patient experiences survey performed in Israel, ongoing all year long to a very large patient sample, and an increasing amount of data becoming available through Clalit's patient portal and Personal Health Record (PHR). Quality of care, patient experiences and financial benchmarks are all part of an online balanced scorecard system available to all managers at all levels of the organization.

To turn this abundance of data into actionable insights, Clalit launched in 2010 its Research Institute, which now holds over 30 professionals of multidisciplinary qualifications – top notch clinicians, epidemiologists, biostatisticians, IT experts, algorithm specialists and public health experts. The institute associates gained invaluable experience in mining and interpreting the organizational database, and creating tools introduced into policy and medical practice. These tools allowed for implementing innovative clinical interventions to tackle key health issues such as reducing healthcare disparities, preventing avoidable readmissions, tackling inadequate treatment adherence, assessing the impact of multi-morbidity, improving control

of key chronic diseases, performing comparative effectiveness real-life studies and using predictive modeling and advanced analytics to allow targeted care in high risk groups. The institute is augmented by its affiliates - leading clinicians from Clalit's 8 districts and 14 public hospitals that initiate and perform advanced studies with the support of the institute.

The Clalit Research Institute has focused in creating data-driven tools for enabling, supporting and assessing innovative clinical interventions to tackle key health and healthcare issues. These include including measuring and tackling inadequate treatment adherence, assessing and tackling the impact of NCDs and multi-morbidity, using predictive modeling and advanced analytics in the point of practice to allow targeted care in high risk groups, and comparative effectiveness real-life studies.

In the presentation at this conference we will review the driving forces and the need for such disruptive innovations, and detail specific examples that allowed for reducing healthcare disparities, preventing avoidable readmissions, and improving control of key chronic diseases. Key conclusion can be drawn from of these case studies to be presented - that integrated data systems allow a wide potential for implementing innovations in care integration. Integrated data allows for innovative patient selection approach, in-depth program planning, real-time implementation support IT tools and real-time monitoring of intervention outcomes thus allowing multi-level effective intervention management.

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Macedonian pharmaceutical bulletin, **62** (suppl) 21 - 23 (2016)
ISSN 1409 - 8695
UDC: 615.12
Short communication

Clinical pharmacy - established paths and new opportunities

Dorothea Rudorf

Massachusetts College of Pharmacy/Health Sciences, 179 Longwood Ave., Boston MA 02115, USA

Clinical pharmacy – development and standards

Over the past 5 decades the role of a pharmacist has significantly changed, from traditionally dispensing medications, to performing clinical services or pharmaceutical care (ACCP - American College of Clinical Pharmacy, 2008). Although there is no international consensus on the definition of clinical pharmacy/pharmaceutical care, it is agreed that a clinical pharmacist, as a member of an interdisciplinary health care team in direct patient care environments, contributes through comprehensive medication management to optimal drug therapy (ACCP, 2008; Frankin and Van Mil, 2005; Van Mil and Fernandez-Llimos, 2013). The American College of Clinical Pharmacy has recently published specific requirements for clinical pharmacists practicing in the USA and around the world (ACCP, 2014).

Clinical pharmacy – specialty services, certification, job satisfaction

Clinical Pharmacy services are performed in various health care settings (e.g. community, ambulatory, hospital) and may differ for patient populations (e.g. pediatric, geriatric). Accordingly, clinical pharmacists may specialize to acquire the unique skills and expertise needed for specific patient groups. Specialization can be in different forms, e.g. on the job training, gradually transitioning into the specialty field, or via specific postgraduate training (Schommer et al., 2008). Clinical Pharmacy specialists practice in many different areas (e.g. cardiology, psychopharmacy, oncology) (CPP - Certification Programs for Pharmacists, 2012; Schommer et al., 2008). Advanced practice pharmacists provide clinical services in primary care clinics addressing common diseases (e.g. hypertension, hyperlipidemia, COPD) and may have prescribing privileges under collaborative practice arrangements with physicians (Murawski et al., 2011).

To demonstrate specialized clinical knowledge beyond licensing standards increasingly more clinical pharmacists seek national, state or organization certification or credentialing, in specific disease state management (e.g. anticoagulation, diabetes, poison information specialist) (CPP, 2012; Schommer et al., 2008).

Survey results indicate that most clinical pharmacy specialists are extremely satisfied with their job and consider direct patient care, interaction with others, autonomy, practicing in a unique environment and applying their knowledge as very attractive aspects of their job. However, long hours (average work time/week 50.6 h) and heavy workload are challenges, yet also welcomed as stimuli for professional growth and innovative services (Schommer et al., 2008).

Clinical pharmacy practice - newer trends

Recent trends are clinical pharmacist involvement in ID subspecialties (i.e. HIV care, Hepatitis C disease, Antibiotic Stewardship programs) and in Transition of Care, and respective practice guidelines have been published (ASHP, 2010; Hume et al., 2012; Schafer et al., 2016; SIDP, 2010).

Pharmacist in HIV and hepatitis C management

Management of HIV disease/AIDS and Hepatitis C provide excellent clinical pharmacy opportunities as both diseases are affecting millions of people, are predominant-

* dorothea.rudorf@mcphs.edu

ly chronic illnesses with major morbidity, known challenges (e.g. poor medication adherence, numerous drug related concerns) and new considerations (i.e. HIV in the aging population or pre-exposure prophylaxis, recent treatment options for hepatitis C, coinfections, high costs) (Schafer et al., 2016; Spooner, 2011). Pharmacist involvement in HIV/Hep C testing, treatment selection and monitoring, management and prevention of drug-related problems, complications or opportunistic infections, and education will provide effective disease state management and optimal patient support.

Antimicrobial stewardship

Antimicrobial Stewardship (AS) is another opportunity for clinical pharmacists (ASHP, 2010; Dellit et al., 2007; MacKenzie et al., 2007). Data show that unnecessary and inappropriate prescription of antibiotics exposes patients to serious adverse drug effects and superinfections (i.e. Clostridium difficile); antibiotic misuse is a critical factor in rising antibiotic resistance and poses a public health threat (CDC, 2013). Regarding improvement of antibiotic use and patient safety as a national priority (CDC, 2013a), and based on evidence that designated programs can optimize treatment of infections and reduce adverse effects (Davey et al., 2013; Ohl and Luther, 2011) the CDC in 2014 recommended that all hospitals implement AS programs (CDC, 2014). Designated ID pharmacist leaders collaborate with ID specialists in infection prevention and control by tracking/optimizing antibiotic prescribing and use, reporting outcomes, implementing policies (i.e. "antibiotic time out", IV to PO changes, dose adjustments) and educating clinicians.

Transition of care

Major medication errors can occur when patients are transferring between different care locations or levels (e.g. admission to, transfer or discharge from hospital) resulting in significant adverse drug events, increased length of stay, high readmission rates and costs (Hume et al., 2012). Official organizations (e.g. US Joint Commission, ASHP/APhA) (ASHP-APhA, 2013; Joint Commission, 2012) have established national patient safety goals requiring accurate patient information in hospitals to prevent readmission rates, and potential payment penalties by insurers (Kristeller, 2014). Transition of Care (TOC) pharmacists plays an instrumental role in facilitating the process of continuing/coordinating therapeutic care (ACCP, 2008; ASHP-APhA, 2013; Hume et al., 2012). Obtaining patient medication histories, performing medication reconciliation, inpatient education, patient discharge counseling and post-discharge follow-up are key activities of the TOC pharmacist.

Clinical pharmacy practice - future trends

It is envisioned that most future clinical pharmacists (and all clinical faculty) will have post-graduate training, will be board-certified specialists, and will be formally recognized and reimbursed as health care providers who ensure optimal drug therapy outcomes (Gubbins et al., 2014; Saseen et al. 2006).

Pharmacy faculty and pharmacists will work in interdisciplinary patient-centered medical homes (PCMH), where an entire team delivers comprehensive and coordinated health care (Smith et al., 2010; Zellmer, 2012) Pharmacists will provide medication therapy management utilizing health information technology and will engage in: handling, administering and monitoring of complex specialty drugs and of medications delivered in innovative dosage forms (e.g. nanoscale devices); digitally monitoring drug levels; and personalized medicine (Gugliemo, 2015). Pharmacy education will need to prepare future clinical practitioners for these innovative and exciting professional practice opportunities (e.g. through interdisciplinary education).

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Macedonian pharmaceutical bulletin, **62** (suppl) 25 - 26 (2016)
ISSN 1409 - 8695
UDC: 613.25
Short communication

Brown fat induction in treatment of metabolic disorders

Mirko Trajkovski

University of Geneva, Medical Faculty, Department of Cell Physiology and Metabolism, Centre Médical Universitaire (CMU), 1211 Geneva 4, Switzerland
University of Geneva, Diabetes Centre, Faculty of Medicine, 1211 Geneva, Switzerland
University College London (UCL), Division of Biosciences, Institute of Structural and Molecular Biology, WC1E 6BT, London, UK

Background

Obesity is a major metabolic disorder leading to various health risks and reduced life expectancy. Food intake, energy expenditure and body adiposity are homeostatically regulated, and malfunctions of this balance can cause increased fat storage and obesity (Farooqi and O'Rahilly, 2005;Murphy and Bloom, 2006). Mammals have two types of adipose tissue (fat): brown and white, with opposing functions. Mammalian white fat is an important regulator of the whole body homeostasis that stores energy in form of triglycerides. The brown adipose tissue catabolises lipids to produce heat, function mediated by the tissue-specific uncoupling protein 1 (Ucp1) abundantly present in the brown fat mitochondria. The brown adipose tissue differentiation can be induced by prolonged cold exposure and beta-adrenergic stimulation which leads to elevated intracellular cyclic AMP (Cannon and Nedergaard, 2004; Young et al., 1984). The classical brown fat is present at distinct anatomical sites, including the interscapular, perirenal and axillary depots. Brown fat cells also emerge in subcutaneous white fat (known as "beige" cells) in response to cold or exercise (Cousin et al., 1992; Guerra et al., 2001), a process referred to as fat "browning". Promotion of increased brown fat development increases energy expenditure and white fat loss, and leads to improved insulin sensitivity and glucose metabolism, without causing dysfunction in other tissues and is associated with a lean and healthy phenotype (Lowell et al., 1993), suggesting the manipulation of the fat stores as an important therapeutic objective.

Gut microbiota regulates metabolic homeostasis

Thegastrointestinal tract is the body's largest endocrine organ that releases a number of regulatory peptide hormones that influence many physiological processes (Badman and Flier, 2005). The intestinal microbiota co-develops with the host, and its composition is influenced by several physiological changes (Koren et al., 2012; Ridaura et al., 2013). The colonization starts immediately after birth and is initially defined by the type of delivery and early feeding. A wide range of pathologies have been associated with alterations of the gut microbial composition (e.g.: asthma, arthritis, autism or obesity) (Sommer and Bäckhed, 2013). The intestinal microbiota can also influence the whole-body metabolism by affecting energy balance (Bäckhed et al., 2004; Koren et al., 20012; Ridaura et al., 2013; Turnbaugh et al., 2006). Transplantation of microbiota from obese human, or animal donors to germ free mice is sufficient to promote increased adiposity and insulin resistance of the new host, suggesting that microbiota alone is sufficient to induce these metabolic changes. The mechanisms and the nature of the phenotypic and morphological alterations that regulate the energy homeostasis of the new host following microbiota transplantation remain poorly understood.

Our recent findings show that cold exposure leads to marked shift of the microbiota composition, referred to as cold microbiota (Chevalier et al., 2015). Transplantation of the cold microbiota to germ-free mice is sufficient to increase insulin sensitivity of the host, and enable tolerance to cold partly by promoting the white fat browning, leading to increased energy expenditure and fat loss. During prolonged cold however, the body weight loss is at-

* mirko.trajkovski@unige.ch

tenuated, caused by adaptive mechanisms maximizing caloric uptake and increasing intestinal, villi and microvilli lengths. This increased absorptive surface is transferable with the cold microbiota leading to altered intestinal gene expression promoting tissue remodelling and suppression of apoptosis - effect diminished by co-transplanting the most cold-downregulatedbacterial strain *Akkermansiamuciniphila* during the cold microbiota transfer. Our results demonstrate the microbiota as a key factor orchestrating the overall energy homeostasis during increased demand ((Chevalier et al., 2015). We recently also showed that the development of functional beige fat is promoted by microbiota depletion either by means of antibiotic treatment or in germ-free mice within the white adipose tissues (Suárez-Zamorano et al., 2015). This leads to improved glucose tolerance, insulin sensitivity and decreased white fat and adipocyte size in lean mice and obese mice. Such metabolic improvements are mediated by eosinophil infiltration and enhanced type 2 cytokine signaling and M2 macrophage polarization in the white fat depots of microbiota-depleted animals. The metabolic phenotype and the browning of the subcutaneous white fat are impaired by suppression of the type 2 signaling and are reversed by recolonization of the antibiotic-treated, or the germ-free mice with microbes (Suárez-Zamorano et al., 2015). These results provide insights into the microbiota-fat signaling axis and the beige fat development in health and metabolic disease. The reduced adiposity, and improved glucose tolerance and insulin sensitivity of the microbiota depleted obese animalssuggestthat browning of the white fat depots by modulation of the microbiota composition could be a new approach for combatingobesity and the associated metabolic disorders.

Conclusion

Microbiota transplantation was reported almost 50 years ago, and has re-gained interest as a treatment option for several pathologies. In the context of the increased obesity prevalence and energy unbalance, our studies showing microbiota changes that promote fat browning, weight loss and increased energy dissipation, imply microbiota as a key player mediating the tight control of the energy homeostasis with large therapeutic potential.

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Macedonian pharmaceutical bulletin, **62** (suppl) 27 - 29 (2016)
ISSN 1409 - 8695
UDC: 004.7
Short communication

Bridging the computer and life sciences: the case of VI-SEEM

Anastas Mishev

Faculty of Computer Science and Engineering, University Ss Cyril and Methodius, Rudjer Boskovic 16, 1000 Skopje, Macedonia

Introduction

Currently, the world of computing is overwhelmed with huge numbers depicting the extraordinary performance of the current computers. This is especially true for the biggest supercomputers in the world, showing off their exa-FLOPS, aggregating peta-bytes of data, measuring and comparing each other on several top charts (Filiposka et al., 2016). But is this their real power? If we compare the best of the best from only 23 years ago (at the time the first top500 (Top500) list was published), with the todays list leader, there is more than 400000 times increase in the peak performance capabilities. One can have all the exas and petas in the world, but the true power of todays supercomputers is in their applicability to solve real life problems. VI-SEEM (VI-SEEM) is one of the examples of trying to put the supercomputers to work on practical problems. It tries to facilitate regional interdisciplinary collaboration, focusing on the scientific communities of Life Sciences, Climatology and Digital Cultural Heritage. Through unification of the existing e-infrastructure into an integrated platform, it strives to better utilize synergies, for an improved service provision within a unified Virtual Research Environment to be provided to scientific communities of high impact in the combined South East Europe and Eastern Mediterranean region.

The project

Building on the success of the previous regional projects and initiatives that helped bridge the digital divide by ensuring access to regional e-Infrastructures, VI-SEEM includes partners from 16 countries in the SEE and EM region, both from the resource providers' and potential users' communities. Bridging the two worlds would bring new value and improve research productivity and competitiveness on the pan-European level.

The general project objective is to provide integrated e-Infrastructure platform for regional cross-border Scientific Communities in Climatology, Life Sciences, and Cultural Heritage for the SEEM region that will be user-friendly and accessible to the fore mentioned communities. This goal will be achieved by linking compute, data, and visualization resources, as well as services, models, software and tools. This Virtual Research Environment - VRE will provide the scientists and researchers with the support in full lifecycle of collaborative research: accessing and sharing relevant research data, using it with provided codes and tools to carry out new experiments and simulations on large-scale e-Infrastructures, and producing new knowledge and data - which can be stored and shared in the same VRE. Through training, user support, application development and porting, the researchers will be able to truly utilize the power of the regional e-infrastructure, to try to solve realistic problems, including computer aided drug delivery, modelling of biomolecules, introduction of novel methodologies into drug development, regional genotype databases development are only a few of the possible applications. Our expectations are that this and similar projects will actually bridge the gap between the computing power and its real applications, for a healthier world and better living.

VI-SEEM for life sciences

Advances in computational infrastructure during the last decade have facilitated the development of biological data analysis for big data and computational biology as key research methodologies in both academia and industry. The use of computers in biology has enabled our better understanding of mechanistic aspects in health and disease and has accelerated the development

of novel therapeutics. In this project, the Life Sciences research community is chosen because of its central role in achieving a higher quality of life in the SEEM region. The aim of the VRE is to create and provide the necessary services over a capable infrastructure to facilitate research for understanding of disease mechanisms and appropriate mitigation methodologies in the SEE and EM populations. Project participants and related institutes will assist in data collection and analysis, run and optimizing computational codes and using the research results to understand the molecular basis of diseases associated with SEE and EM areas with projections to develop personalized therapies.

The Life Sciences research community in the SEEM region could benefit greatly from the e-infrastructures at hand. Large amounts of data need to be stored and be made available to researchers for processing in the compute centres of the region. Therefore, apart from storage resources, fast and reliable networking infrastructure is important for moving large datasets from data archives to the computing centres and also moving simulation results to the researchers' facilities for further post processing and acquisition of results. In terms of compute infrastructure, the models and services to be used by the research groups require capacity and capability computing as well as the provision of computing resources for the installation of user facing services. For example, codes such as NAMD and NWCHEM scale up to hundreds or thousands of cores and can benefit from scalable HPC clusters or supercomputers such as the IBMs BlueGene. Molecular dynamics applications are also known to perform well on GPU systems, while also are being ported to new Intel's Phi accelerator platform. On the other hand, parametric codes for human genome sequence analysis can benefit greatly from the Grid or Cloud IaaS computing model. Finally, user-facing services can be also installed in the IaaS infrastructure that will be available in the project. It is evident that the Life Sciences Scientific Community requires a variety of infrastructure resources all of which are going to be available in the VI-SEEM VRE.

Life science use cases

Some most important and most representative examples of using the regional e-Infrastructure for the needs of the Life Science VRE include:

- Modelling and Molecular Dynamics (MD) study of proteins, membrane proteins and biological model membranes. These three biomolecular entities are responsible for signal transduction and are important drug targets. Therefore, in order to design more efficient drugs and drug delivery systems, a better understanding of the physicochemical interactions that govern biomembrane and protein interfaces is needed.
- Computational simulation of DNA and RNA to enable studying the influence of thermodynamic properties of the DNA/DNA and RNA/DNA duplexes on the transcription and processing of RNA. Computational modelling of the structure, thermodynamics and kinetics of RNA, involved in cancer cell growth
- Computer-aided drug design. By using computational methods and the 3D structural information of the protein target, we are now able to investigate the detailed underlying molecular and atomic interactions involved in ligand: protein interactions and thus interpret experimental results in detail.
- Image processing for biological applications includes experiments by spinning disk confocal microscopy of living cell, which generate images of dozens of GBs per experiment. The generated images require extensive image processing, such as registration, deconvolution, volume rendering, surface rendering, object detection, measurement of shape, size, and intensity of cell objects and automatic object movement tracking of the living cell in 3 dimensions and time.
- Analysis of Next Generation DNA sequencing data to identify disease mechanism pathways and provide patients with timely diagnosis, assessment of risk for developing the disease, targeted and efficient therapy, and give support for possible future reproduction planning.
- Synchrotron data analysis: SESAME is a 3rd generation synchrotron light source that produces very intense pulses of light/X-rays, with wave lengths and intensities that allow detailed studies of objects ranging in size from human cells, through viruses down to atoms, with a precision that is not possible by other means.

The list above is does not limit the possible usage of the resources, only provides some current and ongoing efforts in using the computational, networking and storage infrastructure to aid the Life Science research communities.

The research into computer aided drug design will be given a strong focus during the project, both from the LS researchers, but also from the infrastructure support point of view. Through advances in the drug delivery modelling, novel and hybrid methodologies (Markova et al., 2015) such as molecular dynamics, statistical physics, Monte Carlo etc. will be compared to the traditional methodologies, enabling better understanding of the processes at a very small scale. Through computer aided molecular design (Ng et al., 2015), the simulation results are expected to significantly reduce the clinical trials in anticancer drug research (Kim et al., 2013).

Conclusion

Enabling access to e-Infrastructure through intuitive and user friendly interfaces could bring great benefit to the research communities in the SEE and EM regions. Through virtual collaborative environment, these communities can achieve research excellence on the pan-European and global level. From the point of view of the

e-Infrastructures, strong justification of the investments can be accomplished, demonstrated through real life results. Bridging possibilities of the high end computing infrastructures and the life science scientific communities will produce deeper knowledge of the human biology, better disease understanding, shorter development time of new and targeted drugs with less clinical trials. As in many other cases, the addition of these two will bring much more to the humanity than their simple sum.

Acknowledgment

This work was supported in part by the European Union's Horizon 2020 research and innovation programme, project Virtual Research Environment for Regional Interdisciplinary Collaboration in Southeast Europe and Eastern Mediterranean VI-SEEM [675121].

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Pharmacoeconomy/ Social pharmacy/ Drug information

Macedonian pharmaceutical bulletin, **62** (suppl) 33 - 34 (2016)
ISSN 1409 - 8695
UDC: 615.1
Short communication

Data-driven approaches to tackling medication adherence

Ran Balicer

Clalit Research Institute, Tel-Aviv & Public Health Department, Faculty of Health Sciences
Ben-Gurion University of the Negev, P.O.B. 653 Beer-Sheva 84105, Israel

Introduction

Non-adherence to prescribed medication is a pervasive phenomenon, pronounced more in chronic conditions. Non-adherence to prescribed medication is very common, with approximately half of the patients failing to adhere to the medication regimen. It a complex and multi-faceted phenomenon that involves factors associated with the medication, patient, provider and health system, and thus cannot be addressed with single effort interventions (Osterberg et al., 2015).

Poor adherence to medication regimens has been associated with poor medical outcomes over a range of diagnoses, associated with avoidable admissions costs of $100 Billion a year, in the US alone. Moreover, patient non-adherence is a growing legal concern (Cutler, 2010). In one review, five percent of malpractice cases cite patient noncompliance as a contributing factor, with over a third of these resulting in payment to the plaintiff.

The opportunity in integrated EMR-driven databases

Integrated large clinical datasets offer a unique opportunity to assess the magnitude and impact of the non-adherence phenomenon. In the case studies we will discuss, we used data from Clalit - Israel's largest healthcare organization which serves as insurer/payer and integrated care provider for over half of the Israeli population – over 4.3 million people. Clalit holds abundance of real-time data provided to physicians and nurses, in an actionable, decision-supporting format. Clalit has 100% (single software) Electronic Health Records coverage of ambulatory and hospital care, with an aggregated data warehouse that received feeds from both, on all its members, for well over 1.5 decades. This data included detailed and full demographic, diagnoses (both EMRs and Claims), measures, full laboratory test data, imaging data, patient reported such as smoking status and willingness to quit smoking, cost, both prescription and dispensing medication data, and administrative health services consumption data.

The case of statins adherence

Statins, as one of the most widely-prescribed medications with proven preventive efficacy, have a single indication and are given long term, and thus are a simple candidate drug to assess adherence patterns. In one study we determined the proportion of patients prescribed statins who never fill a prescription, identified who they are, and compared their LDL control to adherent and non-adherent patients. The methods included a retrospective examination among patients prescribed a statin in 2008 and followed through 2010 in Clalit. Statin adherence in patients over age 21 was tracked for 2 years utilizing a new, validated adherence measure based on both written and dispensed prescriptions. Adherence below 20% was considered non-adherence. In this study, we found a total of 67,517 patients received 1,386,270 written prescriptions over the 3-year period. While a traditional adherence measure identified 8000 patients as non-adherent, a prescription+dispensing adherence measure identified 19,000 patients as low adherence patients. Thus, 1 in 6 patients prescribed statins would be overlooked using existing adherence methodologies. Changes in LDL levels of non-adherent patients were 11.9-14.1 mg/dl, compared to 48.3 mg/dl, $p<0.001$ in adherent patients. We thus concluded that non-adherence to statins was a very prevalent problem – more than we have previously assessed, as a large proportion of low-adherence patients may have been overlooked unless full data (prescriptions and dispensing) was available for adherence assessment (Singer et al., 2015).

The case of hypoglycemic drugs adherence

In another study we assessed the attributable impact of adherence to oral glucose medications as a risk factor for poor glycemic control using Clalit's electronic health records data.Adherence to diabetes medications over a two-year period was calculated by prescription-based Medication Possession Ratios for adults with diabetes diagnosed before January 1, 2010. Glycemic control was determined by the HbA1c test closest to the last drug prescription during 2010–2012. Poor control was defined as HbA1c>75 mmol/mol (9.0%). Medication adherence was categorized as "good" (>80%), "moderate" (50–80%), or "poor" (<50%).

Among 228,846 diabetes patients treated by oral antiglycemic medication, 46.4% had good, 28.8% had moderate, and 24.8% had poor adherence. Good adherence rates increased with increasing disease duration, while glycemic control became worse. We used logistic regression models to assess the role medication adherence plays in the association between disease duration, age, and poor glycemic control. There was a strong inverse association between adherence level and poor control (OR=2.50; CI=2.43–2.58), and adherence was a significant mediator between age and poor control.While poor adherence does not mediate the poorer glycemic control seen in patients with longer-standing disease, it was a significant mediator of poor glycemic control among younger diabetes patients. A greater fraction of poorly controlled younger patients (up to one third of them), compared to older patients, could be prevented if at least 80% adherence to their medications was achieved. Therefore, these results suggest that interventions to improve adherence should focus on this younger sub-group (Feldman, 2014).

The case of anti-depressants

In another study we aimed to uncover the impact of adherence to anti-depressant medications (AD) treatment on all-cause mortality. We performed a four-year historical prospective cohort of a total of 251,746 patients aged above 40 years old who were prescribed AD at least once during 2008-2011. Patients were stratified into: non-adherence (<20%), poor (20%-50%), moderate (50% - 80%), and good (>80%) adherence levels. Adherence was measured as a continuous variable representing possession ratio (duration of claimed AD divided by duration of prescribed AD). The association between adherence and Hazard Ratio (HR) for mortality follow a quadratic model in which the lowest HR (0.66 [95% Confidence interval (CI): 0.64 to 0.69]) is at a level of 60% adherence in respect to non-adherence, and leveled off as compared to higher adherence. This shows again that adherence to AD is significantly associated with a corresponding decrease in the risk of mortality, controlling for relevant covariates. Thus physicians from all disciplines should actively improve their patients' adherence to AD since their persistent use is associated with increased survival (Krivoy et al., 2016).

Summary

Our data from these multiple case study suggests that non-adherence is a frequent, impactful phenomenon, which exists across clinical domains and drives control challenges in chronic illness care. In the presentation at this conference we will also review the profound changes brought in the potential of information technology and digital health to allow innovative interventions that may address low rates of medication adherence and the multiple inadequacies in current healthcare systems that drive it. We will discuss medication adherence tracking, using patient health records to engage patients, cost-related aspects of non-adherence and discuss what approaches have shown success and/or promise in addressing this high-impact universal health challenge.

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Macedonian pharmaceutical bulletin, **62** (suppl) 35 - 36 (2016)
ISSN 1409 - 8695
UDC: 615(439)
Short communication

Analyzing pharmaceutical policies: Hungary as a case study

Rok Hren[1,2]

[1]*Carso, d.o.o., a Member of Salus Group PLC, Litostrojska Cesta 46, 1000 Ljubljana, Slovenia*
[2]*Institute of Mathematics, Physics and Mechanics, Jadranska 19, 1000 Ljubljana, Slovenia*

Introduction

In this study, we examined the impact of Pharma Economic Act (PEA), which was introduced in Hungary in 2007. The motivation to analyze this particular legislative measure within a single mid-size country is in its comprehensiveness and in its unique approach toward marketing authorization holders (MAHs). Moreover, recent economic crisis is making such a »laboratory of cost-containment tools« attractive for authorities and payers not only among the Central and Eastern European (CEE) countries, but also among Western (e.g., EU-15) jurisdictions. Final reason for the analysis is availability of detailed data on Hungarian prescription drug market, which are provided to the public by the authorities.

While the National Health Insurance Fund (NHIF-OEP) data provide strong evidence that the PEA resulted in cost-containment of the public expenditure for prescription drugs in Hungary, cost-contaninment itself, however, may not lead to efficiencies either in the prescription drug market, let alone in the overall healthcare market. As it is well known, cost-containment may stifle innovation, thereby reducing the dynamic efficiency; on the other hand, price reductions of off-patent drugs and their generic versions may be insufficent, allowing their prices to remain way above the marginal cost of production and thus promoting static inefficiencies.

Static efficiency

To assess static efficiency, we examined risperidone group (ATC5 group N05AX08). Risperidone group is a suitable proxy for genericized therapeutic group at the time of introduction of the PEA.

Risperidone faced expiry of its intellectual property right (IPR) at the end of 2005, however, in this particular case, the branded firm was unable to follow the price decrease due to the fact that the IPR was still in power in EU-15 jurisdictions and Hungarian price could thereby have a potential detrimental spill-over effect via international reference pricing. To prevent complete loss of revenue, the branded firm launched in 2006 its own authorized generic drug in cooperation with the local partner; in 2008, the local partner launched its own generic, completely abandoning marketing of the authorized generic.

After the introduction of PEA, public expenditure per mg of risperidone decreased in 2008 from 162.7 HUF (€0.54) to 101 HUF (€0.33) for a branded drug and from 155.2 HUF (€0.51) to 92.5 HUF (€0.3) for authorized generic drug. In 2008, public expenditure per mg of risperidone fell further to 37.1 HUF (€0.12) for authorized generic drug. The co-payment per mg increased sharply in 2007 due to the PEA; having in mind that patients suffering from schizophrenia typically poorly adhere to the treatment, such an increase could present potential adherence risk. However, since the average daily dose of risperidone is 4.7 mg, the monthly co-payment would be in total approximately 340 HUF (€1.1), unlikely a financial issue even for such a socially disadvantaged patient group. As we have observed earlier with atorvastatin, price of generic risperidone initially dropped rapidly (by 76% from 2006 to 2008), but in 2009 and 2010 remained virtually unchanged. If we take cost of goods sold (COGS) at 10% of the monopoly price as the rough estimate of marginal cost of production, for a typical branded small molecule launched in 1990s then the potential for further price reduction does exist. Public expenditure with included 12%-rebate was in 2010 for risperidone relatively modest – 871 million HUF (€2.8 million) – yet could still be reduced by about 60%.

* Rok.Hren@carso.si

Dynamic efficiency

To assess dynamic efficiency, we examined a line extension of oral risperidone, which was launched by the branded firm at the end of 2004. This incrementally modified product, however, reached way beyond the usual »evergreening strategies« of branded firms as the product presented the first long-acting atypical antipsychotic (administered once every 2 weeks) and thus addressed the issue of adherence of patients suffering from schizophrenia and associated higher hospitalization rates and costs. The branded firm entered in 2005 into price-volume agreement with NHIF-OEP, which was renewed in 2008. In spite of the controlled nature of growth, the long acting atypical antipsychotic achieved in Hungary diffusion which was globally surpassed only by Spain; in April 2011, its market share in the total antipsychotic market (N05A) was 23.7% (*vs* 26.7% in Spain, 17.1% in France, 13.4% in Italy, 10.9% in Germany and 6.8% in the United Kingdom) (IMS, 2011). One of the former top ranking NHIF-OEP officials mentioned that in his opinion, »majority of patients should be treated by long-acting atypical antipsychotic« and thus corroborated the fact that Hungarian authorities did not oppose the novel technologies in spite of the overall framework of cost-containment.As with oral risperidone, co-payment surged in 2007 and the absolute amount has been with average monthly dose of 75 mg around 525 HUF (€1.7), which is as with oral risperidone likely not excessive. In fact, OEP's own publication found no impact of co-payments on adherence of patients with schizophrenia; another former NHIF-OEP official claimed that in 2006 »25% of schizophrenic patients were treated with two or more atypical antipsychotics, which was clearly a waste of resources«.

Conclusions

NHIF-OEP managed to sharply decrease expenditure for risperidone by implementing the PEA in January 2007. The cost-containment policies did not exclude innovation: an example of a long acting atypical antipsychotic suggests that Hungarian prescription market under the PEA has enabled diffusion of new technologies, on at least per-capita basis comparable to those of G-5 countries. An obvious inefficiency is that the PEA was still financing excessive rents for off-patent drugs, which remain a global challenge for payers. The Hungarian off-patent market reinforces in particular the general notion that regulating generic prices may lead to price convergence and inefficiencies and that generic competition is not sufficiently fierce in regulated environment. Our study has some obvious limitations as we have examined a limited number of products and one might well find the product or a group of products which would deviate from our conclusions.

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Macedonian pharmaceutical bulletin, **62** (suppl) 37 - 38 (2016)
ISSN 1409 - 8695
UDC: 615.2:339.562(4-11)
Short communication

Impact of parallel trade/import of pharmaceuticals in Central East European Countries

Zoran Sterjev*, Rubin Zareski, Katerina Anchevska Netkovska, Zorica Naumovska, Aleksandra Kapedanovska Nestorovska, Aleksandra Grozdanova, Ljubica Shuturkova

Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje, Republic of Macedonia

Introduction

Parallel trade of pharmaceuticals in European Union (EU) started in the '70s as a process in which licensed pharmaceutical distributor in any EU member state can, after obtaining proper authorization, import any drug from another member state, so long as the drug that is being imported is identical to the drug that is locally sourced, that is, it contains the same active ingredient and is produced by the same manufacturer (Kanavos et al., 2005). Since the middle '90s the share of parallel trade grew up to 7-17%, especially in countries like Denmark, Sweden, United Kingdom, Germany and the Netherlands. This process is based on the existence of heterogeneous price regulation systems among different countries in EU. Parallel trade of pharmaceuticals is legal within the EU based on the principle of free movement of goods laid down in Article 28 of the EC Treaty to create a single market. However, it is subject to restrictions to protect industrial and commercial property and human life and health, according to Article 30 (Ginter et al., 2005). Representatives of the parallel trader's consumers and patients while converging prices between different geographic markets and thereby harmonizing the markets for pharmaceutical products. Representatives of the pharmaceutical industry, on the other hand, claim that parallel trade (or "gray market trade") is nothing more than free riding, or piggybacking, on pharmaceutical companies' profits and thereby condemning innovation by reducing the ability and willingness to invest in research and development ("R&D"), while at the same time distorting the supply chain of drugs in low-price countries, which makes availability of certain drugs uncertain for patients (Nilsson, 2013).

In the previous year's parallel trade originally was concentrated on the most important patented products, but now the situation is a different and this process has extended to a wider range of pharmaceutical products. The main problem generated within the parallel trade in CEE countries is a drug shortage. Namely these countries realize the greatest export of drugs resulting in hampering the access to drugs for the local patients.

Republic of Macedonia (RM) as a associate member of the EU is not a part of parallel trade process. There is a similar process named parallel import of drugs. The parallel import of medicines is legally defined and basically it is a modification of the procedure for parallel trade in medicines in the EU Member States. According to our law, PI is import of such medical products that has medical authorization in the R.M under import license issued by the Drug agency from the referent countries, when the importer is not appointed by the MA holder (national decision). The term "Parallel" implies that the MP is imported via pathways that are different to the established one (designated by manufacturer or its original supplier), as competitive channels that are active in the same time/parallely. The countries that are chosen as the reference for the registration of drugs are also chosen as the reference countries for parallel importation. Those countries are: EU Countries, USA, Japan, Switzerland, Canada. Russia and Turkey are added as two another counties to this list. This fact was reason for development of two different and opposing views on the feasibility of introducing of the parallel importation of medicines in our country. On one side was the attitude of some experts and the representatives of the pharmaceutical companies, first of all the innovative pharmaceutical companies, according to which this process will lead to the possibility for the emergence of the counterfeit drugs that will affect the quality of the medicines, the safety in their

* zost@ff.ukim.edu.mk

use and will be the reason for the closure of the representative offices of these companies and the withdrawal of registration files for these drugs from our country. On the other side was the attitude of the managing persons from the Ministry of Health according to whom this process will lead to increased competition, lower drug prices and the proposed legislation and prevent the occurrence of drugs of dubious quality pharmaceutical market.

The aim of this article is to present the effects of parallel trade/import in the pharmaceutical markets in CEE countries.

Methods

Traditional legal search methods were used for identification of impact of parallel trade/import on CEE countries. A literature search was carried out in the PubMed databases, ISPOR databases, Google Scholar and on official web sites of regulatory bodies from CEE countries. A following combination of key words has been used: parallel trade and pharmaceuticals and Central East European Countries or medicines.

Results and discussion

The share of parallel imports in EU increased continually due to a growing focus on specialty drugs (oncology, hematology products, HIV- and CNS-therapeutics) and the capping of price differences at 15 euros per package worth 100 euros or more. The annual turnover of more than 100 parallel traders in 2015 was 5.5 billion in Europe. The parallel trade as a process has some negative effects for the CEE countries. The main is the problem of drug shortage in the countries with smaller prices of medicinal products. Medications imported on other markets come mainly from these countries. They are exported from those particular countries in large numbers, thus hampering the access to drugs for the local patients, causes delays in bringing products to market. For example, the parallel import market of medicinal products in Poland in 2005 was 0.01% of the pharmaceutical market with 40 licenses for this procedure, in 2009 was already 0.7% with 308 licenses, and only in the first three quarters by 2010 it reached nearly 1.1% (Religioniand Czerw, 2012). In Czech Republic the parallel export of medicines is around 185 Mil. € in 2015 (Skoupá, 2016). The oncology drugs are accounted for about 30% of exports in values. In Slovakia, the problem is particularly acute. The medicines price regulation makes Slovakia an attractive destination to buy cheap medicines and export them abroad. Drug sales in Slovakia amount to about €1 billion annually, with re-exports making up as much as 30 percent of the revenue. In Greece in 2012 parallel exports accounted for around 500 million euros of the total value of the pharmaceutical market. During the economic crisis in 2014 the situation it became worst. Based on this situation the Greek government has announced a provisional ban on the parallel exporting of 34 innovative medicines as a emergency measure. Faced with this problem CEE countries from begging of 2013 have been started with introducing of new legal measures to monitor and restrict parallel exports. Slovakia's parallel trade-restricting law has served as the model for a similar regulation introduced in Bulgaria, Romania and Estonia latter.

In R. Macedonia in the previous 2 years period almost 350 different drugs have obtained parallel import authorization. The list includes drugs which are used in oncology, neurology, hematology daily practice, drugs for treatment of respiratory disease, interferons, insulins. List is getting wider and wider. These are drugs that mostly belong to the group of originator medicines and are intended for the hospital usage. But, on the list you can also find generic drugs. In this period parallel importation of medicines in Macedonia led to a reduction in drug prices and to the savings in the health system, while the occurrence of counterfeit medicines and disorders in the quality of imported products are not recorded for now. Based on the data from Ministry of health, to date the total savings enabled through the parallel importation of drugs in RM is 14 million euros.

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Macedonian pharmaceutical bulletin, **62** (suppl) 39 - 40 (2016)
ISSN 1409 - 8695
UDC: 615.2:338.57.055.3(497.7)"2012/2015"
Short communication

The effects of the new methodology application on the method of pricing of drugs

Rubin Zareski*, Ljubica Suturkova, Zoran Sterjev

Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Thereza 47, 1000 Skopje, Macedonia

Introduction

In the previous eight years the Law on Medicines and Medical Devices has been uniquely changed for 12 times, either by a new law enactment or by amendments to the existing laws. The Agency of Medicines regulated the prices of 2700 registered drugs placed on the market in the R. of Macedonia on three levels: unique wholesale price, unique retail price and defined wholesale and retail mark-ups. In December 2011, after a series of attempts to perform regulatory pricing pressure on importers and manufacturers of drugs, the Agency has introduced a new Methodology (Official Gazette of RM No. 156/11, 2011). Core data on the price reductions of the registered drugs were provided by the Macedonian Agency of Medicines. Data are covering the period 2012-2015, during which 8 revisions were made. For the workflow control of the computer model and in order to produce data analysis we have used the programming language Python v3.5. Microsoft Excel was used for additional statistical analysis. This study is the first comprehensive analysis of the result of the implementation of the new Methodology in Macedonia.

Results

The introduction of the new Methodology and its implementation by the Agency for the period 2012-2015 resulted in price decrease of 1386 drugs (including all available registered dosage forms) out of total 2178. In total 680 drugs were lowered by more than 10%. The major price reduction happened in 2014, followed by reductions in 2015, 2012 and 2013. Furthermore, the analysis of the average price changes of the drugs shows that after initial 13% decrease of the prices in 2012, significant percentage of reductions was registered in 2013, followed by declining dynamics in 2014 and 2015.

For objective research reasons we extended our analysis and grouped the pharmaceutical and dosage forms of the drugs. The results shows that since the introduction of the new Methodology until October 2015, a total of 843 drugs with the same INN have reduced prices. Major decrease by generics has been registered in 2014, in total of 355 generic drugs, followed by 2015 with 307 drugs, 2012 with 125 drugs and 56 in 2013. Our next focus was on averaging the percentages of price reduction of evaluated drugs. The results show that the major decrease of 23% was in 2013, followed by 18.4% decrease in 2014, 17.8% in 2012 and 10.8% in 2015. For more precise calculations we have used iterative calculation of the changes of the same generics year over year (YoY). By this same analysis, we have found that major price decrease were registered on glimepiride 88%, fluconazole 87%, ramipril 82%, bicalutamide 80%, torasemide 77% etc.

Detailed analysis of the dynamics of price reduction of the drugs with the same INN shows that major reduction of the prices took place in 2014, when the price of 355 drugs was lowered, with over 32 drugs lowered by 30% or more. Second biggest price reduction took place in 2015 when 307 of the drugs have lowered price, but with lower dynamics of reductions compared to previous years. Moreover, the analysis clearly shows that in 2012, a total of 125 drugs with the same INN have lower prices with 12 drugs lowered on average by 30% or more. Finally, the lowest number of reduced prices of drugs was in 2013 (56 drugs in total), but with the highest average dynamic of reductions of 23% for the period 2012-2015. The average price change was the lowest in 2015 with reductions of only 10.8%, in 2012 with 17.74% and in 2014 with 18.4%.

* rubin@trust.com.mk

Obviously the dynamics of the average price changes of the drugs with the same INN is slowing down YoY, considering the fact that in most of the cases the price reductions are affecting the same generic groups.

Next segment we have focused on was the evaluation of the drugs by the ATC code. Since the introduction of the new Methodology, major price reduction was registered in the Group L with total of 180 drugs and average price decrease of 27%. Next was Group N with a total of 123 generic drugs and an average price decrease of 19%. Group J with 119 drugs and average price reduction of 29% is "the leader" in terms of the relative number of generic drugs and the size of the price reductions. Finally, Group C where the price of total of 69 drugs was lowered in range between 77% and 10%. What is also noticeable is that the average price reduction in this ATC code group is 27%. 2012 tendencies by ATC classification show that the prices of a total of 119 drugs were reduced with average size of 26%. Major price decrease in 2012 by number of generic drugs was registered in Group N and by percentage of reduction in the Groups A and L. In the following year the price of a total of 63 generic drugs by ATC code was reduced with highest average decrease of 28% with major number of drugs in the Groups J, C, A and Group N. 2014 was the year of major changes concerning the number of a total of 276 generic drugs with reduced price by 27.7% as well as dynamics. Major developments are in Group L, with a decrease ranging from 51% to 24%. Second major changes were registered in Group M, reductions of 46% to 22%, Group J with a changes ranging between 58% and 10%, and Group N with a range of reductions between 64% and 11%. In 2015 the price of a total of 197 generic drugs was reduced with major decrease in the Group J with 51%, Group N with 46%, S with 44%, Group H with 35%, Group M with 32% and, Group N with 30% decrease.

Finally we have analysed the ratios between the price reductions and the quantities sold by inserting on X axis average percentages of price reduction calculated by the use of iterative approach and on Y axis sales for 2015 given the year 2012 as basis with 100 points level. The result was a downward slope at rate -0.83x which confirms that the price reduction leads to declining sales of the generic drugs. It is noticeable that out of total 118 generic drugs with lowered prices for the period 2012-2015, in the year 2015 a total of 31 generic drugs have zero sales. Furthermore, 50 out of 118 in 2015 have higher sales than in 2012, but only 19 have increased sale of over 10%. Remaining 31 generic drugs have increase of sale sizing below 10%. Major part of the sales increase was registered for those generic drugs whose price was reduced by approximately 40%, but with the average size of sales stagnating in the range between 100 and 120 level. Finally we have analyzed the price-quantities changes for the period 2012-2015 by dividing the drugs with registered sale increase, and drugs with lowered sales after the prices were reduced. Contrary to the expectations, the group of drugs above level 100 has declining tendency of sale, which reflects the drop in the sales as consequence of continuous price reduction. In the second group, stagnation of the sales quantities is evident (y=0.1x). By excluding the zero sales drugs for the period 2012-2015, we came to the conclusion that the price reductions of generic drugs have insignificantly increased the sales of the drugs, whereby most of the drugs are bellow or on the levels of 2012.

Conclusion

The introduction of the new Methodology in 2011 may look like a win-win solution in which the prices of number of drugs have been reduced, which is preferred policy target of the governments, and the patients have access to cheaper drugs. However, the price reductions of some generics did happen only on some registered dosage forms and for those the sale was dramatically reduced, or zero. The pharmaceutical companies have shifted the sale to the same generics but for different dosage forms, which at the end of the day creates similar profit ratio but it changes the composition of the portfolio offer for the drugs. The decrease of the prices has pushed down the level of sales of the drugs, which is a negative correlation and a clear signal that the supply side is negatively reacting to the price reduction.

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Macedonian pharmaceutical bulletin, **62** (suppl) 41 - 42 (2016)
ISSN 1409 - 8695
UDC: 615.2-027.512
Short communication

Market access of biosimilar medical products – economical, regulatory and clinical issues

Aleksandra Grozdanova*, Katerina Anchevska Netkovska, Zorica Naumovska, Aleksandra Kapedanovska Nestorovska, Zoran Sterjev, Ljubica Shuturkova

Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia

The term "biologicals" most often refers to complex biopharmaceuticals with very specific chemical, physical and biological characteristic due to the nature of their active substance and manufacturing process, immunogenicity and a generally higher variability in their structure. In this heterogenic group of medicines there are different therapeutic proteins like insulin, filgrastim, growth hormone, erythropoietin, interferons and monoclonal antibodies. Often viewed as miracle drugs they offer hope, transforms patient's lives and provide cures and treatments for many severe diseases including diabetes, blood conditions, neurological disorders, autoimmune disorders and cancer. All that hope and promise that this treatments are offering comes with very high price that accounts for most of the biologicals. Data for global sales from 2014 are showing that approximately 80 billons US dollars are spend for this medicines. The predictions are that by 2017 global biological pharmaceutical market will amounts to about 220 billion dollars (Dolinar and Reilly, 2013). This trend of biological spending and growth is putting big financial pressure on the health care budget. The high price of this drugs and the financial crisis that has required healthcare systems to make significant cost reductions and in the same time patent expirations on many of the biological blockbusters were the main factors that drive the interest towards biosimilar medical products (biosimilars). Biosimilars are relatively new but growing segment, offering less costly alternative and enhanced competition to existing biological market. There is a strong interest by healthcare stakeholders in measuring the biosimilar utilization and impact on the market entry. Regulatory issues, manufacturing, safety, pricing, and physician and patient acceptance have a big influence in the developing the biosimilar market. By definition biosimilars are biological medicine similar toor a version of another biological medicine that has already been authorized, with similar active substance and which can prove their similarity towards quality, safety and efficacy (European Medicines Agency, 2014). Biosimilars began to enter European Union (EU) markets in 2007 and increase competition among producers of biologicals. However, the generic approach of substitution is not applied in the case of biosimilars due to their specificity (Weise et al., 2014). In the last years biosimilars have e significant clinical, regulatory and economic impacts in the medical market. At this moment there are 20 biosimilars with marketing authorization in EU, in seven classes as following: 7 Filgrastims, 5 Epoetins, 2 Folitropin alpha, 2 monoclonal antibodies, 1 insulin, 1 somatropin and 1 etanercept. When it comes to the market uptake of biosimilars, in the Consensus paper from EU Comission from 2013 (Consensus Information Paper, 2013) it was clearly stated that the most important conditions for market uptake of biosimilar medicines are driven by factors in the commercial market place. Differences across EU member states in national healthcare systems, have big impact on the biosimilars uptake. Factors influencing the market uptake of biosimilars are local pricing and reimbursement regulation, procurement policies and terms, physician perception of biosimilar medicine and patient acceptance of biosimilar medicines. There are differences in uptake by country, reflecting variations in local healthcare systems. Biosimilar penetration of the accessible market is only relevant when there is significant price difference between the originator and biosimilar, and where treatment options are limited to the molecule for which biosimilars are available. If the therapy area also has products that have been launched more recently, are still patent protected

* agrozdanova@ff.ukim.edu.mk

and which bring differential clinical benefit to patients or segments of patients who do not respond to other therapies, then payers and health systems should more usefully understand the role of biosimilars in the context of the total class of therapeutic options. There are important factors driving biosimilar market performance which play out differently across therapy areas and countries. These approaches in turn are often influenced by budgetary or financing measures imposed on healthcare payers and those with responsibility for managing the drug expenditure budget (Ruiz et al., 2013). Patients can also influence the shape of biologic markets and the uptake of biosimilars through their advocacy voice, typically through patient groups that represent the interests of those suffering from certain diseases. Current differences in the use of biosimilars and competition dynamics across European markets are not just explained by epidemiology and disease factors, but instead reflect local adoption of treatment practices and guidelines influenced by funding decisions and payer actions. These decisions are often made with a narrow focus on medicine costs rather than a broader view that considers the full cost of administering the medicine to patients. Relevant measures and use of real world evidence are needed to bring policy-makers the level of transparency and visibility they need to assess options, make decisions and monitor the results (Wang and Chow, 2012). With respect to the use of biosimilars, these measures may include the appropriate expansion of access to patients who will benefit from biologics, the evolution of medicine cost, as well as the evolution of overall patient treatment cost and health outcomes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 43 - 44 (2016)
ISSN 1409 - 8695
UDC: 615.15
Short communication

The importance of pharmacists in primary healthcare

Lovorka Nikolic*, Una Ivosevic, Natasa Lalic Cunkovic, Mirjana Savicevic

Pharmacy Farmanea, Milosa Pocerca 25, 11000 Belgrade, Serbia

Introduction

Although pharmacists' role to secure quality healthcare and educate patients on disease prevention is by all means very important and presents their role in primary health care, the potential of pharmacists is not recognized by the healthcare authorities in Serbia, as is the case with many other countries (Chandra et al., 2003).

Related to dispensing medicines, pharmacists provide much more than merely detailed information on medicines (Chandra et al., 2003). Community pharmacist must be sure that the patient has received and understood in a proper manner all the information provided, in order to safely and effectively use the medicines and improve the treatment outcome.

In addition to dispensing medicines, patient counseling presents one of the most important services rendered by community pharmacists (Puspitasari et al., 2009). The counseling process includes time, empathy and understanding, an individual approach and open communication with patient (Puspitasari et al., 2009).

By talking with the patients, pharmacists can identify the majority, as well as, solve minor health problems, educate patients on self–medication, proper use of medicines and medical devices and where necessary direct the patient to the doctor.

The objective of this study was to determine in which degree patients/citizens trust in knowledge and expertise of pharmacists subject to their health status, age and education.

Materials and methods

For the needs of this study were used responses to questions from a questionnaire conducted in 26 chosen Farmanea pharmacies in Belgrade, Republic of Serbia, during the period from January 3rd to February 28th, 2014. The questionnaire was anonymous and consisted of 17 closed type questions. The patients filled out the questionnaires in the pharmacy either alone or in consultation with pharmacists. At the beginning of the questionnaire the patients were asked to state their health status, age and education level. The questions referred to reasons and frequency of pharmacy visits and counseling service satisfaction. For the needs of this survey four questions from questionnaire were chosen and the influence of health status, age and education level of respondents was analyzed.

The chosen questions were to give answers to: (1) Most common reasons patients visit pharmacies, i.e. how many of them state counseling for minor ailments as major reason for visit to the pharmacy; (2) To what degree do the patients first consult the pharmacist when facing a minor health problem and (3) Do they subsequently consult the GP; (4) Which health professional do the patients consult on queries related to proper use of medicines or medical devices. The questionnaires were processed by statistic program SPSSV19.

Results and discussion

A total of 3656 fully completed questionnaires were collected. This survey presents and discusses results obtained by analyzing of responses to four questions from the questionnaire depending on the health status, age and education level of respondents.

To the question "Which is the most common reason for your visits to the pharmacy" 21.4% of the patients answered that they visited the pharmacy because of health problem, 27.1% in order to obtain prescription medicine from pharmacy and 51.5% came to buy a specific product.

Related to health status structure of respondents, the analysis showed that healthy persons (23.4%), persons with minor ailments (24.5%) as well as those who didn`t check their health status (27.6%) were those who visited the pharmacy for reason of health problem in a percentage above the general average.

Patients who suffered from serious health problems and severe chronic illnesses in largest number of cases (60.8%

* lovorka.nikolic@farmalogist.rs

and 69.6%) visited the pharmacy in order to get their prescription medicines. Analysis determined that persons who suffered from serious health problems and severe chronic illnesses where in 57% of cases over 65 years of age, hence the most common reason for their visits was as anticipated.

In view of patient age structure, oscillations to general average answers were most noticeable with persons younger than 30 and over 65 years of age. Patients younger than 30 in 26.6% of cases visited the pharmacy for health problem reasons.

Patients over 65 stated obtaining of prescription medicine (59.2%) as the most common reason for visiting the pharmacy which was expected, hence these patients were the majority of those who suffered from serious health problems and severe chronic illnesses.

Related to respondent education level there were no significant oscillations in percentage of patients visiting the pharmacy for health problem reason.

To the question "Which health professional do you first consult when facing a minor health problem", the majority of respondents, 65.3%, stated that they first consult the pharmacist, while 34.7% stated that they first consult their doctor/dentist.

Healthy persons (74.4%), persons with minor health problems (69.8%) and those who don`t check their health status (81.6%) first consult the pharmacist when facing a minor health problem.

Patients younger than 65 in a larger number of cases consulted a pharmacist when having a minor health problem. Patients over 65 years of age were in 67.3% of cases persons who suffered from serious health problems and severe chronic illnesses, who because of possible deterioration of health status or comorbidity in 54.2% of cases consulted their doctor when facing a minor health problem. Furthermore these patients visit their doctors once per month to receive prescriptions for their regular therapy and use this opportunity for physician counseling (Nikolic et al., 2014).

The responses to the question "Do you visit your doctor after pharmacist counseling regarding the same health problem" were analyzed on the answers of those who said that they first consult the pharmacist when facing a minor health problem. Results have shown that 81% of them don`t consult their doctor related to same health problem.

The patient health structure analysis determined that persons who suffered from serious health problems and severe chronic illnesses in a lager degree, in relation to average question response, subsequently consulted their doctor. The stated patient group visits their doctor once monthly in order to get their regular monthly therapy prescription and uses these visits for counseling (Nikolic et al., 2014).

Patients younger than 45 do not consult the doctor after pharmacist counseling in 85.6% of cases, while in 31.7% of patients over 65 years of age subsequently consult their doctor, which was expected bearing in mind their health status.

Results analysis to question "When you hear in a commercial "please consult your doctor or pharmacist on indications, safety precautions and adverse reactions related to medicine or medical device", who do you consult" showed that 61.6% of patients consulted the pharmacist, 15.5% the doctor and 22.9% of patients didn`t consult neither the doctor nor pharmacist.

Responses from persons suffering from serious health problems and severe chronic differed from those of general average, hence they in 22.1% and 24.8% of cases respectively consulted their doctor after watching the advertisement. An interesting fact is that persons with serious health problems consulted the pharmacist in 64.4% of cases after watching the commercial. The category of patients who don`t check their health status especially stands out. They don`t consult any health professional after watching the commercial in 49.3% of cases.

Conclusion

The majority of the respondents recognize the importance of the counseling role of the pharmacist. Healthy persons, persons with minor health problems, persons who don`t check their health status and those younger than 65 years of age visit the pharmacy for counseling on their health issues in over the average number of cases. Persons under 45 years of age don't consult their GP after consulting the pharmacist regarding the same health problem in a high percentage.

The majority of patients on indications, safety precautions and adverse reactions consult their pharmacist after watching a commercial.

This study has shown that the role of pharmacist in primary healthcare is reflected in education on prevention, solving of minor ailments and advising on therapy for patients who are younger, those who consider themselves healthy or are with minor ailments while for patients who are older, with serious or chronic illnesses the primary role of the pharmacist is in therapy dispensing, advising on proper use and monitoring of therapy outcome. This survey has shown that persons who don`t check their health status see the pharmacy as the place of their first choice.

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Macedonian pharmaceutical bulletin, **62** (suppl) 45 - 46 (2016)
ISSN 1409 - 8695
UDC: 615:004.738.5(4)
Short communication

Internet and computer use amongst European pharmacy students

SelenYeğeneoğlu[1]*, Bilge Sozen Sahne[1], Daisy Volmer[2], Afonso Cavaco[3], Maarten Postma[4]

[1] *Hacettepe University Faculty of Pharmacy, Department of Pharmacy Management, 06100 Sıhhiye Ankara-Turkey*
[2] *University of Tartu Department of Pharmacy, Tartu-Estonia*
[3] *Universidade de LisboaFaculdade de Farmacia & iMed. Lisboa, Lisboa, Portugal*
[4] *University of Groningen Faculty of Medical Sciences, Department of Pharmacoepidemiology and Pharmacoeconomics, Groningen Research Institute of Pharmacy, Groningen-The Netherlands*

Introduction

Information technologies are accepted as ubiquitous resources for social and professional information. In particular, Internet use has become important to most daily activities, from work and entertainment to education and participation in society. Also, statistics show that ¾ of all European citizens (EU-27) is using Internet, with Central and Nordic countries as the most active and Southern countries comparably less active (Seybert, 2012). Internet communication is preferred due to speed and ease in many services and activities (Komerik, 2005). With younger generations being the most intense users, students present an interesting group for studying Internet use and potential misuse.

Social networks, online gambling, shopping, banking, watching TV series/movies, and education resources, are known to be the most popular reasons for using Internet among university students (Miller et al., 2010; Turkish Statistical Institute, 2012; Wang et al., 2011; Yılmaz, 2012).

However, no comparative analysis on Internet use has been done so far on pharmaceutical students, which present a rather homogeneous group, with curricula that are equivalent or similar in most European countries (PHARMINE Consortium, 2011). Knowing some differences between Southern and Northern European countries, a comparitive analysis focusing on both differences and similarities between countries and regions seems worthwhile. The present study aims to describe Internet use by pharmacy undergraduates as well as to establish an initial comparison of this use amongst European countries.

Materials and methods

This study is descriptive using a questionnaire-based survey. Pharmacy Schools from Northern, Central and Southern Europe participated. A total of 748 4th year university students from Estonia, Latvia, the Netherlands, Portugal and Turkey were invited to participate.

Notably, included centers were: Ankara, Gazi, Hacettepe Universities; the University of Lisbon; the University of Groningen; the University of Tartu; and Universities of Riga Stradinš and Latvia.

The questionnaire aimed to gather information regarding students' socio-demographics, health status, and Internet use and potential misuse. The questionnaire was pre-tested in each partner country, assuring that no linguistic issues remained. The participants filled out a paper-based questionnaire in the presence of a local coordinator.

Data gathering and structuring was completed for all countries in December 2012. SPSS v15.0 was used for data entry, merging and qualitative and quantitative analysis. Pearson Chi-Square, ANOVA and linear correlations, were applied using the significance level at 0.05.

* selen.yegen@gmail.com

Results and discussion

Out of 748 graduate students, 654 students (87.4%) participated in the study. The majority of the participants were from Turkey (44.5%) and Portugal (32.6%). In all countries, the majority of the students were females.

Students' Internet use is presented in Table 1. Latvian students used Internet at school in a significantly lower frequency (43.1%) than students from all other countries. For all countries, students used Internet more outside (e.g. at home) than inside the school, except in the Netherlands. Turkish students reported less frequent use of Internet at home than rest students in other countries ($p<0.001$). The frequency of Internet access via smart or cell phone was less than 19.0% in Latvia, presenting the lowest frequency of all countries investigated here ($p<0.001$) compared to all other studied countries.

The most often reported purposes for using Internet were communication, academic work, social networking, listening to music and watching movies. Dutch students used Internet for social networking and listening to music more frequently than the others and this difference was statistically significant ($p=0.016$ and $p=0.002$, respectively). Portuguese students used Internet for both watching movies and shopping less frequently than all the others ($p=0.002$ and $p<0.001$, respectively). Internet use for chatting was more popular among the Estonian students than among other pharmacy students ($p<0.001$).

The results of this study provide valuable evidence on how Internet is being used by pharmacy students, recognizing its true potential and actual use in this group in higher education (Garrison and Kanuka, 2004).

This study aimed to look at a coherent educational cohort (i.e. 4th year pharmacy students) between 5 countries: 2 Mediterranean, 1 Central European and 2 Baltic nations. There was a good participation rate (near 90%).

The pharmaceutical profession in Europe is predominantly a female occupation (FIP, 2012; Ruiz et al., 2006). And this predominance was also found in all the surveyed countries.

Beyond the demographic apparent homogeneity, computer and Internet use varied significantly between studied countries, with a lower overall use for the Turkish participants. This lower use can be explained by relatively low computer penetration rates at home in Turkey among the 5five countries (Turkish Statistical Institute, 2012).

Internet use for communication and educational purposes was found not to differ from country to country. This suggests a common electronic ground for studying pharmacy, although information sources may vary. More importantly, results confirmed the central role of computers and Internet for communication and performing academic work. All countries presented high percentages for those activities, which have a clear implication on the development of teaching and learning. Educators should not neglect the progressive, but continuously, swift to online education, while keeping the right balance between media-based/distant and face-to-face teaching (Ruiz et al., 2006).

Social networks have emerged as important tools for maintaining and improving social capital (Johnston et al., 2013; Miller et al., 2010). Although statistically significant differences were found in this sample, high use of social networks was observed in all countries. This issue may take a lot time and interrupt academic working and success. Also social isolation may occur because of intense use of Internet social networking. This can lead the teaching staff to remember their roles and responsibilities: the emergence of an "e-professionalism" concept, the legal and ethical implications of online postings in students' educational decisions, how online personas may blend into professional life – all these factors increase educators' role demands (Cain, 2008).

Conclusion

According to the results, Internet and computer use amongst pharmacy students during their education seems to be equivalent amongst culturally diverse countries.

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Macedonian pharmaceutical bulletin, **62** (suppl) 47 - 48 (2016)
ISSN 1409 - 8695
UDC: 615.2:614.39(497.7)
Short communication

Activities of Macedonian Agency of medicines and medical devices in the improvement of rational use of medicines

Merjem Hadjihamza* Marija Darkovska Serafimovska, Mirjana Donceva

Agency for Medicines and Medical Devices, "St.Cyril and Mehtodius" no.54, 1000 Skopje, Republic of Macedonia

Introduction

The enormous development of new medicines and their permanent introduction in the pharmaceutical market as well as permanent raising of medicines consumption on global level are very challenging and they are not so easy to face with. In particular it is not so easy for developing countries which have low health budgets and rationality in managing this budget is of strategic importance. Most countries are interested to enable access to medicines for their population but at the same time they must establish policies that will promote their rational use.

In Republic of Macedonia the improvement of rational use of medicines is a part of the Health strategy and the National strategy for medicines. Considering the above mentioned findings and being aware that the trend of increasing medicines consumption often caused by their irrational use may affect the public health, the last few years the health authorities in the country, including all stakeholders and the medical experts, have conducted activities at all levels of health care in order to improve the rational use of medicines and public health as well. These activities were preceded by a situational analysis that detected and located the causes of irrational use of medicines, after which Action plan at government level (AP, 2014) were approved. These activities included the educational, social and regulatory dimension of the issue.

In this regard, the Agency of medicines and medical devices conducted inspections in community pharmacies throughout the country.

Objectives

The main purpose of this activity was to check whether the regime of medicines dispensing is in compliance with the legal provisions (LP, 2016) and according to the approved action plan to share educative materials regarding medicines use intended for patients.

Methods

In the first half of 2014 (from January to June) 880 community pharmacies across the country were inspected. Target pharmacotherapeutic groups of medicines to be checked were: antibiotics, anxiolytics, sedatives and antidepressants. The inspection was repeated in the second half of 2014 and the results evaluated.

Results and discussion

Inspection conducted in the first half of 2014 (from January to June) in 880 pharmacies state wide showed that a significant amount of the medicines that belong to above mentioned therapeutic groups were being dispensed without a prescription. These findings were very worrying. The highest consumption was marked in anxiolytics, more precisely medicines that belong to the group of benzodiazepine derivates, followed by sedatives and antidepressants. At the third place were antibiotics (all medicines were for oral use).

The medicines that lead regarding antibiotic consumption are combination of penicillins' including beta lactamase inhibitors more precisely the combination of amoxicillin and clavulanic acid, followed by cephalosporin of first generation (cephalexin) than amoxicillin and ciprofloxacin.

* merjem.haxhihamza@ malmed.gov.mk

What were the most often causes stated by the pharmacies for these worrying data?

The pressure from the patients who demanded to buy the medicine because:

- The elder cannot visit their doctors to take prescriptions for medicines that they use continuously (for example sedatives and anxiolytics)
- People that work, state that they cannot wait to take prescriptions from their doctors, because they know which antibiotic is going to be prescribed to them (most often amoxicillin + clavulanic acid or cephalexin)
- Relatives and friends insisting to get the medicine because they have used it before and got better

During the checkups that have been conducted in the first half of 2014, for all pharmacies where medicines were dispensed without a prescription, a data-record has been written about the established findings and it was pointed out that every pharmacy should work in compliance with the regulatory demands.

Educative brochures regarding rational use of medicines in particular of antibiotics, sedatives and anxiolytics were prepared for patients so they can be informed through their community pharmacies. Considering the low level of health literacy in majority of patients, these brochures contained very clear and simple messages regarding the risks they take by using medicines without medical advice.

During the second half of 2014 Agency of medicines repeated the checkups (from July to December). In most pharmacies it was not recorded at all dispensing of antibiotics, sedatives and anxiolytics without prescription. The comparative analyzes showed the following results: The dispensing of anxiolytics without prescription in particular dispensing of benzodiazepine derivates was decreased for approximately 93% to 95% for all strengths, dispensing sedatives (zolpidem) was decreased for 95% and dispensing of antidepressants (amitriptyline) was decreased for 99.5%. Dispensing without prescription of antibiotics more precisely combination of penicillin's including beta lacatamase inhibitors, (combination of amoxicillin and clavulanic acid 875/125 mg) was decreased for 99% , amoxicillin 500 mg - 97.5% while dispensing cephalosporin's of first generation (cephalexin 500mg) was decreased for 95% which means that conducted comparative analyzes showed significant improvement in proper dispensing of prescription only medicines.

Conclusion

The role of pharmacists is not only dispensing of medicines, the pharmacists are health professionals responsible for improving public health by giving appropriate information to their patients, in order to raise their awareness on the safety risks of improper and irrational use of medicines, educating them and advising on what is best for their well being. The legal provisions must be respected and they are legally binding. The Agency of medicines is very confident that this kind of checkups in community pharmacies should be a continuous process until achieving full implementation of legal provisions. These regulatory and educational activities related to dispensing and rational use of medicines does not mean that the problem of irrational use of medicines is solved once and for all. But on the other hand, exactly these activities are crucial steps in the path of eradication of bad attitude related to medicines use.

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Macedonian pharmaceutical bulletin, **62** (suppl) 49 - 50 (2016)
ISSN 1409 - 8695
UDC: 615.2:659.2(474.5)
Short communication

Sources of medicines information used by Lithuanian community pharmacy patients

Paulius Brazauskas[1], Jurgita Daukšienė[1]*, Romualda Gauryliene[2], Raimondas Radžiūnas[1]

[1] *Lithuanian University of Health sciences, Academy of Medicine, Faculty of Pharmacy, Department of Drug technology and Social pharmacy, Sukilėlių 13, LT-50009 Kaunas, Lithuania*
[2] *Kaunas University of Applied Sciences, Faculty of Medicine, Department of Pharmacy, K. Petrausko g.15, LT-44162 Kaunas, Lithuania*

Introduction

Appropriate and objective information how to make right decision by choosing and using the medication is vitally important (FIP, 2008). The times when health care professionals were expected to take decisions on treatment or new medication without explanation are gone (Coulter, 1999). Nowadays patients access a variety of human, print and electronic sources. It is very important to get know the sources there they pick the information. Given that some of medicine information sources available for the patients could provide misinformation, it is important that patients and health care professionals discuss the medications fully before making decisions. The primary purpose of patient medicines information is to assist the patient and health professional in achieving safe and effective use of medicines. This includes providing information that allows the patient to make an informed decision as to the appropriate selection and use of medicines.

Information about medicines and treatment is available from healthcare professionals, mostly doctors and pharmacists. Other medicine information sources where patient seeks for information are Patient Information Leaflets (PILs), various media sources, etc. Most patients access information from sources that are convenient for them without considering if the information they get is enough confident. It is becoming increasingly important to better understand what places patients report as their sources of drug information (Hamrosi et al., 2014). The aim of the study was to determine the use and perceived reliability of different medication information sources among Lithuanian community pharmacy patients.

Materials and methods

The 385 community pharmacy patients who visited pharmacy to obtain medications or came for consultation in pharmacies in different Lithuanian regions were asked to participate in the study. A method of questionnaire was chosen. The questions about reliability and usability of different medication information sources were included. 60.8% of participants lived in the city (or center of region), 23.6% of participants lived in town and 15.6% lived in village. 73% of respondents were women and 27% were men. The youngest participant in the survey had age of 18 years and the oldest was 84 years. To evaluate results all respondents were categorized in three age groups: 18-40 years old, 41-65 years old and 66-84 years old. The statistical analysis was performed using SPSS (Statistical Package for Social Science) 17.0. Descriptive statistics were calculated to summarize the data. T test and chi-square test were used to analyze differences among groups. Results were considered statistically significant when the p value was less than 0.05.

Results and discussion

All participants were asked about reliability of medical sources that they use and whether these sources are

* jurgita.dauksiene@gmail.com

reliable and confident. Doctor and pharmacist seemed to be most reliable sources among all age groups. Almost all respondents in all age groups (Reliability and usability of both medical information sources were evaluated more than 95% in each age group!) use these sources to receive medical information they require. According to results in the third place little less reliable medical source is pharmacy technician. Mostly it's being used and looks confident for respondents between 66-84 years old. 88.6% of these respondents reported this source as usable and reliable compared to 77.5% in 41-65 years and 81.2% in 18-40 years groups. Patient information leaflet takes fourth place however is the first one among all written medical information sources. Only 50% of respondents in age group between 66 years and 84 years claimed this source as reliable and 25.3% of respondents in same age group reported not using this source of information at all. 84.8% of respondents in 18-40 years age group find this source reliable. Books, family and friends, commercials, magazines, television and internet are less reliable sources to patients. Almost half of all respondents answered they use internet as a source, but information is not reliable. Older patients (age group 66-84) do not use this source to search for medical information. Results of our survey shows that patients are tended to trust healthcare professionals and identifies them as their first choice to receive confident medical information about treatment, taken medications, etc. More than 95% of respondents in all age groups indicate that they use doctors and pharmacists as their sources of medical information and that these sources are reliable. Both sources give accurate and constructive information targeted to patient, his condition and his needs in medical treatment.

Survey revealed that among healthcare professionals pharmacy technicians are to be treated positively due to their interactions with patients. 88.6% of respondents in age group between 66-84 years reported this source as reliable. There are not many studies where importance of their role in consulting patients is investigated however we assume that this specialist plays an important role in providing patient with specific and needed medical information.

Patient information leaflets were indicated as reliable sources. More than 80% of respondents in age groups of 18-40 years and 41-65 years reported to use this source.

Conclusion

Community pharmacy patients reported general practitioner and pharmacist as most reliable and mostly usable sources. Patient information leaflet remains in line with other most usable medical information sources and is number one among all written medical information sources. It is necessary to better adopt patient information leaflet to older patients. As reliability of other written medical information sources is uncertain it is necessary to overview and to control this information.

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Macedonian pharmaceutical bulletin, **62** (suppl) 51 - 52 (2016)
ISSN 1409 - 8695
UDC: 615.214(496.5)"2006/2015"
Short communication

Characterization of typical and atypical antipsychotics use in Albania, 2006-2012

Elda Hoxha[1]*, Vilma Papajani[2]

[1]*Profarma, sh.a, Rr: "Myslym Keta", 1000 Tirana, Albania.*
[2]*University of Medicine, Tirana, Faculty of Pharmacy, Dibra Str., Nr. 371, 1005 Tirana, Albania*

Introduction

Antipsychotics are used to treat psychotic symptoms, agitation and diseases such as schizophrenia and mania (Freudenreich and Goff, 2010).

Nowadays antipsychotics may be classified in two groups, typical and atypical antipsychotics. Typical antipsychotics have been used since the 1950s, while atypical drugs are newer drugs approved for use between 1990 and 2000. Atypical antipsychotics have certain superior therapeutic characteristics compared to typical drugs in terms of efficacy and side effect profile. However, certain clinical trials have failed to prove any superiority in effectiveness. This is because the newer atypical drugs have their own disadvantages, too (Schatzberg et al., 2011).

Atypical drugs are much more expensive than the older typical drugs. Statistics in Albania have revealed that risperidone, an atypical antipsychotic, is among the top ten most expensive drugs (Kola, 2009).

Since their approval, there has been an increase of use of atypical antipsychotics as compared to typical antipsychotic use (Verdoux et al., 2010). Meanwhile, in Albania there are few reports on antipsychotic use. This study's objective is to characterize the use of antipsychotics in Albania.

Materials and methods

Data from National Compulsory Health Care Insurance Fund were used for the study. The Compulsory Healthcare Insurance Fund (the Fund) is a government entity which provides healthcare to insured Albanian citizens and keeps records on medications purchased at community pharmacies by insured citizens (pharmacy claims data). Every citizen who pays a monthly health care contribute to the Fund can purchase medications at a lower price. The total cost of a drug is partially reimbursed by the Fund (reimbursement cost) and partially paid by the patient (patient cost/price). Nationwide antipsychotic pharmacy claims data collected from the Fund from 2006 to 2012 were used for the study.

In the obtained data, antipsychotics were defined by their ATC (Anatomical-Therapeutic-Chemical) code. ATC codes for antipsychotics were used to identify antipsychotics in the pharmacy claims data.

Quantities of antipsychotics were determined as declared in the data, i.e., by their dosage forms, such as capsules, tablets, flacons, ampoules, etc. The amount of typical, atypical and total antipsychotics was calculated for each year of data.

For a more accurate estimation of utilization of antipsychotics, the number of defined daily doses (DDDs) was estimated for each antipsychotic medication. The defined daily dose is the average maintenance dose per day for a drug used for its main indication (World Health Organization, 2003). DDDs were determined using the maintenance daily doses referred to in the British National Formulary. The number of DDDs per 100 000 inhabitants per day was calculated for each antipsychotic every year using the milligrams of antipsychotics utilized. Reimbursement costs in Albanian Lek (ALL) and the mean costs paid by the patient were also estimated. Data were analyzed with Microsoft Excel and SAS statistical software package, version 9.1.

Results and discussion

Typical antipsychotics covered by the Compulsory Healthcare Insurance Fund during 2006-2012 were Chorpromazine, Fluphenazine, Haloperidol, Levomepromazine. Atypical antipsychotics were Clozapine, Olanzapine,

* eldajano@yahoo.com

Risperidone. Lithium is considered as a distinct drug category in the antipsychotics group.

All antipsychotics were available in either one of the oral dosage forms such as capsules, tablets, or oral drops solutions. Fluphenazine and chlorpromazine also existed as solutions for injection of immediate release. Haloperidol and fluphenazine were available also as long-acting solutions for injection.

The purchased antipsychotics totaled 3.36 million in 2006 and increased to 4.78 million in 2012. This is a 42% an increase of total antipsychotic use. Of all antipsychotics, the amount of purchased typical antipsychotics declined gradually from 1.2 million in 2006 to 1 million dosage forms in 2012. The amount of atypical antipsychotics increased from 1.7 million in 2006 to 3.7 million in 2012 (more than doubled). Atypical antipsychotics constituted 60% and 78% of the total amount of purchased antipsychotics in 2006 and 2012, respectively. Quantity of Lithium more than doubled from 362 thousand in 2006 to 729 thousand in 2008.

Numbers of DDDs were estimated for each antipsychotic. Among typical antipsychotics, haloperidol had the highest number of DDDs /day/ 100 000 inhabitants with 40 DDDs in 2006 and rose to 58.8 DDDs in 2012. Chlorpromazine came second behind haloperidol. Other typical antipsychotics use such as fluphenazine, levomepromazine and thioridazine also decreased during 2006-2012. Lithium's number of DDDs increased from 11.5 to 21.9 during the study period.

Among atypical antipsychotics, olanzapine had the greatest increase in use, with a number of DDDs of 18.9 in 2006 and 127 DDDs in 2012. Olanzapine in 2012 had the greatest number of DDDs than any other antipsychotic during the entire 2006-2012 period. Clozapine was among the drugs with the highest number of DDDs during the study period with 32.3 DDDs in 2006 and 57.2 DDDs in 2012. The number of DDDs for risperidone increased also from 28.7 to 46.9 during 2006 - 2012. Number of DDDs for Lithium also doubled during the study period, from 11.5-21.9.

Total reimbursement costs for antipsychotics equaled 252 million ALL in 2006 and 293 million in 2012 (lithium not included). In 2006, the reimbursement costs incurred by atypical and typical antipsychotics were 230 million ALL (91% of total reimbursement costs) and 22 million ALL, respectively. In 2012, the reimbursement costs incurred by atypical and typical antipsychotics were 234 million ALL (80% of the total reimbursement costs) and 59 million ALL, respectively. The reimbursement costs for Lithium were 2.6 million ALL in 2006, and increased to 7.9 million ALL in 2012, a threefold increase during 2006-2012.

For the entire study period, the mean price per typical antipsychotic paid by the patient ranged from 87-105 ALL. This price was on average 5% - 9% of the total typical antipsychotic cost. The mean price per atypical antipsychotic paid by the patient ranged from 815 – 1385 ALL, which on average constituted 11-26% of the atypical antipsychotic total cost during the study period. No distinct trend was noticed for patient costs.

Conclusion

During 2006-2012 there was a 42% increase of total antipsychotic use. While the amount of typical antipsychotics declined, the amount of atypical antipsychotics more than doubled from 2006 to 2012. Among typical antipsychotics, haloperidol had the highest number of DDDs / day/ 100 000 inhabitants, which showed an increase during the study period. Among atypical antipsychotics, olanzapine had the greatest increase in use; its number of DDDs during the study period increased seven-fold. Clozapine was among the atypical drugs with the highest number of DDDs.

Total reimbursement costs increased from 2006 to 2012. There was an increase in the costs of both typical and atypical antipsychotics. Atypical antipsychotics accounted for the majority of reimbursement costs for antipsychotics. Notable is the increase of 2.7 fold of typical antipsychotics reimbursement costs, despite the fact that their use has not changed significantly over the same period. For the entire study period, the mean price per atypical antipsychotic paid by the patient was 10 times higher than the mean price of typical antipsychotics.

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Macedonian pharmaceutical bulletin, **62** (suppl) 53 - 54 (2016)
ISSN 1409 - 8695
UDC: 614.27
Short communication

Developing and implementation of good pharmaceutical practices in a small private pharmacy

Sead Salkoski

Pharmacy Primavita – "Marshal Tito" St. NN, 6300 Struga, Republic of Macedonia

Introduction

Pharmacists and professional healthcare workers face new challenges on daily basis in various aspects in their line of work.

Medication is the most frequent intervention within healthcare systems worldwide. Achieving the best possible outcome of medication for the quality of life of patients should be the primary aim of all health professionals involved in the medication chain, as well as careers and patients depending on their abilities and capacities. Pharmaceutical care is a quality philosophy and working method for professionals within the medication chain. It is indispensable for helping to improve the good and safe use of medicines, thus realizing the full potential of medicines available on the market to achieve the best possible outcome in patients as observed (Cousins et al., 2012).

In the years that have passed since we opened our pharmacy in 2007, we managed to establish work policies while dealing with numerous problems. Even from the beginning, the patients themselves were in the focus of our work due to the fact that it was our firm belief that being professional and honest with our patients will only bring positive feedback on the long run.

In a close collaboration with other professional healthcare personnel, especially the doctors, we created an important bond that has had only positive effects on our patients. Creating trust and a strong belief in our principles was one of the biggest benefits during the first couple of years. Of course, the trust is something we built very carefully and it is long-lasting, and it has to be maintained irrespective of everything.

Throughout the years, as we grew, our main objective was focused on the well-being of the patients. We started with patients with diabetes, making them aware of the importance of eating properly and managing the therapy. We always reminded them how to use and take insulin, or when to take their tablets.

We continued with, perhaps, the most struggling patients – the ones with cancers. We tried to bring some light and positive thinking and, of course, help them with advice on how to improve their immune system and their overall health condition.

The next group of patients with a great need of extra care was the patients with high blood pressure. We are well trained to organize and recognize any drug interactions for this group of patients. Informing them which tablets to drink in the morning and which during the day was the first thing we did for them. Emphasizing the importance of proper dieting and physical activity was the second thing we did, and instructing them how to properly measure their blood pressure was the third. At the end of the day, all of them were happy and pleased with the information and the care they got in our pharmacy.

Materials and methods

The study was conducted by observation of the patient's habits during the months of January to June every year, starting from 2007 to 2014. As shown in another study (Nikolic et al., 2014) 29.09% of patients visit pharmacy seeking advice for minor health problems and 83.5% of patients come to the pharmacy at least once per month. 65% patients said that in case of minor ailments, they consult pharmacist first.

Having this in mind, we took in consideration the number of daily visits by our patients and the time needed for them to be served and answered any questions. The objective was that one patient shouldn't wait more than 5 minutes inside the pharmacy.

* primavita@yahoo.com

Next objective was the total revenue for every fiscal year and the differences between consecutive years in rise of the income parallel to the rise of the number of patients.

Results and discussion

For the first 6 months, we reached an average of 35-40 visits by patients a day. Every patient was treated with great care and we started the implementation of the "Good Pharmaceutical Practice" from the first to the last patient. Very soon, patients started to recognize and appreciate our hard work and constant care. In the next 3 years we reached an average of 80-90 visits per day, in other words, we doubled the frequency of patients visiting and buying different products in our pharmacy.

In 2010 we needed more staff, so we hired our fist pharmaceutical technician. We spent two mounts in training with her so that she could reach out to the patients in the same manner we did. Having a third person working was a big relief for us because we could concentrate more on the patients and on the interaction - "pharmacist-patient". On the long run this brought us more work, meaning more patients. The quality of the service was improved, the waiting period was shortened much more and more patients were visiting our pharmacy. We finished 2010 with an increase in total revenue for 11.6% in comparison to 2009. Compared to 2010, our total revenue in 2011 and 2012 increased 12% and 23% respectively.

We continued with the same trend in 2013. By reaching an average number of 140 visits per day we stayed in line with our goal to become one of the most appreciated and trusted pharmacies in our city. Working in pairs we maintained the waiting time under 4 minutes per patient. In 2014 we averaged 165 visits per day, our goal was to maintain the core stability of our pharmacy and that has always been the patients.

Conclusion

Now, in 2016, having gained a lot of experience, we are ready for challenges. In the middle of this year we plan to create support groups. Those groups should be consisted of struggling patients with the same or similar health conditions that make their life seem unbearable. The concept of support groups is fairly unknown in our city, or maybe even in our country, so we are hoping to bring again some new light in the difficult life of the high-risk patients. We plan to organize meetings with small groups of patients every week. They can share within their own experiences and struggles within the group. By sharing important information they can only help others. This concept, we believe, can have only positive impact in our patients' lives.

We strongly believe that interacting and evolving in some social aspects of people's lives is the future of the modern pharmacist. The continued education and the constant striving for positive changes are our guidelines for the future.

To conclude, providing a high-quality care for our patients and constantly improving their quality of life is a work policy that not only generates immensely satisfied patients, but also generates higher income, which is a result of the increasing number of patients seeking the best possible care, which we, as health workers, constantly provide.

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Macedonian pharmaceutical bulletin, **62** (suppl) 55 - 56 (2016)
ISSN 1409 - 8695
UDC: 615.2:347.77.038
Short communication

The research exemption in Macedonian industrial property law and its effects on the extent of patent protection for drugs

Jadranka Dabovic-Anastasovska[1]*, Nenad Gavrilovic[1], Katerina Ancevska-Netkovska[2]

[1]*Faculty of Law "Iustinianus Primus", University "Ss Cyril and Methodius", blvd. Goce Delcev 9b, 1000 Skopje, Republic of Macedonia*
[2] *Faculty of Pharmacy, University "Ss Cyril and Methodius", blvd. Majka Tereza 47, 1000 Skopje, Republic of Macedonia*

Introduction

Industrial property rights, as is the case with other intellectual property rights, are generally defined in terms of exclusive entitlements granted to the holder of such rights. The exclusivity is granted, in most legal systems, upon a registration of the relevant industrial property right in an administrative procedure. By granting industrial property rights, competent authorities of the state offer certain monopoly which, construed in terms of different legal theories (Dabovic-Anastasovska and Pepeljugoski, 2012), is limited. Legally speaking, the term of the exclusivity granted is limited and, after its expiry, the relevant industrial property right lapses and enters the so called public domain. Such is the case also with patents as industrial property rights. The term of protection is 20 years from the filling of the application for protection (Article 33 of the Agreement on Trade-Related Aspects of Intellectual Property Rights (hereinafter: the TRIPS)). The Republic of Macedonia is a party to the TRIPS and this requirement is implemented by national legislation (Article 74(1) of the Industrial Property Law (hereinafter: the IPL)).

Materials and methods

The IPL recognizes the granting of patents for inventions of drugs, in terms of Article 27(1) of the TRIPS. When a patent for an invention of a drug is granted, its holder is authorized to undertake certain exclusive entitlements and to claim such exclusivity relating to third parties (Article 28 of the TRIPS and Article 89 of the IPL). On the other hand, said exclusivity is not absolute bearing in mind that applicable law may reserve certain entitlements only until the public interest becomes affected. Therefore, the exclusivity granted is limited by applicable law. In terms of Article 30 of the TRIPS, its Members may provide limited exceptions to the exclusive rights conferred by a patent, provided that such exceptions do not unreasonably conflict with a normal exploitation of the patent and do not unreasonably prejudice the legitimate interests of the patent owner, taking account of the legitimate interests of third parties. When patents for inventions of drugs are concerned, third parties interests are converged in terms of the interest of public health.

Article 91(2) of the IPL provides that the exclusive entitlements of the patent holder in terms of the IPL shall not relate to the undertaking activities for research and development of the subject matter of the protected invention, in particular: manufacture, use, offer for sale, export or import of the protected invention, including also the activities for obtaining approval for placing on the market of drugs for human and veterinary medicine or products for protection of plants. This is the case of the so called "Bolar clause", introduced in terms of the TRIPS (Neethu, 2015), which allows for fast introduction of a generic drug after the patent term by permitting technical preparation for registration of the same drug from an alternative source before the patent has expired (Dukes, 2006). Its brooder acceptance came after the 1984 Hatch-Waxman Act (35 U.S. Code § 271(e) (1)) was adopted in the US following the decision in Roche Products v. Bolar Pharmaceutical 733 F.2d 858 (Fed. Cir. 1984) where the court held that Bolar's acts (obtaining the active ingredient from a foreign manufacturer and beginning studies for filing an application for a generic drug before the Food

* jadranka_dab@yahoo.com

and Drug Administration) constitute an infringement of Roche's patent (and not an exception in terms of an experimental use) which had not lapsed yet (Haracoglou, 2008; Kretzschmar, 2014). In the EU, a similar exemption from a patent infringement exists in terms of Article 10(6) of Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use, as amended in 2004 (Barnden, 2008; De Stasio and McClay, 2004), although its scope is under dispute (Cohen and Peirson, 2013).

Results and discussion

The public health argument considers the "Bolar clause" in terms of the right of access to drugs (Van Puymbroeck, 2010). One should, on the other hand, bear in mind the scope of the "Bolar clause" and its scope and possible limitations are dependent on national law. For example, the Court of Justice of the European Union is yet to reach a decision in case C-661/13 Astellas Pharma Inc. v Polpharma SA Pharmaceutical Works on whether EU law considers the research exemptions also in terms of acts by which a third party for purely commercial reasons offers or supplies to a manufacturer of generic medicinal products a patent-protected active substance which that generic pharmaceutical undertaking has planned to use for conducting studies or trials for a marketing authorization under medicinal product law (Ostrowska and Minde, 2014; Straus, 2014). Also, in a dispute before the WTO it was previously found that one may undertake acts generally reserved for the patent holder in terms of obtaining approval for placing on the market of drugs but one cannot undertake activities of stockpiling patented goods during a certain period before the patent expires (Canada – Patent Protection of Pharmaceutical Products, WT/DS 114/R, March 17, 2000).

Conclusion

A "Bolar clause"-like exception to patent infringement, as regulated by the IPL, can therefore be deemed as generally consistent with the TRIPS. On the other hand, one may also find that the wording of Article 91(2) of the IPL is broader than EU law, while also taking into account the ambiguities of the latter. The IPL, in those terms, seems to be enacted in the spirit of the 1984 Hatch-Waxman Act as it expressly covers the acts of manufacture, offer for sale, export or import. Here one may even dispute the alignment with the TRIPS. Although this may prove to be inadequate when legal rules are concerned, the Macedonian pharmaceutical market will probably not be largely affected by it. Truth be told, the mainstream pharmaceutical sector will function notwithstanding the provisions of the IPL. In any case, intentionally or not, the wording of the "Bolar clause"-like exception of the IPL may be treated as an argument for the need of a clarified and more developed EU range rule, as the Republic of Macedonia is a candidate country for membership in the Union.

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Macedonian pharmaceutical bulletin, **62** (suppl) 57 - 58 (2016)
ISSN 1409 - 8695
UDC: 615.2:339.562(497.7)
Short communication

Parallel pharmaceutical trade in Macedonia – pros and cons

Goran Koevski[1]*, Jadranka Dabovik Anastasovska

Faculty of Law "Iustinianus Primus", University "Ss Cyril and Methodius", blvd. Goce Delcev 9b, 1000 Skopje, Republic of Macedonia

Introduction

As from beginning of 2012 a legal frame for parallel trade (imports) of medicines was introduced in the Republic of Macedonia. Since this regulatory reform raised a lot of practical dilemmas our aim is to clarify some of them. Parallel imports are legitimate goods that are placed into circulation in one market and then imported into a second market without the authorization of the patent holder, but, at the same time, allow existence of competition for a drug which is still under patent protection (Religioni and Czerw, 2012). They are neither generic versions of a brand name drug, nor are pirated copies that form part of the "black market" (Brougher, 2014). They have the same active ingredient, in the same amount and the same dosage form as the locally sourced drugs.

The driving force for parallel trade is the price difference between the source (exporting) and the destination (importing) country. This kind of arbitrage has been for decades legally practiced in the Europe Union (EU) under the common norms of the primary EU law, in particular European competition law on free movement of goods (Articles 34, 101 and 102 of the Treaty on the Functioning of the European Union - TFEU) and represent instrument for creating competition for any medicine during its patent life (Miyase, 2011).

Parallel trade in the European pharmaceutical sector is widespread. In its 2009 Final Report following its inquiry into the pharmaceuticals sector, the European Commission (EC) noted that the turnover of parallel traders was between EUR 3.5 billion and EUR 5 billion in Europe, that is, between 2% and 3% of the overall market (EC report, 2009). Drugs facing competition from parallel imports are found to have on average 17% to 21% lower prices than they would have had if they had never faced such competition (Granlund and Miyase, 2011). There is a vast body of case law regarding competition law issues in parallel trade of medicines: The Istituto Chemioterapico Italiano S.p.A. and Commercial Solvents Corporation v Commission of the EC; The Bayer AG v Commission of the EC; GlaxoSmithKline Services Unlimited v Commission of the EC; Syfait and others v GlaxoSmithKline; AstraZeneca v Commission of the EC; Hoffmann-La Roche & Co. AG v Commission of the EC; etc. (Jones and Sufrin, 2008).

Parallel imports, nevertheless, might differ from locally-sourced drugs in color, taste or shape. Due to the differences in country-specific labelling requirements or standard package sizes, parallel imports might thus be repackaged or relabeled. The ECJ has also issued decisions related to this issue: Bristol-Myers Squibb; Upjohn; Merck Case; Boehringer Ingelheim I and II Cases. Two recent cases on the repackaging of pharmaceutical goods by parallel importers reconsidered the exhaustion of rights principle in the context of free movement of pharmaceuticals: the Welcome Foundation Ltd v Paranova Pharmazeutika Handels GmbHand Orifarm and Paranova v Merck Sharp and Dohme (Armengod and Baudenbacher, 2009). Parallel imports are inevitably related to the pharmaceutical industry which itself is subject to rigorous patent system (Folland et al., 2013).

Although territorial by nature, patent law is faced with the challenge of protecting and managing patent rights worldwide. Those rights are governed by two main organizations: the World International Patent Organization (WIPO) and the World Trade Organization (WTO). One of the agreements that countries must ratify upon joining the WTO is the Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPS). Parallel imports are allowed under Article 6 of the TRIPS Agreement (Brougher, 2014). The legality of parallel imports steams from the

* gkoevski@gmail.com

territorial exhaustion of intellectual property rights (IPRs). Regional exhaustion applies in the EU, meaning that IPRs are exhausted upon first sale anywhere in the EU (Granlund and Miyase, 2011). On EU level there is continuous struggle between different associations representing interests of various stakeholders.

The European Association of Euro-Pharmaceutical Companies (EAEPC) as professional and representative voice of pharmaceutical parallel distribution in Europe should be mentioned first. Its position is that the despite the specifics of the pharmaceutical market, parallel distribution of pharmaceuticals is a legitimate commercial practice and consistent with the goals of the EU single market (EAEPC, 2016). On the other hand, the European Alliance for Access to Safe Medicines (EAASM) represents the stance that the main academic and policy arguments should not be only driven by the economics of parallel imports, but rather many other issues of equal importance should be considered as well (EAASM, 2014). Hence, pharmaceutical companies should be permitted to implement practices responding to parallel trade as long as these measures are not contrary to the EU competition rules (Forrester and Dawes, 2008). For example, parallel imports may cause negative effects such as: drug shortage in the exporting countries; the drug quality may be damaged due to the transportation, inappropriate storage or repacking; disruption of already established producer – exclusive distributor relations on a particular market; cuts in research and development investments and negative effects in launching new medicines (Monti, 2007; Religioni and Czerw, 2012); consumers might consider parallel imports to be imperfect substitutes for the locally-sourced drugs, increased risks relating to the entry of counterfeit medicines into the legitimate supply chain, etc. (Hawkins, 2011). Thus, practices for gaining exclusivity extensions by pharmaceutical companies should be justified. Those practices are: non-patent exclusivities, combining two or more successful drugs into one tablet and marketing it as a new product, continuation application practice, strategic patenting, etc. (Brougher, 2014; Gupta et al., 2010).

The case of the Republic of Macedonia

As mentioned before the concept of parallel imports in Macedonia was introduced in 2012 by amending the 2007 Law on Drugs and Medical Devices. The parallel trade is not defined by the 2010 Law on Protection of Competition. However, Macedonian competition legislation is fully in line with the EU one, meaning that EU practices are fully effective in national context. Macedonia is member of WTO since April 2003. Up to December 2015, 186 approvals have been issued for parallel imports by the Drug Agency in Macedonia. Turkey emerges as an exclusive exporting country in all imports. Four drug wholesalers are engaged in parallel imports (MALMED, 2016). However, there is still no significant official evaluation as regards the economic effects of these imports on the Macedonian pharmaceutical market.

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Macedonian pharmaceutical bulletin, **62** (suppl) 59 - 60 (2016)
ISSN 1409 - 8695
UDC: 618.19-006.6-052
Short communication

Evaluation of reliability and validity of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) questionnaire (Albanian version) among breast cancer patients from Kosovo

Selveta Shuleta-Qehaja[1]*, Aleksandra Grozdanova[2], Aleksandra Kapedanovska Nestorovska[2], Zorica Serafimoska[2], Ljubica Shuturkova[2], Zoran Sterjev[2]

[1]Kosovo Medicines Agency, Rrethiispitalit (QKUK), 10000 Pristina, Kosovo; 2
[2]University "Ss Cyril and Methodius", Faculty of Pharmacy, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlayet al., 2015).The diagnosis and subsequent treatment of cancer is often associated with considerable psychological and social difficulties for patients (Cankurtanet al., 2008). Quality of life (QoL) has become a part of the evaluation criteria for cancer therapy besides the classical biomedical criteria. It is the most frequently used outcome measures in oncology research (Lee et al., 2000). In Kosovo there is very little information available about the QoL of cancer patients.

The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) is a questionnaire to assess the quality of life in cancer patients. The QLQ-C30 questionnaire has been used worldwide (Aaronson et al., 1993). The Albanian QLQ-C30 version has been translated by EORTC, and to our knowledge this is the first evaluation of the questionnaire.

Materials and methods

A random sample of 62 breast cancer women patients, who were attending the Institute of Oncology of the UCCK were included in the study. The study period was between the end of February 2014 until the middle of May, 2014. The study was approved by ethics committee from University Clinical Centre of Kosovo (UCCK). Patients were informed about the purpose of the study questionnaire and were voluntarily included in the study and gave the verbal informed consent. The personal interview with patients was conducted by the clinician. Obtained results were analyzed with current published literature.

Results and discussion

62 women with breast cancer were interviewed. The patients' mean age was 50.0 (SD 10.9), with a range of 32–80 years. Most of the patients were married (91.9%) and had completed primary or secondary education (71%), while 16.1 % had completed high school/university. Most of patients had undergone chemotherapy (96.8%) and 82.3% of patients had undergone surgical treatment-mastectomy. The disease stage of patients was as following: 11.3% stage 0-I, 30.6% stage II, 58.1% stage III-IV, while 29% had distant metastasis.

Mean scores for the GH/QoL, physical, role, emotional, cognitive and social functioning subscales were above 33 suggesting there were no problems regarding their functioning. Mean scores of symptom scales were less than 66 for almost all symptom scale and/or items suggesting lack of severe symptomatology among patients with exception for financial difficulties with mean score of 92.47 indicating financial problems for women with breast cancer.

Eight subscales achieved the acceptable standard of

* sshuleta@yahoo.com

reliability (≥ 0.70), exception was only the cognitive scale which was 0.54. The highest score of internal consistency was 0.96 for GH/QoL.

The convergent validity testing showed that all correlation coefficients between an item and its own subscale were ≥ 0.40 with the exception of item 5 (self care) with (ρ = -0.22). In Table 3 are presented Spearman's correlation coefficients between each item and its own subscale. The correlation coefficients ranged from -0.22 to 0.98. Item 7 (hobbies/limited leisure activities) had strongest negative correlation with its corresponding role functioning subscale, as well as item 25 (memory difficulties) with its corresponding cognitive functioning subscale. Items 29 (physical condition) and 30 (general QoL) had the strongest positive correlation with their corresponding GH/QoL as well as item 14 (nausea) with its corresponding nausea and vomiting subscale. The results for item discriminant validity were satisfactory, with exception for item 5 (self care) which showed higher correlation with other subscales (role functioning, social functioning, GH/QoL), vomiting and pain than with its corresponding physical functioning.

Generally the QLQ-C30 subscales showed moderate to strong correlation with each other. Exception was a strong relationship between fatigue with GH/QoL (ρ = 0.73), fatigue with physical functioning (ρ = 0.72) and between pain with fatigue (ρ = 0.70).

Findings of known group comparisons according to the disease stage generally showed that patients with advanced stages of breast cancer (stages III-IV) had higher symptomatic scores than those in early stages. However, none of these differences was statistically significant. In terms of education level, patients with high school/university reported higher cognitive functioning compared to other subgroups (p = 0.03), other functional scales differences were not statistically significant. Interestingly, patients with secondary school showed better GH/QoL than patients with high school/university (p = 0.009).

Conclusion

In this initial study the EORTC QLQ-C30 (version 3.0) in Albanian language for breast cancer women patients was found to be reliable and valid. Although the sample size was small and in the study were included only women with breast cancer it is still important for its preliminary information on validity and reliability of the EORTC QLQ-C30 in Albanian language. Studies with larger number of patients, including male patients as well as other groups of cancer patients and test-retest reliability are recommended to assess the results of this initial study.

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Macedonian pharmaceutical bulletin, **62** (suppl) 61 - 62 (2016)
ISSN 1409 - 8695
UDC: 615.15:614.254
Short communication

Additional services as a basis for the concept of pharmaceutical care

Martin Gigovski[1]*, Yiannis E. Polychronakis[2]

[1]*City College, University Of Sheffield 3, LeontosSofou Street, 546 26 Thessaloniki, Greece*
[2]*University of Salford, Salford Business School, Salford, M5 4WT, United Kingdom*

Introduction

The concept of Pharmaceutical Care (PC), perceived as a responsible pharmacist's philosophy, aiming to supply the community with quality medicines and reliable services, (EU, 2012), involves the provision of a complex set of pharmacist's actions with patients and healthcare professionals for the improved patient's health condition (Edmundsa and Calnan, 2001).

Additional services, competent activities provided by pharmacists, represents the basis of the PC (Puspitasari, 2009), where one of the services is counseling, a cognitive ability of pharmacists to transfer valuable information and establish a rationale relationship with their patients. Counseling, combined with other competences produces additional defined services, the Medication Therapy Management (MTM) and Disease management, approaches that are managing the disease for achieving positive outcomes (McGivney, 2007). The significance of MTM lies in the ability of the pharmacist systematically to achieve the desired therapeutic effects, starting from the therapy review, continuing with the patient's personal record, based on a healthcare management plan (APhA and NACDS Foundation, 2008). This is one way how the pharmacists could bring mutual collaboration on a desired level with other healthcare providers, as well. Counseling and MTM enables the determination of the therapy regimes (Al Rahbi, 2013), and playing inevitable role in the health system. Particular important for the health system is the creation of an individualized management programs for elderly patients with chronic diseases, where community pharmacists are considered one of the best healthcare providers, especially for elderly patients with chronic diseases (Nash, 2001).

Research studies have found that in patients, receiving the additional services for their health improvement generates satisfaction with the received attention (Tinelli, et al., 2007). One particular example of successful implementation of additional services is United Kingdom (Paudyal, et al., 2011), where pharmacists deliver The New Medicine Service, Medicine use review and Appliance Use Reviews (Wells et al., 2013), with whom pharmacists treat minor ailments with non-prescription medicines, provide information, guidance and education on patients for self-medication (Anderson, 2000).The significance of the PC is practically shown through the generated savings of 600 million dollars for the compliance and additional 700 million dollars for the improved prescribing (Etemad and Hay, 2003), with only 40 dollars an hour, for what the PC concept is remunerated.

The aim of this study is to evaluate the current practices of pharmacist in Macedonia, regarding the possibilities of future representation of PC through the additional services.

Materials and methods

Quantitative method was used for the data collection method, using anonymous structured closed ended 33 questions questionnaire, divided in several groups. The sampling population was pharmacists working in pharmacies in Macedonia. The questionnaire was constructed and distributed through the web site platform Survey Monkey (Palo Alto, CA, United States). The interest in entering the survey occurred in total number of 85 participants, where from 67 completed the survey. Generated data was analyzed using the statistical program SPSS v. 20 (IBM Corporation, Armonk, New York, US).

The demographic data showed that majority of surveyed were females (83.6%), over 90% between 18-45

* margig33@yahoo.com

years old, with 80% of surveyed with professional experience below 10 years. Majority of participants were from Skopje and South West Macedonia, 53.7% and 34.3% respectively.

Results and discussion

The survey showed that 90% of surveyed are familiar with the PC, expressing at the same time the need to redefine their community roles.Regarding the additional services, 92.5% consider to have the competence to provide additional services, for what they have positive respond to patients request for additional services, equally in patients with acute and chronic diseases, out of which, 85% are asking additional advices. Respectively, 60% of surveyed have the practice to follow up their patient's medical condition, thus in a way they are practicing additional services, with 40% giving an example of a particular service they provide. Majority of those services are advices for improved diet and healthy life style, better therapy management and counselling for possible medicine interactions. Mostly, time spend for additional services is between 5-10 minutes, with only 36% of pharmacists devoting below 5 minutes and in only 9% of cases, between 11-15 minutes.The collaboration of pharmacist with doctors is at only 30%, but with a mood at 70% of surveyed, that interaction to be improved. Almost all surveyed, 98%, have expressed that proper regulation and remuneration by the health system is important for the practical implementation of additional services.According to 55% of the surveyed, all stakeholders can benefit from PC, where pharmacies, could expect high level of loyalty from their patients and improved economic results.

Conclusion

The basis of PC, as a contemporary concept lies in the successful introduction of additional services, for what the pharmacist have the knowledge and capabilities to provide them to their patients. The research found, that majority of surveyed pharmacist are well known with the concept and its essence. Practically, for the patient's interest, they already provide certain services, with which they are helping their patients in therapy management and improved medication results, for what pharmacist are being awarded with high degree of patient's loyalty, which is essential for the functioning of the pharmacies. On the other hand, for the complete introduction of the PC, there is need for a state's support, especially in the part of remuneration with which the pharmacies will be stimulated to deliver even more qualitative approach for their patients and made them economically more stable and independent.

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Macedonian pharmaceutical bulletin, **62** (suppl) 63 - 64 (2016)
ISSN 1409 - 8695
UDC: 615.2:347.77.038(497.7)
Short communication

Protection of public interest in the area of health through compulsory licenses of patents for pharmaceuticals under the Macedonian legislation

Neda Zdraveva[1]*, Valentin Pepljugoski[2]

[1]*Faculty of Law "Iustinianus Primus", University "Ss Cyril and Methodius", blvd. Goce Delcev 9b, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Law, FON University, Str. Kiro Gligorov b.b., 1000 Skopje, Republic of Macedonia*

Introduction

The Macedonian Law on Industrial Property (hereinafter: LIP) in general terms defines the patent as an industrial property right by which inventions are protected (Article 3, par. 1, line 2) and does not restrict patenting pharmaceuticals. In the period for which the patent is granted (20 years, with possibility for additional protection period of up to 5 years for pharmaceuticals) the patent holder enjoys the exclusive rights to use the protected invention in production, marketing goods manufactured as per the protected invention and to dispose the patent. These rights in the same time mean that the patent holder enjoys the right to prohibit third parties to use the patent in production or in trade, including production, offering for sale, export, or import and storage of products for those purposes, without license (LIP, Article 89, par. 1 and 2, in line with Article 27 of the Agreement on Trade Related Aspects of Intellectual Property Rights (hereinafter: TRIPS).

By principle rule, the license is obtained by agreement between the patent holder and the third party (licensee) and the scope of the rights transferred to the licensee is mutually agreed by the parties (Dabovic - Anastasovska, 2009). Exception to this rule exists in the cases of the compulsory license.

Materials and methods

A compulsory license is an authorization granted by competent state body, in Macedonia the court, a third party to use the patented invention without the permission of the patent holder (Dabovic-Anastasovska and Pepeljugoski, 2012). This research examines the national legal system of compulsory licensing in reference to the one established under the TRIPS and how are the main functions of the system enabled.

Results and discussion

The TRIPS does not limit the grounds for granting compulsory licenses; however it provides that countries can only use those grounds which are allowed by their national legislation. The conditions under which a compulsory license is granted are to be regulated in the national legislation in accordance with the TRIPS (Article 31). This possibility is seen as one of the TRIPS' flexibilities (Van Zimmeren and Van Overwalle, 2011)

Countries, including Macedonia, have specified different grounds for issuing compulsory licenses (Article 97 par. 1 and 2) and these include public health reasons. The LIP further details the compulsory license for the needs of public health, specifying in Article 102 par. 1, that the court (that is the curt with jurisdiction for settlement of disputes in the field of industrial property, may grant compulsory patent license i.e. a supplementary protection certificate for the needs of production and sale of pharmaceutical products, where such a product is intended to be exported in the importing countries with problems in public health. The LIP, in this context, defines pharmaceutical product as any product in the pharmaceutical industry, including the medications for human use, which include any substance or mixture of substances designed for treatment or preven-

* n.zdraveva@pf.ukim.edu.mk

tion of human diseases including any substance or mixture of substances which may be applied to people in terms of renewal, improvement, or adjustment to their physiological functions by causing pharmaceutical, immunological, or metabolic effects or by giving the medical diagnosis, including active components and accessories to diagnose outside the alive human body (Article 102, par.3).

When deciding upon issuing compulsory license, as per Article 102, par. 2 of LIP, the court is obliged to take into consideration the WTO General Council Decision of 30 August 2003 on the implementation of par. 6 of the Declaration on the TRIPS agreement and public health of 14 November 2001 (hereinafter: Council Decision and Doha Declaration respectively).

The Doha Declaration in par. 6 recognizes that WTO members with insufficient or no manufacturing capacities in the pharmaceutical sector could face difficulties in making effective use of compulsory licensing under the TRIPS Agreement, and instructs the Council for TRIPS to find an expeditious solution to this problem. Following this the General Council made a Decision, noting that, exceptional circumstances exist justifying waivers from the obligations set out in paragraphs (f) and (h) of Article 31 of the TRIPS with respect to pharmaceutical products, establishes the system for granting compulsory licenses for pharmaceuticals. The use of this special compulsory license requires formal notification to the WTO. There are three types of notification: 1. importing member's one-off general notification of intention to use the Par. 6 System (not required for least-developed country members); 2. importing member's specific notification of the details of the needed pharmaceutical products and other details required under the Par. 6 System; and 3. exporting member's notification of grant of a compulsory licence for export and conditions attached to it. The features of the system have been adequately transposed in LIP (Article 103-115). The Council Decision will be replaced by the amendment to the TRIPS i.e. introduction of Article 31bis, when it is accepted by two thirds of the membership (the deadline is set to 31.12.2107; Macedonia accepted it on 16.03.2016).

Conclusion

As noted by the General Council Chairperson's statement inter alia the established system for compulsory license for pharmaceuticals should be used in good faith to protect public health and, without prejudice to par. 6 of the Decision, not be an instrument to pursue industrial or commercial policy objectives. It is seen that compulsory license may also constitute an important tool to promote competition and increase the affordability of drugs, while ensuring that the patent owner obtains compensation for the use of the invention (Correa, 2000). It is also argued however, that the present system as such creates a series of obstacles preventing effective access to drugs (Gupta, 2010). When assessing the functionality of the system one must take into consideration the opposing views and of the pharmaceutical companies (Reichman, 2009). It is to be noted that, as per available literature none of the SEE countries have granted compulsory licenses for the public health (Mešević, 2014).

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Macedonian pharmaceutical bulletin, **62** (suppl) 65 - 66 (2016)
ISSN 1409 - 8695
UDC: 615.15:614.254
Short communication

Attitudes of pharmacists about professional practice and work with patients in regard to reaching high level of adherence

Milica Zeković[1]*, Dusanka Krajnović[2], Valentina Marinković[2], Tatjana Stojković[2], Ljiljana Tasić[2]

[1]*Center of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, Tadeusa Koscuska 1, 11000 Belgrade, Serbia*
[2]*Department of Social Pharmacy and Pharmaceutical Legislation, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia*

Introduction

Adherence is exceptionally important for efficacy and safety of pharmacotherapy and has significant impact on clinical, economic and humanistic therapy outcomes. Inadequate level of adherence is a widespread and everlasting problem with possible immense and long-term consequences affecting not only patient but also the health care system. For that reason the issue of adherence remains considerable challenge for health care professionals in clinical practice. Community pharmacists, being most accessible to the public, are perfectly positioned to help patients understand the value of their medications, the importance of prescribed regimens and to underline the need for adherence. A survey research has been conducted with an objective to examine attitudes of pharmacists about professional practice and work with patients in regard to reaching high level of adherence.

Materials and methods

The cross-sectional survey has been conducted in April and May 2014 during courses for pharmacists' professional education organized at the Faculty of Pharmacy, University of Belgrade. Validated questionnaire named Scale of general attitudes and beliefs of pharmacists regarding their work with patients was applied. (Jocić and Krajnović, 2014). The questionnaires have been distributed to pharmacists while they were registering for the course and they could submit them at a place specially intended for that purpose all the time while the lectures lasted. Participants completed questionnaires anonymously and all gave their informed consent for taking part in the survey. Statistical analysis has been performed with a software package IBM SPSS Statistics 21. Since it has been determined that distributions of all numerical data deviate from the normal ones, statistically significant difference and correlation has been analyzed by adequate non-parametric methods of inferential statistics (Mann-Whitney test, Kruskal-Wallis ANOVA test, Spearman's rank correlation). Threshold of statistical significance has been set at the conventional level of $p \leq 0.050$ for all analyses. This study has been approved by the Ethics Committee for Clinical Trials of the Faculty of Pharmacy, University of Belgrade.

Results and discussion

A total of 483 questionnaires have been distributed while 447 have been returned. Nevertheless, 14 questionnaires have been excluded since they were partially or inadequately completed, thus the total sample of survey participants in the research represents 433 pharmacists, the response rate 89.64%. There were 312 survey participants (72.05%) who were employed in state owned community pharmacies and 121 participants (27.94%) employed in privately owned ones. The average age of survey participants was 34.7±9.8 years. Although there were predominantly females (374 or 86.37%), pharmacists in this research did not significantly differ regarding age (ZU=-1.19; p=.234), nor the work experience in pharmacies (ZU=-.86; p=.390).

The total of 83.60% of survey participants deemed that patients were interested to be educated about medi-

* zekovicmilica@gmail.com

cines, medical devices or health products which they utilize, while 76.75% of pharmacists claimed that they cooperate with patients in regard to prescribed therapy. One of the pharmacists' responsibilities and an important part of professional activities in a pharmacy is the provision of information and expert advice to public. In accordance with this, 87.43% of survey participants have stated that they always explain patients how to use certain medicine or medical device when dispensing them in a pharmacy, while 83.60% of them claimed to give information regarding health maintenance and improvement on daily basis. Information, valuable pieces of advice and instructions which are given by pharmacists must be relevant, accurate, timely, objective and completely adapted to needs, specific characteristics and state of a particular patient. An effective patient education and counseling process optimizes the chance that patients will make informed decisions, use medications properly, and meet therapeutic goals. In the conducted survey, pharmacists employed in state owned and privately owned pharmacies have not demonstrated significant discrepancies in agreeing with the following statement "Patients understand my instructions as to use of medicines and medical devices" (ZU=-1.37; p=.171). Average survey participant in both groups has answered that he mainly agrees with this claim.

The majority of study participants have demonstrated a relatively high degree of agreement with claims from the questionnaire which relate to the importance of the role of pharmacists as members of a team of health professionals. Actually 94.70% of survey participants agreed with the claim "Patients rely on pharmacists more and more regarding the use of medicines". Statistically higher degree of agreement has been recorded (ZU=-7.91; p<.001) in the group of pharmacists employed in privately owned pharmacies with the claim "Information which I give to patients are of high significance for the therapy outcomes" in comparison to their colleagues from state owned pharmacies, although it should be stressed that average survey participant in both groups has responded that he completely agrees with this claim.

Survey results indicate that pharmacists are aware that successful development of adequate interpersonal relation with patients highly depends on their professional communication skills. Inadequate verbal and/or non-verbal communication, confusing messages, insecurity and lack of consistency all have a negative impact on the quality of pharmaceutical care and can significantly endanger the realization of the desired degree of adherence. Therefore, it is important for pharmacists to realistically evaluate their communication skills and continuously improve them in the course of professional practice. Although most pharmacists have shown a high degree of agreement with the claim "I think that my behavior during our interaction influences the patient motivation" by Spearman's rank correlation it has been determined that with higher age and longer work experience the degree of agreement with the stated claim decreases (ρ=-0.196; p<0.001).

During pharmacist-patient interaction some disagreements or differences in approaches to solving certain situations or problems are inevitable. Researchers have shown that there is a correlation between professionally negative characteristics of pharmacists (such as aggressiveness, insensitiveness, tendency to conflicts) and low level of satisfaction and adherence of the patient (Ranelli, 2000). Survey participants have on average responded that they completely disagree with the claim "I have conflicts with patients on daily basis". Furthermore, pharmacists employed in state and privately owned pharmacies did not differ when agreeing with the claim "I think that I do not have much understanding for patients" (ZU= -.65; p=.513). Average survey participant in both groups has expressed complete disagreement with this claim.

Conclusion

Due to specific competences and unique position of the most accessible health professionals pharmacists have an especially significant role in adherence optimization. They have a professional and ethical obligation to perform expert review of the overall patients' therapy, give information and council, identify potentially existing factors predisposing low adherence level, apply individually tailored strategies for adherence enhancement and continually monitor patient cooperation and therapy outcomes. In order to empower them to successfully fulfill all those responsibilities it is necessary to insist on the development of adherence-related competences within the educational and programs of continual professional training, as well as to evolve consciousness about the significance of that aspect of the therapy management. It is necessary to pay special attention to improvement of communication skills and professional virtues which could contribute to establishment of successful relations with patients based on cooperation, trust and mutual respect.

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Macedonian pharmaceutical bulletin, **62** (suppl) 67 - 68 (2016)
ISSN 1409 - 8695
UDC: 613.88-053.6:614.2-051
Short communication

The role of pharmacists and other health professionals in promotion of reproductive health of young people

Bijana Vasić[1,3]*, Dragana Đuričić[2,3]

[1] *School of Pharmacy and Physiotherapy, Donska 27- 29,11000 Belgrade, Serbia*
[2] *Medigrooup General Hospital, Milutina Milankovica 3, 11070 Belgrade, Serbia*
[3] *Association of pharmacists in education, Mileve Maric Ajnstajn 74/27, 11000 Belgrade, Serbia*

Introduction

Many studies that have been conducted indicate the necessity of educating the youth on reproductive health. Promotion of youth reproductive health includes all measures, activities and efforts of the health service and the entire community to protect, maintain and improve health. Health workers have a special place in preservation of reproductive health.

The aim is to highlight the importance of the role of pharmacists and other health workers in the education of young people about the importance of reproductive health. Based on these methods, opportunities for further development and improvement of health literacy of young people about reproductive health have been analyzed.

Materials and methods

For analysis we used results of survey conducted on 240 students of Pharmacy and Physiotherapy school during school year of 2013/2014 and also surveys that were about knowledge and opinions of youth on reproductive health from project "Health education on reproductive health".

Results and discussion

Young people, through teaching, acquire information and knowledge about reproductive health, as well as the awareness of active participation in decisions related to it. School has to achieve educational and behavioral goals on reproductive health, through the contents of the basic, necessary and optional subjects. Young people learn to analyze information, and based on them develop critical thinking. The conducted study suggests that most students of first and fourth grade of secondary school of Pharmacy and Physiotherapy have basic knowledge of reproductive health and use of emergency contraception (Vasić, 2015). The worst results were in matters related to the efficacy and mechanism of action of emergency contraception. The synergy of the education and the health systems achieves preservation of reproductive health of youth. The role of the carrier of the activities related to the reproductive health of young people is assigned to counselors within the school health centers. Private health centers should be specially engaged in education of youth. Counseling centers perform medical and educational work with young people. The acquisition of knowledge, attitudes and skills of young people on reproductive health is carried out through group work, under special programs. Through multiple levels, in a short period of time and by active learning, training on the topics of risk behavior, lifestyles, is carried out. Preventive counseling activities can be conducted through brochures and manuals. Counseling centers for young people provide a sense of security, privacy and confidentiality. Confidentiality gives a special sense of security. Health workers should communicate with young people and create confidence for the open discussion about problems (Rasević, 2014). The most appropriate form of transfer and acquisition of knowledge represent a group work and active learning methods through the workshop proceedings. The recommendation of the World Health Organization from 2002 "The Community is to promote the value of counseling, parents be encouraged to support the youth and adolescents

* bixy74@gmail.com

to be informed and involved in the activities of the institution".

It is alarming that health workers have the least impact on young people in the preservation of reproductive health. At a low level, there is a family, which should influence the formation of positive attitudes about sexuality. In the project "Health education on reproductive health" of 950 second grade students of secondary schools in Vojvodina (10 high schools), average age of 16,9 years, the most common source of information about sex life is the media in 675 (71.0%) of students and peers-608 (64.0%) of students. Less common sources of information are parents-152 (16.0%) of students, the school-85 (9.0%) of students and health workers-38 (4.0%) of students (Ukropina et al., 2014).

When it comes to students of the first and fourth grade of Pharmacy and Physiotherapy School, knowledge about emergency contraception is satisfying, although there is a small percentage of students who had not heard of it. For information about emergency contraception students of first and fourth grade have the least confidence in health professionals (doctors and pharmacists), and maximum in magazines and friends. There are differences in the responses of first and fourth grades in the awareness of the emergency contraception tablets and the source of the information. 15 first grade students (12.3%) haven't heard about emergency contraception, while the number of informed students received information from various sources: 35 students (28.8%) from the magazine, 39 students (32.6%) from a friend / relatives, 10 students (8.4%) from pharmacists, 6 students (5.32%) from doctors and 15 students (12.6%) from other sources. The fourth graders showed better informed about emergency contraception. 8 students (6.9%) haven't heard of it, and the other students had different sources of information: 50 students from magazines (41.5%), 41 students (33.8%) from a friend / relative, 9 students (7.9%) pharmacists, 7 (5.6%) students from doctors, 5 students (4.6%) from other sources (Vasić, 2015).

The results suggest that we should work on overcoming prejudice and creating communication between students and health workers. Students need to be provided with timely and accurate information about emergency contraception, and the best source for this is health workers. Health workers are in similar position when it comes to sources of information about sex life in the project "Health education on reproductive health." In this sensitive period of maturation young people should be given the correct message, so the responsibility of health workers is high. The results of research conducted in Pharmacies in Belgrade form May to June of 2014 on 60 people of reproductive age (15-49) are similar. A total of 25% of respondents have heard of emergency contraception: 21.3% from friends / relatives, 3.3% from doctors and 30 % from other sources (Milosavljević et al., 2014).

Conclusion

Test results related to reproductive health of young people show the need for further education of young people in relation to reproductive health, better cooperation between young people and health workers and greater involvement of the educational system in preserving the health of young people. Reproductive health of young people is at risk because of lack of education on contraception and sexuality. It is alarming that health workers have the least impact on young people in the use of emergency contraception and preservation of reproductive health. Recognized attitudes and gaps in knowledge of students about emergency contraception impose an obligation on healthcare professionals (doctors and pharmacists) in educating young people about emergency contraception with the aim of preserving and improving reproductive health. Pharmacists are the most accessible health care professionals in the primary health care level, and their role in the education process of youth is very important.

It is necessary that health centers, pharmacies and educational institutions in the municipalities organize public discussions on reproductive health and its preservation. A permanent solution for information, education and promotion of reproductive health and prevention of sexually transmitted diseases and HIV should be found. It is especially important that the socially disadvantaged and marginalized groups are informed as well. At the macro level, the reproductive health of young people should be part of the policy of the whole society in order to prosperity. Joint action of employees in education, health, local government units and families can preserve the health of young people.

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Macedonian pharmaceutical bulletin, **62** (suppl) 69 - 70 (2016)
ISSN 1409 - 8695
UDC: 615.035.7
Short communication

The use of drugs outside of approved application

Svetlana Golocorbin-Kon[1]*, Mladena Lalic-Popovic[1], Nebojsa Pavlovic[1], Maja Đanić [2], Natasa Milosevic[1], Branislava Rakic[1], Momir Mikov[2]

[1]*Department of Pharmacy, Medical faculty, University of Novi Sad, Hajduk Veljkova 3, Novi Sad, Serbia*
[2]*University of Novi Sad, Medical faculty, Department of Pharmacology, Hajduk Veljkova 3, Novi Sad, Serbia*

Using the drugs in compliance with the marketing license specifying the formulation, dosage and age-category, issued by a relevant authority is referred to as *on-label* use. However, the practice of *off-label* prescribing is very common. It implicates the use of the medication in a manner not listed in the approved prescribing guidelines with respect to indications, age-category, dosage regimen or administration route (Christopher, 1993). EMA, FDA. and other national regulatory authorities, as well as the ALIMS are responsible for ensuring the quality, safety and effectiveness of the drug put on the market and its compliance with the approved guidelines. However, such authorities generally do not regulate the application of drug in everyday practice. The doctors are entitled for freedom in prescribing drugs. Such prescription-freedom must be in accordance with fundamental postulates of medicine – doctor's responsibility and care for patient's wellbeing. The physicians must keep in mind their professional liabilities and responsibilities towards relevant national legislation and obey the medical ethical principles. In line with novel scientific accomplishments, the doctors should prescribe an *off-label* drug only if this off-label prescription is the safest and most effective therapeutic option for the patient (Beck, Azari, 1998). The aforementioned facts clearly indicate that the *off-label* practice of drug prescribing is legal and very common in many countries worldwide. Based on the outlooks from the available international literature, it is evident that *off-label* prescribing is legitimate (Killick, Berghe, 2010). The question that inevitably arises is when is the *off-label* prescribing an appropriate approach? Regrettably, a universal answer does not exist. The physicians are entitled and responsible to estimate what could be considered the "appropriate" *off-label* application in each individual case. Contrary to drug prescribing, which is regulated by somewhat more flexible regulations, promotion of drugs is strictly regulated by national laws. The European regulations governing the marketing of medicines do not offer a universal definition for "appropriate application of the *off-label* drugs", yet defining the number of situations where *off-label* prescription is allowed: products currently undergoing clinical trial, exceptions from EU Directive and, *off-label* use under the individual decision of a treating physician while applying appropriate procedure to protect patients' health (Sackett et al., 1996). In the majority of EU member countries, the patient's right is to obtain information about available alternative treatments to that proposed by a treating physician, that is, available *on-label* therapies when a doctor suggested an off-label treatment option. Similar to the neighboring countries, the issue of *off-label* prescription of drugs in Serbia has not been addressed in relevant legislation. Although the *off-label* prescription practice is evident from the data on everyday medical practice, the ALIMS has not yet publicly communicated any official data. Contrary to Great Britain where the registry of *off-label* drugs applied in pediatrics or neonatology is available, neither regulations pointing to the *off-label* drug prescription nor relevant registries of *off-label* medicines exist in our country. Moreover, promotion and advertising of *off-label* medication is not regulated, i.e., directly prohibited by any law or subordinate legislation except for the general prohibition on advertising and promotion of drugs other than OTCs. Advertising of a prescription drug to professional community is allowed under conditions stipulated in the license, and in accordance with the previously approved summary of drug characteristics. One of the major reasons behind *off-label* prescribing by the physicians is the unavailability or lack of licensed, effective and safe therapeutic options for particular conditions and diseases. Sometimes, *off-label* prescribing is the ultimate choice of the doctors, particularly after the ap-

* magistrakon@orcon.net.nz

proved treatment options have failed. The lack of therapeutic alternatives for specific patient populations, often results in *off-label* drug prescribing to unapproved patient population in spite of potential contraindications stated in the SmPC or PIL). Consequently, *off-label* prescription is often practiced in pediatric, geriatric and obstetric practice. Another common reason for prescribing medicines outside the limits of their original license is convincing evidence on their effectiveness and safe application in particular situations. Physicians' decisions on *off-label* prescribing are often justified by the clinical and scientific facts relying on EBM The EBM concept represents "the conscientious, explicit, and judicious use of current best evidence in making decisions about the care of individual patients", that is, "integrating individual clinical expertise with the best available external clinical evidence from systematic research" (Weynantset al., 2010). This very definition of EBM encourages some physicians in prescribing *off-label* drugs, provided that such application is appropriate in given circumstances. The doctor should decide about the best available therapeutic option for the patient while providing patient's consent for its application. *Off-label* drug prescription is highly prevalent in medical practice. Most frequently, the drugs are prescribed outside their licensed indications or to different age categories. The studies revealed that *off-label* prescribing is more common among specialists than in general practitioners. Psychiatric and malignant diseases are often associated with *off-label* prescribing, which is not surprising, having in mind, that unknown etiology and factors influencing disease progression require highly complex approach to the treatment and selection of an effective therapeutic. Application of drugs outside their original license is evident in obstetrics, psychiatry as well as in the treatment of some infectious diseases. *Off-label* prescribing is common in pediatrics practice, which is due to the specific age of the patients. The most commonly prescribed *off-label* drug categories include drugs used in the therapy of cardiovascular diseases, anticonvulsive drugs, antipsychotics, antidepressants and antiasthmatics. Numerous studies conducted in various therapeutic fields, among different age groups and geographic regions indicated that the newborns are the population that is most commonly treated with medications that are beyond the license for this particular age group. According to these studies, the percentage of children who received at least one off-label or unregistered drug range between 36%-92% at pediatric departments, 80%-97% at neonatology department and 11%-37% in primary health care. *Off-label* prescription is highly prevalent in oncology. According to available data, particular antineoplastics are more frequently associated with unlicensed and *off-label* drug prescriptions than with the licensed one. However, the drawbacks of *off-label* prescription practice should not be neglected. Potential problems associated with the *off-label* drug prescription include the following: adverse reactions associated with *off-label* drug prescription, increased responsibilities of health care providers in view of patient wellbeing, impossibility of compensating health care expenses due to application of *off-label* drugs, promotion of *off-label* drugs by the manufacturers. Major drawback of the *off-label* drug application is an increased probability of adverse effects of the drug. It is attributed to the fact that safety and effectiveness of an *off-label* drug have not been confirmed. The, implementation of more precise legislation defining appropriate prescription and application of *off-label* drugs as well as stipulating the responsibilities of all parties participating in such therapeutic approach is highly demanded in our country. Creation and regular update of a registry of *off-label* drugs applied in daily healthcare practice is of vital importance.

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Macedonian pharmaceutical bulletin, **62** (suppl) 71 - 72 (2016)
ISSN 1409 - 8695
UDC: 616.37-006.6-085.277
615.277:616.37-006.6
Short communication

The impact of clinical effectiveness of gemcitabine on quality of life in patients with pancreatic cancer in all stages

Zana Ibraimi[1]*, Ilir Kurtishi[2], Ardiana Murtezani[3], Agim Shehi[4], Edita Alili[5]

[1]*Department of Pharmacy, Faculty of Medicine, University of Prishtina, St."Bulevardi Deshmoret e Kombit"n.n. 10000 Prishtina, Kosovo*
[2]*Institute of Oncology, University Clinical Center of Kosovo, St."Bulevardi Deshmoret e Kombit" n.n. 10000 Prishtina, Kosovo*
[3]*Physical Medicine and Rehabilitation Clinic, University Clinical Center of Kosovo, St."Bulevardi Deshmoret e Kombit" n.n. 10000 Prishtina, Kosovo*
[4]*Institute of Public Health, Faculty of Medicine, University of Tirana, St. "Aleksander Moisiu" no. 80, 1000 Tirana, Albania*
[5]*Department of Pharmacy, Faculty of Medicine, State University of Tetovo, St."29 Nëntori" n.n. 1200 Tetovo, Macedonia*

Introduction

Pancreatic cancer is an aggressive disease which usually causes no symptoms in its early stages, making it difficult to diagnose. Initial symptoms may include severe pain in the back or stomach area, unexpected weight loss, jaundice, feeling sick, diarrhea, weight loss and loss of appetite, which can severely reduce a patient's quality of life (QoL). As such, there are rarely more than a few months between diagnosis and death. In this context of limited survival, quality of life assumes great importance and it`s improvement must be the main treatment goal. Gemcitabine is a chemotherapy treatment that is toxic to cancer cells by stopping a part of the cancer cell replicating itself. It has been considered the standard treatment for locally advanced or metastatic adenocarcinoma of the pancreas, because it has a wider spectrum of antitumor activity due to its different cellular pharmacology and mechanism of action. Gemcitabine is metabolized intracellularly to two active metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). The cytotoxic effects of gemcitabine are exerted through incorporation of dFdCTP into DNA with the assistance of dFdCDP, resulting in inhibition of DNA synthesis. The aim of this study is to assess the clinical effectiveness of gemcitabine on quality of life in patients through different stages of pancreatic cancer.

Materials and methods

Fifty pancreatic cancer patients, suffering from local, advanced or metastatic pancreatic adenocarcinoma, as histologically proven by the Oncological Institute of Kosovo, were recruited in a trial during the period, 2014/2015. They were treated with gemcitabine of 1000 mg/m2 once weekly for seven weeks, followed by one week of rest during the first cycle and subsequently 1000 mg/m2 once weekly for three weeks followed by one week of rest until relapse or intolerable toxicity. The quality of life was measured by the Functional Assessment of Cancer Therapy-Hepatobiliary cancer (liver, bile duct and pancreas) (FACT-Hep) scale. The FACT-Hep contains specific subscales assessing physical well being (seven questions), social/family well being (seven questions), emotional well being (six questions), functional well being (seven questions), and additional concerns (18 questions) (FACT-Hep, 2007). The collected data were analyzed with STATA Version 11 program. Baseline characteristics were analyzed with t-tests for continuous data and Pearson's $\chi 2$ was used for categorical variables. The focus of the analysis was to test whether significant differences existed between the age of patients, components of FACIT, surgery, radiotherapy, stage of cancer, quality of life, and clinical effect of the treatment. A P value of less than 0.05 shows the criterion for statistically significant results.

* zibraimi@yahoo.com

Results and discussion

From 50 patients, 26 (52%) were newly diagnosed and 24 (48%) had prior treatment history. Median age is 62 years (range 18–79 years), 28 males (56%), 22 females (44%). A majority, 34 patients (68%) had stage IV disease at diagnosis, 10 patients (20%) had stage III, and 6 patients (12%) had stage I-II at diagnosis. Only 8 patients (16%) underwent surgery, where 3 (6%) of them had whi pple`spancreaticoduodenectomy, while 5 (10%) had gastroenterostomy. None of them went through radiation therapy and all of them 50 (100%) were treated with chemotherapy –gemicitabine. Overall QOL, FACT-Hep, p-value shows significance of correlation between components of FACIT and stage of tumour. Physical well being shows significant p-value (p=0.0000). Social/family well-being shows significant p-value (p=0.003). Emotional well-being shows significant p-value (p=0.0001). Functional well being shows significant p-value (p=0.0000). Additional well-being shows significant p-value (p=0.0000). All of these scales were significantly associated with survival, after inspecting the effects of stage at diagnosis. On average, about 50% of patients (25) with pancreatic cancer survived beyond 12 months. About 4% (2 patients) lived 3-6 months, 6% (3 patients) 6-9 months, and 34% (17 patients) had a median of survival from 9-12 months. Two patients (4%) are still alive, which means they have survived over 18 months. Median survival time for the entire cohort was 10.3 months (range 1–24 months), p-value shows significance of correlation between survival rate and stage of tumour, respectively (p=0.0003). The best available evidence relating to the use of gemcitabine as a first line therapy has been shown to be well tolerated and to have a mild toxicity profile. Pancreatic cancer is the seventh most common cause of cancer death, according to Institute of Oncology, Kosovo. Three-quarters of deaths are in people over 60 years old. The findings in this study shows that the clinical effect of gemcitabine on the QoL of patients with pancreatic cancer may improve it. This is in accordance with several other studies (Bourgade et al., 2013; Burris et al., 1997; Halm et al., 2000; Kamar et al., 2003; Kuwahara et al., 2012) which shows beneficial effects of gemcitabine on overall QOL and psychological distress.

Conclusion

This study demonstrates that gemcitabine can improve quality of life in advanced pancreatic cancer patients. There is evidence of a very small survival possibility and an improvement in QoL. The QoL satisfaction of patients with pancreatic cancer measured by the FACIT-Hep, provides helpful information and they may have significant implications, as well as relief in getting clinical decisions.

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Macedonian pharmaceutical bulletin, **62** (suppl) 73 - 74 (2016)
ISSN 1409 - 8695
UDC: 615.065(497.2)
Short communication

Ad-hoc comparative analysis of regulatory safety information and web-based data for recombinant medicines for assisted reproduction techniques

Svetoslav Stoev[1]*, Hristina Lebanova[2], Emilia Naseva[3], Vladimir Atanasov[1], Ilko Getov[1]

[1]*Faculty of Pharmacy, Medical University-Sofia, 2"Dunav" Str., 1000 Sofia, Bulgaria*
[2]*Pharmacy College, Medical University- Pleven, 1 "Kliment Ohridski" Str, 5800 Pleven, Bulgaria*
[3]*Faculty of Public Health, Medical University- Sofia , 8 "Byalo more" Str, 1527 Sofia, Bulgaria*

Introduction

Spontaneous reporting of adverse drug reactions (ADR) is one of the most effective ways to monitor the safety profile of a medicinal product during its post-authorization life-cycle. Patients are direct participants in the spontaneous reporting system in Bulgaria since 2012 (Lebanova et al., 2015). However the rate of ADRs reported by patients to the competent authorities is still low. On the background of the significant under-reporting of ADRs through official channels, new medically orientated internet forums have given voice to patients who share information about their therapy, including experiences with adverse events (Ginn et al., 2015 and Lardon et al., 2015). As a result some risks, which could be vital, are being discussed online.

The effectiveness of several different gonadotropin preparations, used for ovarian stimulation of women, undergoing therapy for assisted reproduction technologies (ART) has been widely discussed. Being focused on the comparative effectiveness studies of menotropins and FSH, derived by recombinant technologies, the issue of safety of these preparations is neglected and reduced only to the lack of ovarian hyper stimulation syndrome (OHSS) and multiple pregnancies, which are usually set as safety endpoints in clinical trials (Lebanova et al., 2015). However, as far as the safety monitoring of these medicines is concerned, there is still not enough and reliable post marketing data for the safety profile of stimulation hormones.

Objectives

To compare ADRs of recombinant medicines, used in ART, identified in consumer reviews from online forums and the corresponding data, mentioned in the Summary of Product Characteristics (SmPC) and the data, collected from the Bulgarian Drug Agency reporting system.

Materials and methods

Both Google, social media and selected medical web-based forums search was conducted using the following key words in Bulgarian language: Adverse drug reaction, side effects, assisted reproduction, in vitro fertilization (IVF), ovarian stimulation. In addition a search for the INN and the trade names of medicines from the focus group was performed - Corfollitropin, Follitropinalfa, Follitropin beta, Urofollitropin, Menotropin, human menopausal gonadotropine. It was conducted a systematic review of the SPCs of the medicines, approved by the BDA, current to the date of the consumers' web posts. A leading aim of the current research was harvesting the information from user archives and posts to identify both expected (i.e., mentioned in the SPC) and unexpected ADRs.

As most of the lexicon, used by forum participants, while sharing their experience with investigated medicines in the collected messages, does not correlate with the medicinal terminology, some kind of "reference list" was created for the purpose of this study to refer user content slang to the medical explanation of a clinical condition.

* slavi1702@gmail.com

Results and discussion

The study identified 201 valid reports with resemblance to adverse events, among a sample of 1000 posts collected from 25 specialized forums, posted between November 2013 and May 2015. In comparison, there are no adverse events reported to the BDA for the same period for the studied drugs. Forum discussions, used for extracting the consumer reviews are locked for changes and/or deletion by moderators.

The analysis of the user generated content showed that the majority of reviews and comments, which possibly could contain adverse event information are connected with the application of recombinant gonadotropins. The most often recombinant preparation, being detected in uploaded comments is Follitorpin beta (Puregon®) - 34% of all collected on-line posts, followed by Follitropinalfa (Gonal F®) - 25% of all the messages, concerning stimulation hormones and Corifollitropinalfa (Elonva®), which is less discussed and was found in only 13% of the investigated posts. The rest 28% of the analysed web content, obtained during the study, is connected with on-line discussions concerning purified urinary gonadotropins and are not object to the current article.

An important fact that should be noticed here is the descriptive statistics mentioned above does not claim to reflect significantly the frequency of ADRs, observed in the real clinical practice. Current dissemination of user-related content can only result in conclusions about the frequency of on-line discussion of the mentioned recombinant stimulating hormones, without pointing a tendency of prescribing or valid statements for benefit/risk ratio of these medicinal products. What should be considered here are biases like prescribing preferences of physicians at the time of the on line discussions, social-economic status of the patients, who are authors of the on-line comments, and the attitude of treated women to the Internet as source of medical information.

The analysis of signals, detected in the user comments showed that social media based signals issued potential unexpected adverse events for all of the investigated recombinant medicinal products. Regarding Follitropinalfa, social media users electronically share descriptions of 4 conditions that can be possibly associated with and following the administration of the product: weight gain, ovarian pain, frequent urination and emotional instability. None of this possible ADRs has been listed in the section 4.8. “Adverse Drug Reactions” of the SmPCs, where the following possible adverse events are categorized in accordance to their frequency: ovarian cysts, stomach ache and gastrointestinal disorders, ovarian torsion, thromboembolism, mild to severe hypersensitivity, reactions, including anaphylactic reactions and shock. Comparison of the two lists showed that both internet signals and the SmPC provide information for two potential adverse reactions – headache and the OHSS syndrome.

Consumers, who discuss electronically their experience with Follitropin beta cite 7 conditions, they suffered while using the medicine and can be read in the ADR section of the SmPC– local reactions at the site of injection, OHSS, headache, gastrointestinal disorders, pelvic pain, generalized hypersensitivity reaction, mood swings. In contrast, user-generated web signals offer 4 unexpected conditions, which can be possible adverse reactions: emotional instability, hot flashes, weight gain, and excessive hair growth.

In support of the hypothesis that social media can be a valid source of reliable information about adverse reactions to recombinant gonadotropins, the current study found a significant parity between signals, provided on-line by users and the approved product characteristic. Both sources were found to offer reports for the following conditions, associated with the Corifollitropin administration: headache, dizziness, nausea, back pain, OHSS, pelvic pain, pelvic discomfort, breast tenderness. All the collected user reviews were checked for statements, corresponding with all the rest ADRs, mentioned in the SmPC: hot flushes, abdominal distention, vomiting, diarrhea, constipation, spontaneous abortion, ovarian torsion, adnexa uteri pain, premature ovulation, breast pain, fatigue, injection site hematoma, pain at the injection site, irritability, increased level of Alanine aminotransferase and Aspartate aminotransferase, procedural pain, but none of them were cited during the on-line discussions. Instead there were signals, detected which contribute to the list of conditions, potentially connected with the product by describing palpitation, joint pain and fatigue and sleepiness, which might prove to be unexpected ADRs.

Conclusion

The rate of reporting of ADRs by the official pharmacovigilance channels is still extremely low, especially for hormones, used for ovarian stimulation during fertility therapies. At the same time internet forums and chat rooms can be a source of valuable first-hand information for disorders, resulting from application of hormones mentioned above.

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Macedonian pharmaceutical bulletin, **62** (suppl) 75 - 76 (2016)
ISSN 1409 - 8695
UDC: 615.33(497.115)"2015"
Short communication

Results from PPS of antimicrobial prescribing in University Clinical Center of Kosovo

Denis Raka[1]*, Kreshnik Hoti[1], Naim Morina[1], JetëmiraBytyçi[3], Albiona Rashiti[2], Zana Deva[3], Besa Bahtiri[3], Lul Raka[2,3]

[1]Pharmacy Department, Medical Faculty, University of Prishtina "Hasan Prishtina", St. Deshmoret e Kombit p.n., 10000 Prishtina, Kosovo
[2]National Institute of Public Health of Kosovo, Prishtina, St. Deshmoret e Kombit p.n., 10000 Prishtina, Kosovo
[3]Medical Faculty, University of Prishtina "Hasan Prishtina", St. Deshmoret e Kombit p.n., 10000 Prishtina, Kosovo

Introduction

A major concern of public health worldwide is the emergence of antimicrobial resistance due to inappropriate use of antimicrobials and their impact on increasing morbidity, mortality and costs (Herrera, 2013; Huttner et al., 2013). To combat this phenomenon there is a consensus that coordinated action and measures are needed (Huttner et al., 2013, Maddocks, 2013). A simple, feasible way to provide information on antibiotic use on hospital level is conducting Point Prevalence Surveys (PPS). This survey is a part of a worldwide project on antibiotic surveillance called Global PPS, which will enable to make result comparisons between hospitals in Kosovo and abroad.

The aims of this survey were to monitor patterns of antibiotic use in the hospital and to identify targets for quality improvement within the aim to optimize antimicrobial prescribing for children and adults.

Materials and methods

The survey was implemented in June 2015. The survey included all inpatients (adults, children and neonates admitted on medical, surgical and intensive care units) at 8 o'clock. Detailed data were collected only for patients who were being treated with antibiotic on the day of survey. These data included gender, age, weight (children and neonates), antibiotic used (type, dose, dosage, route of administration), type of treatment (empiric, targeted), reason for treatment (community acquired infection CAI, hospital acquired infection HAI, surgical or medical prophylaxis), use of treatment protocols, reason in notes. Surgical prophylaxis was encoded as single dose, 1 day or more than one day prophylactic prescribing. Since the audit of prophylaxis was conducted for previous 24 hours, surgical departments were not audited on Monday. Data were collected from patient's medical record, including treatment cards, laboratory results and other official medical documents. Daily hospitalizations and outpatients, defined as ambulatory care patients and emergency admissions on the day of the survey were excluded from the survey. Denominator data included all eligible inpatients on the day of survey.

All the data were entered online using Global-PPS web based tool. After that data were validated and analyzed.

Results and discussion

On the day of the survey 1468 beds were available, with an occupancy rate of 63.56%.

The total number of patients included in the survey was 933: 768 (82.31%) adults, 77 (8.26%) pediatric and 88 (9.43%) neonatal patients.

Overall, from the total number of hospitalized patients, 325 (34.8%) patients received at least one antibiotic: adults 32.3%, children 54.5% and neonates 39.8% of respective patients.

Top 3 diagnoses for treatment, excluding prophylactic treatment, NICU (Neonatal Intensive Care Unit) and NMW (Neonatal Medical Ward) patients, were Pneu (25.2%), Bron (15.4%) and TB (13.8%).

The majority of patients received parenteral antibiotics (89.2%). 26.3% of patients had multiple antibiotic diagnosis and 26.7% were identified as multiple antibiotic patients.

95% of patients that received antibiotics were treated

* denisraka@hotmail.com

empirically.

The most prescribed group of antibiotics in UCCK were other beta-lactam antibiotics with 51.8% (ATC J01D), followed by aminoglycosides 15.1% (ATC J01G) and other antibiotics 14.4% (ATC J01X).

Overall 32 different types of antibiotics were prescribed; cefazolin was the most prescribed one (21.9%), followed by ceftriaxone (16.0%) and gentamicin (7.8%). Analysis of quality indicators of antibiotic prescribing for adults and children in our survey showed that there were no treatment protocols and no stop/review dates documented. On the other hand, reason in notes was documented in most of antibiotic prescriptions.

Community acquired infections (CAI) constituted 85.3% of antibiotic prescriptions, whereas hospital acquired infections (HAI), 14.7%. Prophylactic treatment was more pronounced in surgical prophylaxis 74.7%, in comparison with medical prophylaxis 25.3%.

Our survey showed that in UCCK duration of prophylactic treatment was more than 1 day in all cases.

Antibiotic use among children is considerably higher than 36.7% result from the ARPEC PPS study of 2012 (Verspoten et al., 2016) and results from the Antibiotic Resistance and Prescribing in European Children Point Prevalence Survey (ARPEC-PPS, 2011) conducted in European (35.4; 95% CI: 33.6-37.2%) and non-European hospitals (43.8%; 95%CI: 41.3-46.3%) (Versporten et al., 2013).

What stands out from our results is high percentage of antibiotics used for the treatment of tuberculosis (13.8%), which suggests high prevalence of tuberculosis in Kosovo and it is a concerning fact for our public health system.

Parenteral antibiotic administration was significantly greater than European results from (ECDC, 2013; ESAC, 2009) where parenteral prescription for adults and children was 70.6% (country range: 47.8-91.4%).

Empiric prescription of antibiotics was unacceptably high. Reasons for this may be various, i.e. Clinical Microbiology Laboratory is a part of Public Health Institute and not within the hospital, and another contributing factor may be the lack of sufficient collaboration between clinicists and microbiologists. Antibiotic prescription based on susceptibility testing is considered as an important factor in raising the success rate of treatment as well as in shortening the time of hospital stay.

In Europe prescribing patterns are characterized by very common use of the combination penicillins, mainly co-amoxiclav (11% of all antimicrobial agents) in 79.2% of 17 European Hospitals (2000 - 2005) (Zarb et al., 2011).

Our survey showed that cephalosporin's were the most used group of antibiotics in UCCK, which is not in line with recommendations of WHO. High level of cefazolin prescription, an older 1st generation cephalosporin, deviates from the recommendation on ceftriaxone use from the cephalosporin group.

Results show quite high aminoglycoside prescription (15.1%). Actual recommendations on their prescription suggest to be as restrictive as possible and in strict therapeutic monitoring (Avent et al., 2011).

Overall, it is noted that antibiotic prescription patterns in UCCK are in contradiction with approved WHO recommendations, which recommend prescription of narrow spectrum antibiotics, targeted therapy and implementation of restrictive policies (WHO, 2011).

Also, of great concern is the fact that medical and surgical prophylactic use of antibiotics in UCCK is more than 1 day in all recorded cases, which suggest the need for establishing proper prophylactic protocols.

Conclusion

Antibiotic prescription in UCCK hospitalized patients deviates from rational prescribing standards. Data from this survey will be an important tool in identifying goals for quality improvement in UCCK and Kosovo and to support preparation of guidelines and protocols for prudent use of antibiotics.

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Macedonian pharmaceutical bulletin, **62** (suppl) 77 - 78 (2016)
ISSN 1409 - 8695
UDC: 615.33(497.115)
Short communication

Antibiotic prescribing in regional hospital Prizren

Denis Raka[1]*, Kreshnik Hoti[1], Naim Morina[1], Jetëmira Bytyçi[3], Albiona Rashiti[2], Zana Deva[3], Besa Bahtiri[3], Lul Raka[2,3]

[1]Pharmacy Department, Medical Faculty, University of Prishtina "Hasan Prishtina", St. Bulevardi Dëshmorët e Kombit p.n., Prishtina, Kosovo
[2]National Institute of Public Health of Kosovo, St. Bulevardi Dëshmorët e Kombit p.n., Prishtina, Kosovo
[3]Medical Faculty, University of Prishtina "Hasan Prishtina", St. Bulevardi Dëshmorët e Kombit p.n., Prishtina, Kosovo

Introduction

Antimicrobial resistance is a major public health problem and continuing progress in the treatment of many infections is threatened by the growing resistance of pathogens to antimicrobial agents (Versporten et al., 2016). Judicious use of antibiotics is essential to slow the emergence of antibiotic resistance in bacteria and extend the useful lifetime of effective antibiotics (Goossens, 2009).

The importance of surveillance data with regard to antimicrobial use and resistance at local and national levels and the role of antibiotic stewardship is emphasized by the 2011 European Commission Action Plan against rising threats from antimicrobial resistance (COM, 2011).

A simple and feasible way to assess antibiotic consumption is using a point prevalence survey (PPS).

Kosovo is a part of global efforts to combat irrational antibiotic use and accompanying bacterial resistance through the Global PPS program. As part of this national and global program, we conducted the survey in regional hospital Prizren.

The study aimed to describe antibiotic prescribing practices among hospitalized patients in regional hospital (RH) in Prizren, among adults, children and neonates and to identify targets for improvement of the quality of antibiotic prescribing.

Materials and methods

The survey was implemented in June 2015. The survey included all inpatients from all wards admitted at 8 o'clock. Detailed data were collected only for patients who were being treated with antibiotic on the day of survey. These data included gender, age, weight (children and neonates), antibiotic used (type, dose, dosage, route of administration), type of treatment (empiric, targeted), reason for treatment (community acquired infection CAI, hospital acquired infection HAI, surgical or medical prophylaxis), use of treatment protocols, reason in notes. Surgical prophylaxis was encoded as single dose, 1 day or more than one day prophylactic prescribing. Since the audit of prophylaxis was conducted for previous 24 hours, surgical departments were not audited on Monday. Data were collected from official patient's medical record. Daily hospitalizations and outpatients, defined as ambulatory care patients and emergency admissions on the day of the survey were excluded from the survey. Denominator data included all eligible inpatients on the day of survey. All the data were entered online using Global-PPS web based tool, after what data were validated and analyzed.

Results and discussion

Total number of patients included in the survey was 232. 47% of them received an antibiotic, 200 adults (43% treated with antibiotics), 18 children (83.3% treated with antibiotics) and 14 neonates (57.1% treated with antibiotics). Two most frequent diagnoses for treatment with antibiotics were ENT (ear-nose-throat infections) and TB (tuberculosis) with 17.7% each. Parenteral antibiotic prescription was 95.8% in total. Empiric prescribing was dominant (91.3%) in comparison to targeted treatment. Most prescribed groups of antibiotics in RH Prizren were other beta-lactams 57.1% (ATC J01D), followed by aminoglycosides 18.3% (ATC J01G) and penicillins 15.9% (ATC

* denisraka@hotmail.com

J01C). Top 3 AB prescribed were ceftriaxone 42.9%, gentamicin 9.3% and ampicillin 8%. Investigation of quality indicators for antibiotic prescribing in adults and children in RH Prizren showed a consistent lack of treatment protocols and documentation of stop/review date of antibiotic treatment. Reason for treatment was documented in almost all cases. Community acquired infections were the main reason for treatment (93.9%); health care infections (6.1%). 71.2% of prophylactic prescribing was for surgical prophylaxis, and 28.8% for medical prophylaxis. In terms of duration of prophylactic treatment, all patients were treated for more than one day. Regional hospital Prizren is a secondary type hospital and one of the largest in Kosovo.

Antibiotic use in pediatric (83.3%), neonatal (57.1%) and in adult (43%) patients is very high, when compared with numbers from ECDC report, according to which 35% of patients received at least one antibiotic (country range 21.4%–54.7%) (ECDC, 2013), results from ARPEC PPS from 2012, according to which the mean antibiotic use in European children was 36.7% (Verspoten et. al, 2016) and results from (ARPEC-PPS, 2011) conducted in European (35.4; 95% CI: 33.6-37.2%) and non-European hospitals (43.8%; 95%CI: 41.3-46.3%) (Versporten et al.,2013). Antibiotic use in children hospitalized in RH Prizren is significantly higher than in European hospitals, especially in medical wards (83.3% vs. 39.5%, according to ARPEC-PPS 2012). What stands out in RH Prizren is the top place for tuberculosis treatment, alongside ear-nose-throat infections, which is quite concerning.

When compared to European hospitals, RH Prizren has a very high percentage of parenteral antibiotic prescription (95.8%). Studies conducted in Europe show that parenteral prescription in adults and children is 70.6% (country range: 47.8-91.4%) (ECDC, 2013; ESAC, 2009).

Total empiric prescribing was very high resulting from different reasons common in Kosovo's health system, such as low capacities of clinical microbiology laboratories, lack of collaboration between clinicians and microbiologists, scarce funding of laboratories etc. As prescription based on susceptibility testing is one of the cornerstones of prudent antibiotic use we recommend urgent measures to improve this anomaly.

Excessive use of ceftriaxone (42.9%) is an indicator of a irrational antibiotic prescription which needs to be addressed, due to impact on triggering bacterial resistance, particularly for ESBL strains (extended spectrum beta-lactamase) (Skrlin et al., 2011). Results show high rate of aminoglycoside prescribing, which are the second most prescribed group with 15.1%. Actual recommendations recommend that prescription of this group of antibiotics be as restrictive as possible and in strict therapeutic monitoring. Narrow spectrum antibiotics (benzylpenicillin, erythromycin, azithromycin, clarithromycin), targeted treatment and implementation of restrictive policies on antibiotic prescription, recommended by WHO, are not applied in RH Prizren. The lack of treatment protocols and documentation of stop/review date greatly impacts the quality of antibiotic prescribing, so we recommend urgent measures to correct these quality indicators.

Conclusion

Antibiotic prescribing in RH Prizren is not according to standards. Data gathered from this survey will help to identify targets to improve the quality of antibiotic prescription within the hospital.

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Macedonian pharmaceutical bulletin, **62** (suppl) 79 - 80 (2016)
ISSN 1409 - 8695
UDC: 615.357.37(497.731)"2011/2014"
Short communication

Analysis of consumption of insulin in the municipality of Stip from 2011 to 2014

Dijana Atanasova*, Aleksandra Petrova, Elena Drakalska, Marija Atanasova, Bistra Angelovska

Facutly of Medical Sciences, University "GoceDelcev"- Stip, KrsteMisirkov 10-A, 2000 Stip, R. Macedonia

Introduction

Diabetes mellitus is a syndrome characterized by chronic hyperglycemia and disorder of the metabolism of carbohydrates, protein and fat associated with a relative or absolute lack of insulin secretion and insulin action. Diabetes is one of the most common endocrine disorders, with a tendency of increased growth. It is a consequence of modern lifestyles and the increasing number of internal, genetically conditioned and external etiological triggers. Diabetes is not only medical but also economic and social problem (Brunton, 2008).

Diabetes mellitus - type 1 is a disease of the young people - children, adolescents and adults. Insulin is part of their daily treatment because without it the outcome is fatal (Haycox, 2004; Heise et al., 2004).

Diabetes mellitus - type 2 covers 90% of all cases, 90% of insulin therapy, while the rest use the pill therapy. Average daily dose of insulin is about 40 IE (International Units) (Alberti and Zimmet, 1998).

The optimal insulin therapy should induce physiological insulin secretion. Insulin analogues have different absorption, distribution, metabolism and elimination in terms of human insulin. This enables the analogues to get approximately closer to the human insulin (Nathan et al., 2009).

Insulin that are required for patients in the Municipality of Stip are obtained and issued by the hospital pharmacy at PHI Clinical Hospital Stip. Until 2012 year they were obtained under the Law on public procurement, through tenders organized by the hospital. Since 2012, insulins strips to measure blood sugar, insulin needles, pens and kits are obtained through centralized procurement by the Ministry of Health.

The purpose of this paper is to realize the changes in the variety and number of consumed units of insulin PHI Clinical Hospital Stip given the type of mostly used insulin per producer, strips to measure blood sugar, insulin needles, pens and whales in Stipdiabetes center in the period from 2011 to 2014.

Materials and methods

The research and evaluation of the spent insulin's is based on the data of the consumption of insulin in the period from 2011 to 2014 received from the Hospital pharmacy in PHI Clinical Hospital Stip.

The data is arranged by type and origin of insulin preparations, summed by year and made a comparison of consumption and variety. The results are expressed in number of packages.

Descriptive method was used in data processing.

Results and discussion

According to data from the Diabetes Centre in Stip, insulin's consumption in 2011, 2012, 2013 and 2014 continues to grow. The hospital has purchased various types of insulin, according to the events, and generics manufacturer.

Insulin consumption in 2011 was 34932 insulins units from different manufacturers with different types of action, with 16 trade names. Over the coming years, the consumption increased. In 2012 was 36932 packages from 16 trade names, in 2013 increased to 41597 packages from 12 trade names, while in 2014 the consumption of insulin reaches up to 46460 from 11 trade names . The highest consumption in all four years has insulin NovoMix "30" / Flexpen 3 ml / 300, which is a generic Insulin aspart, and belongs to a group of

* dijana.15623@student.ugd.edu.mk

insulin's with average-long acting and it is combined with long-acting insulin's. It's consumption in 2011, 2012, 2013 and 2014 was 9656, 13573, 18426 and 15950 respectively.

Since 2013, there was reduced range of insulins used under the trade name and manufacturer, as a result of the centralized supply of insulin from the Ministry of Health. This measure is taken because of the steady rise in consumption of insulin, which is probably due to the increased number of patients with diabetes, early detection and timely same passage of patients with insulin dependent diabetes treated with insulin. With centralized procurement there is provided lower range of branded insulin, low prices, total costs and reliability in the supply of sufficient quantities to meet the increased needs.

In the research there are processed the spending strips to measure blood sugar, needles and other accessories. In 2012 and 2013, we issued a number of different types of strips to measure blood sugar depending on the type of the glucometar. While in 2014 was issued only one type of strips for measuring blood sugar and it is called Trueressult. In 2012 there are issued 63600 boxes of strips to measure blood sugar, while in 2013 consumption increased by 29% and amounted to 89650. In 2014 there are issued 115620 strips to measure blood sugar levels from Trueressult and that consumption increased by 22%. The highest consumption of strips to measure blood sugar levels in 2012 are from Roche Accucheck Nano 1/50 and it was 28250.

Consumption needle apparatus for measuring blood sugar in 2011 was 67711. In 2012 the use of the needles increased by 16.8% and amounted to 81391, in 2013 increased by 22.6% and amounted to 105178 needles. In 2014 we have reduced the use of needles by about 10% and it amounts to 94639 needles.

The rising use of bands for measuring glucose in the blood and needle apparatus for measuring the sugar is partly due to the growing number of diabetics, and it is a part of a program of measures introduced by the Government of the Republic of Macedonia by introducing centralized procurement of insulin and stripes for measuring blood sugar.

Conclusion

Consumption of insulin in 2011, 2012, 2013 and 2014 PHI Clinical Hospital Stip continues to grow. The highest consumption of fast-acting insulin, while the consumption of insulins intermediate - long action in the last two years completely replaced with insulins medium - long-acting combined with long-acting;

Since 2013, decreased variety of insulins used under the trade name and the manufacturer, which is a result of the centralized supply of insulin from the Ministry of Health

Increasing use of strips to measure blood sugar and needle apparatus for measuring glucose on blood level which is partly due to the growing number of diabetics, part of a program of measures introduced by the Government of the Republic of Macedonia by introducing centralized procurement of insulin strips to measure blood sugar.

By tracking the consumption of insulin improves the basics for quality procurement and reliable supply of quality and reliable insulin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 81 - 82 (2016)
ISSN 1409 - 8695
UDC: 615.035.7(497.11)
Short communication

Medication errors in the health care delivery-a review of the literature

Tatjana Stojkovic[1], Valentina Marinkovic[1], Dusanka Krajnovic[1], Milica Zekovic[2]*, Ljiljana Tasic[1]

[1]*Departmant of Social Pharmacy and Pharmaceutical Legislation, Faculty of Pharmacy, University of Belgrade, 450 VojvodeStepe, 11 000 Belgrade, Serbia*
[2]*Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, 1 TadeusaKoscuska, 11 000 Belgrade, Serbia*

Introduction

Over the last decade, medication errors have become an issue of major concern in health care and a critical component of quality management (Bonnabry et al., 2008). These types of failures may arise at any stage of the health care delivery process, and various policy documents have been published, stressing their frequency and impact. However, despite the growing interest indecreasing the rate of medication errors, a low level of awareness and knowledge has been noticed among health care professionals. Therefore, this paper aims to provide a short review of the concept of medication errors, with a special attention given to their definitions, types and causes, as well as to the current regulatory requirements regarding their reporting and monitoring in the Republic of Serbia.

Material and methods

A comprehensive search of available electronic databases Medline and Web of Science has been carried out from database inception to December 2014. A following combination of key words has been used: (medication errors OR adverse drug events) AND (definitions OR taxonomy); (medication errors OR adverse drug events) AND (incidence OR frequency OR types OR causes OR classification). Additionally, applicable laws and regulations of the Republic of Serbia related to the issue of errors' reporting and monitoring have been examined.

Results and discussion

Patient safety and medication errors represent a significant part of the quality of health care, as a wider concept. A 1999 landmark report "To err is human" brought this issue to the forefront for both professional and general public, stating that "At least 44,000 people, and perhaps as many as 98,000 people, die in hospitals each year as a result of medication error that could have been prevented" (Kohn et al., 1999). In addition to such morbidity and mortality rates, it was estimated that medication errors resulted in considerable health expenditures as well.

Various definitions of medication errors have been identified, such as the one from the National Coordinating Council for Medication Errors Reporting and Prevention (NCCMERP), stating that this type of failures represents "any preventable event that may cause or lead to inappropriate medication use or patient harm while the medication is in the control of the health care professional, patient, or consumer" (NCCMERP, 1998). However, authors have identified heterogeneity of definitions and terminology in this area, which significantly hampers the synthesis of existing scientific knowledge and a comparison of the results. Therefore, it would be necessary to develop an international taxonomy, in order to facilitate errors' reporting and the synthesis of data obtained.

Medication errors may occur at any stage of the medication delivery process, such as prescribing, dispensing and administration. Prescribing errors include prescribing faults, a type of failure which results in an irrational, inappropriate or ineffective prescribing, and prescription errors, as omissions in filling the prescription that results in erroneous specifying one or more data. The

* zekovicmilica@gmail.com

second type of potential failures are dispensing errors, which could be defined as any discrepancy between the medication order and delivery made by pharmacists in a hospital or community pharmacy setting. According to the study results, these errors occur at a rate of 51.5 million out of 3 billion prescriptions filled annually in the United States i.e. 4 per day in a pharmacy filling 250 prescriptions daily, respectively (Bootman et al., 2006), whereas the commonest types are dispensing the wrong medicine, strength, form or quantity, as well as improper labeling. Lastly, administration errors include failures made by nurses during administration of medicines to hospitalized patients, as well as errors made by patients during the use of medicines. This is mostly caused by a low level of adherence, health illiteracy, inadequate way of drug storage and accidental medication mix-ups caused by visually/audio similar names and/or packages. Elderly patients are considered to be particularly vulnerable, owing to the polypharmacy, physical disability and cognitive limitations.

Various critical system elements that may have an impact on medication errors occurrence have been identified, such as incomplete collection and use of demographic and clinical patient data; insufficient health care professionals' knowledge of medications; poor and ineffective communication among health care providers and the absence of collaborative practice; sound-alike/look-alike medications and/or their improper labeling; inadequate working environment such as poor lighting, high noise levels, cluttered work space and work overload; inadequate and/or insufficient health professionals'training about interventions for the safety improvement; the lack of adequate education of patients regarding their own health status and treatment; and finally, the lack of supportive strategies for reporting, analysis and reduction of medication errors in health care facilities (Cohen, 2007).

Nowadays, the underreporting of medication errors has been recognized as a tremendously significant problem. In the Republic of Serbia, this aspect of risk management has been regulated by the Rulebook on indicators of quality of health care (Official Gazette of RS, No 49/2010). All health care providers have been obliged to monitor and report defined indicators, including those related to the patient safety, such as the rate of adverse drug reactions reported, the number of prescriptions with administrative and professional mistake (prescribing faults and prescription errors), as well as the rate of dispensing the wrong drug. However, a significant trend of missing data regarding the patient safety indicators has been noticed in annual reports on improving the quality of health care, due to inadequate reporting. Owning to this fact, the same conclusion that our health care professionals do not realize the importance of failures' monitoring and reporting has been drawn for several years, emphasizing the necessity of raising their awareness related to the significance of recording all failures incurred so as to prevent them from happening again. In addition, it is necessary to conduct research on the types and causes of medication errors, which has not yet been undertaken in Serbia. This may allow the proactive detection of systemic weaknesses and implementation of appropriate risk-reduction strategies.

Conclusion

Raising awareness of the issue of medication errors has resulted in an increase in the number of studies on incidence, types and causes of prescribing, dispensing and administration errors. However, the identified heterogeneity of operational definitions disables the synthesis of existing findings in this area. Furthermore, even though risk management phases, including monitoring and recording of medication errors, have been regulated in many countries, experience from Serbia indicates the necessity of further health care providers' education regarding these activities, since underreporting still remains one of the most significant barriers for the prospective risk management and errors prevention.

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Macedonian pharmaceutical bulletin, **62** (suppl) 83 - 84 (2016)
ISSN 1409 - 8695
UDC: 615.22.035:546.289
Short communication

Analysis of coordination compound of germanium with nicotinic acid as potential cardioprotector

Violetta P. Narokha*, Iryna V. Nizhenkovska, Olena V. Kuznetsova, Olga V. Afanasenko

Bogomolets National Medical University, Department of Pharmaceutical, Biological and Toxicological Chemistry, 01004 Kiev, Pushkinska str. 22, Ukraine

Introduction

Cardiovascular diseases (CVDs) are the foremost health problem in the world today and hold the lead in mortality causes. This is why the search and development of new effective pharmaceutical substances for the protection of cardiovascular system are among the priority directions of scientific research. A lot of drugs that are used for correction of cardiovascular system disturbances can be in certain manner placed among membrane-stabilizing substances that reduce the accumulation of free radicals and reactive oxygen species (ROS) in cells. Among the most attractive are original complex substances of germanium with organic bio ligands that are characterized by wide spectrum of pharmaceutical activity and low toxicity.

Aim

The goal of this work was to determine a potential cardioprotective activity of a new coordinational compound of germanium and nicotinic acid (МІГУ-1) that was synthesized in laboratory of Department of General Chemistry and Polymers of Odessa I.I.Mechnikov National University under the supervision of professor Seifullina I. I. using a model of experimental chronic heart failure (Kresyun et al., 2004).

Materials and methods

Studies were conducted in adult male Wistar rats (weight: 180-220 g). The animals were maintained (including euthanasia) pursuant to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (2016/63/EU) The animals were fed a normal, balanced diet and had free access to water in the animal house (vivarium) of the Bogomolets National Medical University (Kyiv City, Ukraine). Experimetal chronic heart failure (CHF) was induced by intramuscular injection of doxorubicin (DXR) (manufactured by Kyivmedpreparat, OJSC, Ukraine 5 mg/kg once a week for 5 weeks. Investigated compound was administered intraperitoneally daily for 5 weeks. Control group consisted of animals that had solution of nicotinic acid (NA) (niacin, BP, crystal powder (substance), manufactured by Aarti Drugs Ltd, India) administered to them intraperitoneally daily for 5 weeks, 10 mg/kg. Intact animals had physiological solution administered to them intraperitoneally daily for 5 weeks. After the end of experiment the animals were decapitated under light ether anesthesia, followed by extirpation of a heart for biochemical analysis. Index of antioxidant protection system (APS) of a cell, products of peroxide oxidation of lipids (POL) and oxidative modification of proteins (OMP) of cardiocytes were measured using spectrophotonometry and fluorescent methods (Nizhenkovska,2009. Kolesov et al.,1984, Chevari et al.,1985, Dubinina,1995). Amounts of protein in tissue were measured using Lowry method (Lowry et al., 1951). Results were computed using the Student t-test or Mann-Whitney test for distribution-free quantitative sampling (n=10) and estimated with $p<0,05$.

Results and discussion

DXR belongs to anticell antibiotics and is one of the most used drugs during chemotherapy of cancerous growth. On the other hand it is widely known that DXR is characterized by a several negative side effects, first of all – big cardiotoxicity (Klaunig et al., 2010).

* v.narokha@ukr.net

Oxidative stress is caused by an imbalance between excessive ROS formation and decrease in the body's antioxidant defense. ROS play a dual role in a variety of physiologically normal and pathological conditions. In physiological concentrations, ROS transmit signals of external and internal environment of the body through regulatory metabolic cascades, act as mediators and redox messengers in various cellular processes and intracellular signalling systems. At the same time, in certain pathological conditions, excessive ROS accumulation promotes cell death through induction of oxidative damage to cellular macromolecules, such as lipids, proteins and DNA. Increased damage from ROS determines the cell fate through induction of cell cycle arrest and apoptosis. Normal tissues, balancing between synthesis and elimination of ROS, maintain intracellular redox homeostasis (Olson et al.,1981; Saleem et al., 2014).

As opposed to free-radical processes in the body, there is an antioxidant system consisting of a complex network of protective mechanisms for cells, tissues and organs that preserve and maintain body homeostasis. Balance between these two opposing components, in a state of physiological optimum, keeps peroxidation at a certain low level, preventing chain oxidative process, and describes the antioxidant status in the body. One of the ways to prevent oxidative stress is antioxidant system activation, the components of which, in small concentrations, can inhibit the excessive free radical generation.

In this work animals were observed to have an intense formation of ROS that leads to failure of exchange of lipids and proteins of cardiocytes in experimental animals under the chronic DXR intoxication. Administration of NA and МІГУ-1 led to decrease of toxic effect of DXR on heart tissue. It was proven that МІГУ-1 complex has more pronounced antioxidant properties compared to NA.

Conclusions

Results of experiments prove expediency and efficiency of МІГУ-1, member of a new class of chemical compounds – coordination compound of germanium and nicotinic acid, in preventing problems that develop during chronic heart failure.

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Macedonian pharmaceutical bulletin, **62** (suppl) 85 - 86 (2016)
ISSN 1409 - 8695
UDC: 615.03(497.6)
Short communication

Importance of clinical pharmacist in system of health care in Bosnia and Herzegovina

Vedina Čordalija[1]*, Fahir Bečić[2], Tea Mušić-Dreković[3], Esma Karahmet[4]

[1] *Psychiatric hospital of Canton Sarajevo, Nahorevska 248, 71000 Sarajevo, Bosnia and Herzegovina*
[2] *Faculty of pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000Sarajevo, Bosnia and Herzegovina*
[3] *Mobis pharm d.o.o. ,BišćePolje bb, 88000 Mostar, Bosnia and Herzegovina*
[4] *Private pharmacy"Vedypharm", Panjina Kula 78, 71000 Sarajevo, Bosnia and Herzegovina*

Introduction

Clinical pharmacy as defined by European Society of Clinical Pharmacy is a branch specialized for health which includes all activities and services of clinical pharmacists to ensure development and growth of rational, proper and safe administration of drugs (ESCP, 2010).

Our country, Bosnia and Herzegovina, is a member of European Association of Hospital Pharmacists (EAHP), and we also have Hospital Pharmacy section. Needs for health care are growing, as well as the health departments` costs due to demographic changes, aging of population (number of people older than age of 65), needs for more and more resources because of other health services and polytherapy (use of multiple drugs in the therapy). Also, there are appearances of new diseases, growing number of diseases that require long-term and expensive treatments, arrival of modern medical procedures and expensive drugs with progress of technology. That is the reason why the Bosnia and Herzegovina government and the Ministry of health have decided to conducte reforms in health department for reduction of the costs and system sustainability.

Nowadays, clinical pharmacists have more roles than it was in the past. Pharmacists as health care professionals are expected to be more included in process of prescription, drug administration as well as tracking of efficiency and safety of therapy, which means an equal role of pharmacists and physicians in order to achieve a better effect in patient treatment.

One of the modern roles of pharmacist is conductance of rational pharmacotherapy in developed boundaries and pharmaceutical care, focused on direct relation of pharmacist and the patient, meaning an individual approach to each patient, collecting data about drugs used by the patient from the disease history – therapy list, as well as by patinet direct contact.

Individual approach to patient is foundation of rational pharmacotherapy for achieving optimal therapy effect with minimal side effects (Bačić Vrca at al., 2000). Patients` individual and constant medical status tracking (ex. drug concentration in body fluids) (Crnković and Bačić Vrca, 2013), enable determination of eventual interactions and side effects. However, the certain role of hospital pharmacists has not been into the practice as much as it should be in Bosnia and Herzegovina hospitals and clinics.

The purpose of this paper is to show the importance of clinical pharmacist in health care system, including pharmacoeconomic aspects, patient safety and to establish the role of clinical pharmacist in hospital pharmacy in Bosnia and Herzegovina.

Materials and methods

Type of this research is of retrospective and descriptive character. Official reports from Psychiatric hospital of Canton Sarajevo for period of three years (2013, 2014 and 2015), that contain all relevant data about drug consumption and financial reports were used. Those reports were analyzed and obtained results were compared.

* cordalija.vedina@gmail.com

Results and discussion

Drugs in hospital pharmacy of Psychiatric hospital of Canton Sarajevo that have been dispensed from this department to other department in hospital is based on their needs.

Since 2015 we've have new procedures that leads to rationalizing pharmacotherapy. We have noted less drug consumption per patient, thus resulting with lower drug cost.

Pharmacoeconomy analysis, as it was previously mentioned was done for period of three years (2013, 2014 and 2015) and it showed significant reduction in drug usage. Namely, analysis has shown that in 2013 in Psychiatric hospital in Canton of Sarajevo 91.285 BAM (Bosnia-Herzegovina Convertible Mark) were spent, or it was 41% of the budget. The same analysis for 2014 showed that 80.337 BAM were spent or 37% of the budget, while in 2015 in Psychiatric hospital in Canton of Sarajevo 48.880 BAM were spent or 22% of the budget for analyzed period.

Comparison of traditional system of drug distribution with new established system distribution per patient where pharmacist can control the dosage, interval of dosage, clinically significant interaction of drugs etc., resulted with significantly lower usage of drugs expressed in daily defined doses (DDD)/100 thus indicating enhanced quality in drug distribution with new established system. Clinical pharmacists helped to reduce polytherapy, medical mistakes and gives recommendations based on guidelines for engaging on untreated conditions or stoppage of unneeded therapy. The most common mistakes that might happen are drug administration in wrong time, missing out drug dosage and wrong dosage.

Official Gazette of Sarajevo Canton in 2015 mentioned for the first time Decision on the positive, the hospital and in-house compounded list of medicines in Sarajevo Canton, that contained drug distribution in hospital pharmacy and modern role of clinical pharmacist, which means that even cantonal government had accepted the importance of clinical pharmacist in rationalization of pharmacotherapy and reducing the costs for drugs (Official Gazette of Sarajevo Canton 39/2015).

Conclusion

Clinical pharmacists as health care professionals have very important role in system of health care, with their knowledge and skills, thus improving the quality of health care by rationalization of pharmacotherapy leading to reduction of polypragmasy, reduced costs and improved quality of patient's time in hospital.

As it can be seen from the results, new procedures implemented in 2015, have brought positive results in reducing costs for drugs in our hospital pharmacy for nearly 50%, thus pointing to the importance of clinical pharmacists.

We should pay attention that in current sitiation clinical/hospital pharmacist in Bosnia and Herzegovina does not have the role that they should really have in order to work with physician and other health care professionals as a multidisciplinary team. So, the hospital management and Ministry of health should strengthen the importance of role of clinical pharmacist in hospital usage of drugs as well as that head of department of hospital pharmacy should be pharmacist who held specialist degree in clinical/hospital pharmacy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 87 - 88 (2016)
ISSN 1409 - 8695
UDC: 613.84(474.5)
Short communication

Perception about health promotion and smoking cessation counselling among community pharmacists in Lithuania

Jurgita Dauksiene [1,2], Greta Pavasaryte[1], Aurelija Batakyte[1,] Gediminas Daukšys[3], Daisy Volmer[4]

[1] *Lithuanian University of Health sciences, Academy of Medicine, Faculty of Pharmacy, Department of Drug technology and Social pharmacy, Sukilėlių 13, LT-50009 Kaunas Lithuania*
[2] *Kaunas University of Applied Sciences, Faculty of Medicine, Department of Pharmacy, K. Petrausko g.15, LT-44162 Kaunas Lithuania*
[3] *Kaunas University of Applied Engineering Sciences, Faculty of Engineering Science, Tvirtovės al. 35, LT-50155 Kaunas, Lithuania*
[4] *University of Tartu, Faculty of Medicines, Institute of Pharmacy, 1 Nooruse Str., 50411 Tartu, Estonia*

Introduction

Health promotion has been described by the World Health Organization as a process of enabling people to increase control over and improve their health (WHO, 2008). In primary care, community pharmacists are well accessible health care providers who could easily support health promotion initiatives at the individual, community and societal levels. A number of studies in UK, Australia and other countries give evidence that pharmacist interventions are willingly accepted by patients. Tobacco smoking is the main risk factor for several leading causes in death including cancer and heart disease and also the leading cause of early death. Today, community pharmacies provide different extended services connected to health promotion and smoking cessation could be listed as one of these services. It is important to emphasize that counselling about smoking cessation consists of different type of communication: identification of patient´s willingness to quit smoking, evaluation of his/her health condition and providing information about nicotine replacement therapy. Earlier international research has demonstrated that quality of smoking cessation consultation given at community pharmacies could be improved by covering all above listed counselling aspects.

Materials and methods

Cross-sectional survey among community pharmacists and assistant pharmacists was undertaken in January till June 2015. The participants were recruited using universal sampling from six different districts of Lithuania. Questionnaires were distributed to randomly selected community pharmacies and collected one week after the first visit. All pharmacists and assistant pharmacists who worked at the selected pharmacies were asked to fill in the questionnaire. The questionnaire consisted of 26 closed and open questions about health promotion and smoking cessation at community pharmacy and 12 questions about demographics of the respondents. The statistical analysis was performed using Statistical package for Social Science (SPSS v. 17.0). Descriptive statistics were calculated to summarize the data. T test and chi-square test were used to analyze differences among groups. Statistical significance was set at the level $p<0.05$.

Results and discussion

In total there were 450 questionnaires distributed and 352 returned as completed (response rate 78.2%). Majority of the respondents (97.2%) agreed that health promotion is very important to patients and half of them (53.4%) considered that community pharmacies could provide this type of services. Although even 69.3 present

* jurgita.dauksiene@gmail.com

of pharmacy professionals think that smoking cessation information is available for the pharmacy patients in many various resources, but only 31,9 percent of the respondents think that patients who want to quit smoking can do it by themselves without any professional consultation and the only condition for stop smoking is a wish. 73.3 percent of all the respondents agrees that community pharmacy is the place where comes patients with smoking cessation questions, but only 33.3 percent of the respondents strongly agreed with a statement that that "I am a qualified professional who can help people who want to stop smoking" (26.5 percent disagreed with this statement). Most of the pharmacist (85.3 percent) also disagreed with a statement that "It is nothing wrong to smoke few cigarettes per day". Respondents have experienced interest towards smoking cessation consultation one to two times per day and mostly it took up to five minutes to communicate with patient. Despite on 79.8% of the respondents reported having professional experience on smoking cessation consultation, a number of barriers connected with pharmacist (professional competence and communication skills); management of pharmacy (payment for extended services, selection of nicotine replacement products, possibility for private consultation, lack of time for communication with patients) and governmental activities (dissemination of information on tobacco use risks and reimbursement of nicotine replacement therapy) were listed. More frequent information sources about smoking cessation for pharmacists were professional journals and books (76.1%), formal education (university/college) and internet (53.6%) and the information provided from industry of nicotine replacement therapies 4 (2.3%), the patient's motivation (91.1%), pharmacist´s competence (78.6%) and duration of the consultation were named as the main factors for successful communication. Pharmacists who had longer working experience and previous status of smoking were more keen on providing smoking cessation consultation ($p<0.05$). Nicotine gum (94.4%) and nicotine patches (86.3%) were indicated to be the most effective medications for ceasing smoking.

Conclusion

Community pharmacists in Lithuania have positive perception towards health promotion activities at community pharmacy. Most of the respondents declared existing professional knowledge for provision of this type of services. Smoking cessation counseling is everyday practice at community pharmacies in Lithuania. Improving pharmacists´ education in patient counseling and health promotion including smoking cessation would increase quality of extended community pharmacy services in the future.

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Macedonian pharmaceutical bulletin, **62** (suppl) 89 - 90 (2016)
ISSN 1409 - 8695
UDC: 615.035.7-053.2(496.5)
Short communication

ACE inhibitors, calcium antagonists, β– blockers products authorized in Albania and their availability for pediatric groups

Briseida Dosti*, Ledjan Malaj

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Medicine-Tirana, Str. Kongresii Manastirit, No. 133, 1005 Tirana, Albania

Introduction

In order to provide medical assistance to children, physicians often have prescribed unauthorized medicinal products due to lack of suitable and authorized medicine for children (Cuzzolinet al., 2006). This attitude seems to lead to an increased rate of adverse drug reactions and medical errors (Auby, 2008). Moreover, tablets have been authorized for children below the age of 6 years, even though they may be not able to swallow tablets (Cohen et al., 2009). It is very important to study at what rate authorized medicines are really adequate for use in children.

The first objective of this study was to evaluate the number of medicines and active ingredients for pediatric population that are authorized and marketed in Albania, for five selected groups. The second objective was to evaluate the age-appropriateness of the selected medicines evaluated on two aspects: dose capability and suitability of the pharmaceutical form for use in children.

Materials and methods

From database of medicines of Albanian National Agency of Medicines and Medical Devices in Albania, till 10 April 2015, was identifies authorized medicines. For this study were selected the following active ingredients: ACE Inhibitors: Captopril, Ramipril, Enalapril; Calcium antagonists: Nifedipine, Verapamil, Amlodipine; β– Blockers: Propranolol, Carvedilol, Atenolol.

This database doesn't allow extraction of marketed products and active ingredients classified based on age-appropriateness. For this reason were the evaluated Summary of Product Characteristics (SmPC). From SmPCs that were used sources information document, were analyzed sections: pharmaceutical form, therapeutic indications, posology, route of administration, suitability of dosages and pharmaceutical forms for use in children.

A special focus was dedicated to age-appropriateness of selected products. First investigated aspect was if recommended dose is prescribed to children based on classification of pediatric age: preterm newborn infants, term newborn infants, infants and toddlers, children and adolescents. Second investigated aspect was if approved pharmaceutical form, for selected products in this study, were suitable for use in children. For solid forms (such as tablets and capsules) were evaluated presence of score line and possibility of opening or not of contain of capsules and mixing of it with liquid.

Based on all prescribed methodology use for this study were collected these data: authorized indications; authorized age-group; pharmaceutical form; authorized dosage; presence of score line in tablets or film coated tablets; information on possibility for opening capsules contains.

Results and discussion

In total was studies 118 authorized medicinal products in Albania; 5 products containing Captopril, 18 products containing Ramipril, 17 products containing Enalapril, 17 products containing Nifedipine, 9 products containing Verapamil, 19 products containing Amlodipine, 1 product containing Propranolol, 24 products containing Carvedilol and 8 products containing Atenolol.

Evaluation of the SmPCs of products suggesting that only 3 active substances are authorized for use in children. Furthermore, all these products were for solid oral intake forms and all were tablets (n = 118, 100 %). Investigation of the SmPCs for age-appropriateness of these products

* brizidosti@yahoo.com

based on recommended dose and suitability of forms for use in children showed that all products are authorized in following pharmaceutical forms: 78.5 % tablets, 7.4 % coated tablets, 12.4 % modified-release film-coated tablets and 1.7 % film coated tablets. For any active ingredient weren't found authorized liquid formulations, which are more appropriate for use in children.

Evaluation of authorized solid (tablets, film coated tablets, capsules) pharmaceuticals forms for the presence score line, in other to archives smaller dosages shows these results: 40.7 % of medicinal products were without score line (n = 48), 53.4 % have score line in one side of the tablet (n = 63), 3.4 % have score line in both sides (n = 4) and for 2.5 % (n = 3) information wasn't available in checked SmPCs.

Conclusion

This study shows lack of availability of pediatric medicines for selected products and shows that pediatric medicines may not be age-appropriate. Therefore, are needed more efforts to increase the number of drugs authorized for the pediatric groups.

The development of medicines for use in children require that a specific active ingredient need to be available in different dosage forms and strengths. The dose capability was considered an important criteria. A medicine is either dose capable or it is not. However, the suitability of pharmaceutical forms is not as absolute. According to EU reflection paper tablets and capsules are only suitable from the age of 6 years. However, recent studies have shown that small tablets can be swallowed by young children. Also, some capsules can be opened and their content can be mixed with liquids, such as water. This make possible easy intake comparing with other solid forms.

Healthcare personnel's should consider that by using formulation not appropriate for children may cause administration errors, lack of therapeutic compliance and unexpected side effects. In order to reduce the risk of any of below problems, they are encouraged to search between marketed products the most appropriate medicine for treating groups of pediatric population.

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Macedonian pharmaceutical bulletin, **62** (suppl) 91 - 92 (2016)
ISSN 1409 - 8695
UDC: 615.12(474.5)
Short communication

The characteristics of non-chain community pharmacies in Lithuania and their owners' attitude towards professional autonomy

Jurgita Dauksiene[1,2]*, Edita Tiurninaite[1], Edita Kizeviciene[1,2], Aiste Balzekiene[3]

[1] *Lithuanian University of Health sciences, Academy of Medicine, Faculty of Pharmacy, Department of Drug technology and Social pharmacy, Sukilėlių str.13, LT-50009 Kaunas, Lithuania*
[2] *Kaunas University of Applied Sciences, Faculty of Medicine, Department of Pharmacy, K. Petrausko str. 15, LT-44162 Kaunas, Lithuania*
[3]*Kaunas University of Technology, Faculty of Social Sciences, Arts and Humanities, Institute of Public Policy and Administration, A. Mickevičiaus str. 37-1106, LT-44244 Kaunas, Lithuania*

Introduction

In recent years, the number of independently owned pharmacies has declined even as the total number of pharmacies in Lithuania has increased. The studies indicate that current factors affecting public pharmacy are not conducive to independent pharmacies development. Global trend is that that the traditional model of a pharmacist owning his/her own pharmacy is replaced by chains of community pharmacies .With increasing corporate ownership, there is concern that this trend will adversely affect the profession's ability to influence pharmacy practice and practice change. "Dual loyalty" of pharmacists (to the employer and to the patient) is present in all sectors of pharmacy practice. The professional autonomy is defined as "the right and privilege granted by a governmental authority to a class of professionals, and to each licensed individual within that profession, to exercise independent, expert judgment within a legally defined scope of practice, to provide services in the best interests of the client. It is very important to pharmacy practice because of the change in the profession vision: the movement from a mainly medication compounding and supply to a patient-care function is observed. A code of Ethics for Pharmacists regulates the priority of patient need and wellbeing, but corporate ownership can force the pharmacist to the enhancement of supply side" (FIP, 2014). The risks associated with the decrease of health professional autonomy are poorly perceived by consumers, policy makers, health insurance executives, hospital and health care administrators, and many health-care practitioners. The aim of the study is to determine the characteristics of non-chain community pharmacies and to analyze their owners' attitude towards professional autonomy.

Materials and methods

The research was conducted from January to December, 2015. The research projects was divided into 2 stages. In first part the independent non - chain pharmacies were identified and classified, and then only the owners of independent pharmacies who owned 4 or less pharmacies were asked to participate in the survey, using structured questionnaire. Questionnaire included the questions about attitudes towards professional autonomy and characteristics of their pharmacies. The evaluation of professional autonomy consisted of two parts: pharmacist' professional autonomy benefits (1) for society and (2) for the profession of pharmacy. In total there were 121 questionnaires distributed, out of which 102 were returned fully completed (response rate 84.3%). The statistical analysis was conducted using SPSS (Statistical Package for Social Science) 17.0. Descriptive statistics such as frequencies, means and ranges were calculated to summarize the data. T test and chi-square were used to analyze the differences among groups. Results were considered significant when the p value was less than 0.05.

* jurgita.dauksiene@gmail.com

Results and discussion

81.4% of the respondents were women and 91. % of respondents had a Master's degree in Pharmacy. In most cases, respondents indicated that they manage one pharmacy (71.6%). Over half of respondents (56.86%) do not offer additional services in their pharmacies (i.e. blood pressure measurement, the determination of glucose concentration in the blood, etc.). Evaluating their pharmacies respondents provided the following answers – nearby treatment facility (55.89 %), is not far away from a direct competitor (58.82%), is not nearby intensive flow of people (85.29 %), is not a niche pharmacy (77.45%). Individual non-chain pharmacies owners claim highly qualified professionals work in their pharmacies (100%). Respondents strongly agreed and agreed that pharmacists, whose professional autonomy is unrestricted, (1) can meet the patients expectations better (94.12%), can apply professional knowledge more competently (90.20%), can collaborate with other health care professionals more actively (87.25%), contribute to better health care system (92.16%) and (2) have greater public confidence (68.63%), avoid a conflict of interest when they recommend a product to patients (82.35%), ensure pharmacist profession cultural improvement and maintenance of professional honor (92.16%). According to respondents, the main benefits of independent pharmacy are motivated staff (39.31 %) and patient's stability (28.32%). The main difficulties in the management of an independent pharmacy are unequal discounts from wholesale (47.2%) and tax burden (25.6%). 43.14% of respondents hoped that they will be able to keep their pharmacies in the coming 5 years. This study highlights distinct 'independent' expression of professional identity and suggests the need to assess the value of independent community pharmacy as being different from but complementary to the service provided by multiples/large chains.

Conclusion

This study showed that all owners of independent community pharmacy work in their pharmacies as pharmacists or administration staff. Pharmacy owners perceive professional autonomy a high level.. They believe that it has great influence to their professional practice, enhance job satisfaction and bring added value to the patients. Despite all difficulties in the management and tough competition with non-chain pharmacies, the majority of independent community pharmacies owners value the professional autonomy and this encourages them to keep business.

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Macedonian pharmaceutical bulletin, **62** (suppl) 93 - 94 (2016)
ISSN 1409 - 8695
UDC: 616.12-008.64-052:316.728
Short communication

The impact of socio-demographic and lifestyle factors in patients diagnosed with heart failure

Pamela Gruda[1]*, Mihal Tase[2], Mirjeta Beqiri[3], Suela Këlliçi[4]

[1] *Representative Office of Novartis Pharmaceuticals, Rruga "Hoxha Tahsim" No.23, Tirana, Albania*
[2] *Internal Medicine Department, University Hospital Center, Rruga e Dibres 372, Tirana, Albania*
[3] *School of Business Administration, Gonzaga University, 502 E. Boone Ave Spokane, WA 99258-0009*
[4] *Faculty of Pharmacy, University of Medicine, Rruga e Dibres, 371,Tirana, Albania*

Introduction

Heart failure can be defined as an abnormality of cardiac structure or function that results in the inability of the heart to deliver oxygen at a rate sufficient to meet the requirements of metabolizing tissues, despite normal filling pressures (McMurray et al., 2012). Heart failure is a major cause of morbidity and mortality. The aim of this study is to assess the impact of socio-demographic and lifestyle factors in patients diagnosed with heart failure as lifestyle interventions could have substantial power to improve patient's health. The prevalence of heart failure is expected to increase by 25% by 2030 (Heidenreich et al., 2011). Heart failure is a life-threatening disease and it should be considered a global priority. There is little evidence about heart failure patients in Albania, hence the necessity of carrying out such a study.

Materials and methods

We surveyed 200 patients, who were hospitalized at the department of Internal Medicine in QSUT "Nënë Tereza", Tirana during the period from January 2015 until June 2015. The methodology consisted of the use of both primary and secondary data. This study was mostly prospective, but it had some retrospective elements. We used Chi-squared test to find statistically significant correlations between socio-demographic and lifestyle factors with heart failure. When $p<0.05$, the correlation was statistically significant, while when $p<0.1$, the correlation was considered marginally significant.

* pamelagruda18@gmail.com

Results and discussion

In total, 200 patients were included in this study, 160 of them were diagnosed with heart failure. 117 (58.5%) of them answered the questionnaire during their stay in the hospital, while for the other 83 (41.5 %) patients, we obtained their data from their clinical files. From 200 patients included in this study, females represented 52.5% and males represented 47.5%. Meanwhile, among patients diagnosed with heart failure, females and males represented equally 50%. This ratio is in accordance with other studies in the world, where the prevalence of heart failure in females and males is similar. This can be explained with the fact that females live longer than males, even though males have higher incidence than females (Mehta and Cowie, 2006). The mean age of 200 patients was 66.9, while the mean age of patients diagnosed with heart failure was 70.1. 81% patients lived in the city and 19% lived in the village. From 117 patients that were asked about their level of education, 31.6% patients answered that they had finished pre-elementary school or less, 23.1% patients had finished elementary school, 35% had finished high school and 10.3% had been graduated. Furthermore, 53.8% of the patients interviewed indicated their income as low, 45.3% as average and 0.9% as high.

Another question that was asked to patients was if they performed any physical activity; in regards to this question, 41.9 % answered Yes and 58.1 % answered No. To the question if they kept a healthy diet, 44% answered Yes and 56% answered No. Also, patients were asked if they smoke and the responses were as below: 7.7% were active users, 36% used to smoke in the past, but they no longer did and 56.3% had never smoked. With regards to the use of alcohol, 72.6% of the patients answered that they never used

it, 23.9% answered that they used it rarely and 3.4% answered that they used it frequently.

From the Chi-squared test was found a strong correlation between age and heart failure (p=0.001). Heart failure becomes more common with increasing age. This result is consistent with studies in North America and Europe, where few patients with heart failure are 50 years of age or under and more than 80% are 65 years of age or over (Bui et al, 2011) Another strong correlation was also found between physical activity and heart failure (p=0.001). Patients that did physical activity had lower risk to develop heart failure than patients who did not. This correlation has also been found from other studies in the world. Furthermore, physical activity is one of the elements where there can be intervention to prevent heart failure (Young et al., 2014)

Our study didn't find any correlation between income level, smoke, place where patients lived and heart failure (p-values, respectively: 0.262, 0.563 and 0.787). On the other hand, there was a correlation between the level of education and heart failure (p=0.041). Lastly, the results of this study indicated a marginally significant correlation between healthy diet, alcohol use and heart failure (p-values, respectively: 0.084 and 0.068).

Conclusion

Based on our findings, it can be concluded that physical activity and healthy diet should be part of patient's lives, because they have an important role in preventing heart failure. Health professionals should be the ones to promote a healthy lifestyle, which has a direct impact on the development of heart failure and the disease recidivism.

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Macedonian pharmaceutical bulletin, **62** (suppl) 95 - 96 (2016)
ISSN 1409 - 8695
UDC: 628.4.045/.046:615]:502.13(100)
628.4.045/.046:615]:502.13(497.11)
Short communication

The approach to the pharmaceutical waste management in the world and in Serbia

Svetlana Goločorbin-Kon[1]*, Mladena Lalić-Popović[1], Nebojša Pavlović[1], Maja Đanić[2], Jelena Cvejić[1], Velibor Ilić[3], Momir Mikov[2]

[1]*Medical Faculty, Department of Pharmacy, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia*
[2]*Medical Faculty, Department of Pharmacology, toxicology and clinical pharmacology, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia*
[3]*Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia*

Waste represents an enormous loss of resources in the form of both materials and energy. The amount of waste produced can be seen as an indicator of how efficient we are as a society, particularly in relation to our use of natural resources and waste treatment operations. Historically, waste management systems were introduced to protect public health.

In the 1970s and 1980s, waste management systems focused on controlling outlets to air, water and groundwater. In recent years, the focus has been increasingly on utilizing waste as a resource. Even though several measures have been introduced to reduce waste quantity, waste volumes still increase. Environmental pollution by pharmaceutical waste can become a hazardous waste if it is not disposed of in accordance with law and if you are irresponsible and out of control delayed and thrown in the trash container, poured into drains or buried in the ground. Hazardous waste at its origin, composition or concentration of hazardous substances can cause harm to the environment and human health. Considerable concern has been raised regarding potential effects on human health due to presence of pharmaceuticals from environmental to drinking water. Thus, drugs and their degradation products can pollute underground waters or enter the food chain. Some studies show that several common drugs were present in the final effluent in concentrations high enough to potentially affect ecosystems (UK Water Industry Research, 2014). Expired household drugs often end up in wastewaters or in municipal wastes. In accordance with the EU regulations, throwing away unused drugs has been forbidden since 1994 (European Union: European Council Directive 94/67/EC, 1994). Yet, it was established that a third of all sold drugs in Germany, as well as around 25% of those sold in Austria is disposed of with other household waste or ends up in wastewaters (Greiner and Ronnefarhrt, 2003). A survey showed that 17.7% of respondents dispose of the expired and surplus drugs by flushing them down the toilet. More than half the respondents of the USA-made study threw drugs into wastewater, while only 23% of the respondents had returned drugs to the pharmacist (Bound and Voulvoulis, 2005).

Research conducted in other countries has shown that unused drugs are mostly stored in households and that they are most often thrown into municipal waste or spilled into wastewater. All of the previously mentioned facts are important from the aspects of estimate of ways in which drugs get into the environment. Due to the continuous release of active substances, their bioactive metabolites accumulate in the ground and sediments and have an unfavorable impact on natural ecosystem (Tong et al., 2011).

In Serbia, particularly in Novi Sad, most of expired drugs citizens have returned to the special containers placed within the pharmacies of Public Pharmacy Institution of Novi Sad, Autonomous Province of Vojvodina.

At the Public Pharmacy Institution of Novi Sad, was established Pharmacy Waste transfer station in 2011. There were collected and classified drugs from 34 pharmacies that people dispose in containers placed in pharmacies. It can be seen that the percentage of deferred and unused medications is large. Boxes and blisters that make pharmaceutical waste, indicating that some of the prescribed

* magistrakon@orcon.net.nz

drugs, because most of the drugs are from the list of prescription, half- used and unopened. It would be a huge harm that drugs were not used and did not contribute to improving therapeutic outcomes, were not collected as pharmaceutical waste, and accidentally found exposed to the influence of atmospheric factors and reaches the soil or water, which is an immense and immeasurable danger to the environment and for human health.

In addition to the organized collection and classification, pharmacists at Public Pharmacy Institution of Novi Sad organized and proper storage of pharmaceutical waste for transport and destruction are is done by registered companies. This waste with appropriate documentation is exported and destroyed in the neighboring countries that have organized incinerators such as Hungary and Poland.

IPA grants supported transport and destruction of expired drugs stored in Pharmacy Waste transfer station 2011 to 2013. From 2013, more than 5 tones of expired drugs are stored in Pharmacy Waste transfer station and still waiting to be destroyed.

The Law on pharmaceutical waste in Serbia has not regulated who is responsible for payment of transport and destruction of pharmaceutical waste. Most of stored drugs in Pharmacy Waste transfer station in Public Pharmacy Institution of Novi Sad are drugs for cardiovascular diseases (angiotensin converting enzyme inhibitors, diuretics, beta-blockers, calcium channel Blockers), antipsychotics, analgesics (diclofenak), antibiotics, drug for diabetes (metformin, sulfonylureas, insulins), sleeping pills (barbiturates) and some antineoplastics.

The action performed by pharmacists within Public Pharmacy Institution of Novi Sad contributed to the disposal of a part of pharmaceutical waste arising in the territory of Novi Sad and its surroundings, but it is not sufficient, since remained uncovered other possible " producers " of pharmaceutical waste such as hospitals, outpatients department, within the dental, medical centers, institutes, private pharmacies, veterinary clinics and others. It is necessary a large-scale action that will lead to the organized collection of pharmaceutical waste from all sorts of "producers" and then the waste must be classified and kept until destruction.

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Macedonian pharmaceutical bulletin, **62** (suppl) 97 - 98 (2016)
ISSN 1409 - 8695
UDC: 616.379-008.64-085.252.349.7-052-047.22(469)
Short communication

Type 2 diabetes risk assessment in patients of a Portuguese community pharmacy

Esperança Maria Simoes da Silva[1], Maria Margarida Duarte Caramona[2]*

[1]*Communnity pharmacy, Farmácia Rocha, Rua do Brasil,70, 3030-775 Coimbra, Portugal*
[2]*Pharmacology and Pharmaceutical Care Goup, Faculty of Pharmacy, Institute for Biomedical Imaging and Life Sciences (IBILI), University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal*

Introduction

More than 1 million Portuguese have diabetes and of these, about half don't know to have the disease, which progresses silently until appear the first complications.

Given the extent of this problem there is the need to find integrated approaches across society in an effort to prevent and identify individuals at risk.

There is a significant opportunity for a coordinated and synergistic action between pharmacies and primary care regarding the promotion of primary and secondary prevention of diabetes.

Aim

This study aim was to assess the risk of diabetes type 2 in the patients of the community pharmacy and subsequent personalized assistance, in particular the recommendation for the medical consultation of patients with high and very high risk of type 2 diabetes. Has also aimed sensitize the population to the problem of diabetes, inform and educate about the risk factors and promote ways to control them.

Materials and methods

For two weeks between, 14 to 28 November 2015 the pharmacy assessed the risk of type 2 diabetes of its patients. Promoting information and education about the disease, informed about the risk factors and strategies to control, advising also individually each patient to adopt the most appropriate measures, maintaining healthy lifestyles. Prompting the patient for the necessary monitoring, including medical, in situations where there was a greater risk of diabetes.

Thus, all patients over 18 years old, who visited the pharmacy were invited to participate in the study.

It was made a qualitative interview to all patients included in the study, and all data was collected in order to follow the design observational study.

Patients diagnosed with diabetes or taking medications for diabetes and pregnant women were excluded

We applied the questionnaire "Evaluation Sheet Diabetes Risk Type 2" of the Portuguese national program for diabetes, (adapted from the WHO International Diabetes Program, with the international score purpose, DGS 2008) to determine the risk of getting type 2 diabetes within 10 years.

Questionnaire variables were: gender, age, weight and height, waist circumference, anti-hypertensives in taking, family history of diabetes, habits and lifestyles and history of high blood glucose levels.

The questionnaire consists of 8 Likert questions, allowing immediate calculation of the score based on the responses. Since the scale determines the risk of having type 2 diabetes within 10 years. A score less than 7 points, was considered very low risk, the patient was only warned to keep styles and healthy life. A score between 7 and 11 points, was considered low risk, although the risk is slight the patient was warned to continue maintaining healthy lifestyles. A score between 12 and 14 points, was considered moderate risk, the patient should adopt healthy lifestyles and review their risk factors. A score between 15 and 20 points, was considered high risk, the patient should in the next doctor's appointment tell the doctor about his risk

* caramona@ci.uc.pt

of diabetes. Finally a score higher than 20 points, was considered very high risk, the patient was informed that he must consult his doctor and inform it about his risk of diabetes.

In light of this evaluation, each of the assessed patients had specific information about his individual risk and about the needs and behaviors that should adopt regarding the prevention of diabetes. In cases where the risk was highest was recommended seeking medical care.

The risk factors considered for diabetes were: age above 45 years old, overweight (BMI> 25 kgm^2) or obese (BMI> 30Kgm^2), waist circumference more than 94 cm for men or 80 cm in women , sedentary lifestyle (less than 30 minutes a day of physical activity), family history of diabetes in first grade, hypertension or dyslipidemia, previous history of cardiovascular disease, blood glucose abnormalities in fasting and previous impaired glucose tolerance, prior gestational diabetes, and use of drugs predisposing to diabetes.

The data presented after performed the statistics analysis include descriptive information from the patients and the narratives of qualitative data.

Results and discussion

In these two weeks, we included 85 patients. The sample was mostly formed by women, 69.7%. There were 26.7% patients younger than 45 years old, 27% had between 45 and 54 years old and 46.6% were over 55 years old. It was found that 51.1% had a BMI lower than 25kgm^2, but 63.3% of women had a waist circumference exceeding 88 cm and 34.6% of men exceeding 102 cm. As for physical activity 50% of men said to practice at least 30 minutes a day and only 41.6% of women did it. However 93.0% ate any vegetables or fruit every days. About 43% of the sample was taking anti hypertensives. As for family history of diabetes type 1 or type 2, 48.8% of subjects had no history and 48.8% did not report any episodes of high blood sugar in any health examination. However we obtained 18.6% of patients with very low risk, 39.5% with low risk, 20.9% with moderate risk, 18.6% with high risk and 2.3% with very high risk.

This assessment score has potential as a tool to identify a high risk group of DM2/pre-DM2 among community pharmacy patients, especially, when used together with information from health care providers. There is evidence that intensive lifestyle and various pharmacotherapeutic interventions decrease the incidence of DM2 or even can delay the progression of DM2 among persons with prediabetes

Therefore this population mainly formed by women with a sedentary lifestyle and overweight may gain if modify their lifestyles. Since they have non-modifiable factors such as age and gender, pharmacist can help in order to persuade the patient to lose weight and decrease of abdominal circumference. With a non pharmacological intervention this patients can improve their diabetes assessment and also the pharmacist may give a good help to obtain this objective.

Conclusion

The participation of pharmacy, translated into a large number of patients at risk of diabetes evaluated in this initiative is a critical success factor for the demonstration of Pharmacy relevance in the development of new forms of collaboration recoverable by the health system. Besides the contribution of this initiative to combat diabetes, we are also making an important step in the development of differentiating initiatives pharmacy as indispensable to a network of modern health care, efficient and citizen-centered

There was a strong recommendation for medical consultation of patients with high risk and very high of type 2 diabetes and was intended to sensitize the population to the problem of diabetes. Information and education about the risk factors of diabetes is one way to avoid in the future more diabetic patients and to avoid the excessive waste of money with their treatment. Having a better understanding about the disease, the evaluation of the risk factors to prevent the illness and also to control it, have certainly pharmacoeconomical repercussion in the wastes of national health systems economy and mainly advantage for the population that use the pharmacy services and accepts the daily work of the pharmacists.

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Macedonian pharmaceutical bulletin, **62** (suppl) 99 - 100 (2016)
ISSN 1409 - 8695
UDC: 615.33(497.115)
Short communication

A study of the public knowledge of use of antibiotics in Kosovo

Arijana Deshishku[1]*, Merita Berisha[2], Linda Duraku[3], Arjeta Deshishku[4], Bujar Fetahu[5]

[1] *Pharmacy "Reis Pharm-A", Hasan Prishtina Rd., Obiliq , Kosovo*
[2] *Institute of Public Health, Faculty of Medicine, University of Prishtina, Kosovo*
[3] *Pharmacy "Pentagon" Mitrovca - Kosovo*
[4] *Dental Clinic "B.F.", Fushe Kosove - Kosovo*
[5] *Main Center of Family Medicine, Fushe Kosove, Kosovo*

Introduction

Infections posed a serious threat to human life, killing millions of people until the discovery of antibiotics. The term antibiotics, which means "against life", is derived from the fact that an antibacterial drug is extracted from living things and used to kill or attenuate bacteria. However, there is a tight correlation between longer duration and multiple courses of antibiotic uptake and higher rates of bacterial resistance.

In recent decades, the emergence and spread of bacterial resistance to antibiotics is an ongoing threat worldwide, which presents a significant threat to public health globally in the 21st century. The increase of antibiotics resistance will decrease their therapeutic effectiveness, increase treatment failures and, as a result, lead to longer and more severe illness episodes with higher costs and mortality rates.

As more strains of bacteria become resistant to an ever-larger number of antibiotics, our drug choices will become increasingly limited and expensive and, in some cases, nonexistent. If this trend continues unchecked, a wide range of modern medical procedures, from basic dental care to organ transplants, likely would be accompanied by a much greater risk of developing a difficult-to-treat or untreatable antibiotic infection. The safety of many modern medical procedures is dependent on the ability to treat bacterial infections that can arise as post treatment complications.

The aim of this study is to measure the levels of knowledge of the population about the effectiveness of antibiotics and risks associated with their unnecessary use.

Materials and methods

A cross sectional survey using a validated questionnaire, conducted among the general public in Kosovo, during the months of October and November 2014. The English version of the questionnaire from Eurobarometer (2008) (Special Eurobarometer 407, Antimicrobial Resistance, May – June 2013), was adapted and translated into Albanian. A random convenience sample of 392 people was included in this study. The questionnaire consisted of 6 questions.

The respondents in the sample are mainly from Prishtina as the largest city, the capital of Kosovo that has a variety of locations, people from the town or villages. The questionnaire application was made in different places like in some private pharmacy, family medical centers and laboratories, people on the streets, in some Dental Clinics, Medical Private Clinics, Homes and the University. The data was digitally stored and analyzed using Microsoft Excel. Descriptive statistics were carried out by providing the number and percentage of each of the demographic variables as well as knowledge about the questions of antibiotics.

Results and discussion

From 392 patients, 194 were males (49.49%) and 198 females (50.50%). Median age is 51 years (range 15 - 74 years).

The subjects in the survey were asked to rank from their knowledge of antibiotics as low, middle or high. From 392 respondents 184 (46.94%) said that they knowledge of antibiotics is middle, 149 (38.01%) said that they have low

* anadeshishku@gmail.com

knowledge of antibiotics and 59 (15.05%) said that they have high knowledge of antibiotics.

The study also has one declaration and asked the respondents if they 'agree' 'disagree' or 'don't know' that all of us have a responsibility towards the effectivity of antibiotics. 249 (63.52%) of the respondents agreed with this declaration, 86 (21.94%) said they did not know and 49 (12.50%) did not agree with this declaration. A further 8 (2.04%) respondents did not want to answer this question.

Respondents were asked if each of the following four statements about antibiotics (Antibiotics kill viruses; Antibiotics are effective against colds and flu; Unnecessary use of antibiotics makes them become ineffective; Taking antibiotics often has side-effects, such as diarrhea) was 'true' 'false' or 'don't know'.

Almost half of the respondents 176 (44.90%) said that it is true that antibiotics kill viruses, 116 (29.59%) said that it is false that antibiotics do not have effectiveness on viral infection and 100 (25.51%) responded they do not know if antibiotics kill viruses.

A large majority 210 (53.57%) of those polled gave the correct answer that the overuse of antibiotics reduces their effectiveness. Just 71 (18.11%) gave us the wrong answer that it is 'false' and 111 (28.32%) 'do not know' if that is true or not.

Antibiotics are effective against cold and flu which is 'false'and the right answer to this question was obtained from only 116 (29.59%) of respondents. More than half of the respondents answered wrong by stating that is 'True' that antibiotics are effective against the cold and flu 220 (56.12%) whereas 56 (14.29%) others answered that they don't know.

Respondents were asked whether taking antibiotics often has side effects such as diarrhea. 241 (61.48%) of the respondents gave the correct answer that antibiotics can produce side-effects. There is more uncertainty over this issue than the preceding ones: 130 (33.16%) of the respondents were unable to give an answer to this question. A slightly smaller proportion 20 (5.10%) gave an incorrect answer. This is in accordance with several other studies (Awad and Aboud, 2015; Mouhieddine et al., 2014; Sirijoti et al., 2014; Widayati et al., 2012;)

Conclusion

The finding in this study showed that the majority of the population had a moderate level of knowledge and attitudes in relation to antibiotics, by using them for wrong reasons and in the wrong way. We believe that this study may be useful in increasing people's knowledge for the correct use of antibiotics and their awareness concerning the risks of inappropriate use of them, because of the developing resistance of antibiotics.

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Macedonian pharmaceutical bulletin, **62** (suppl) 101 - 102 (2016)
ISSN 1409 - 8695
UDC: 616.895.4-052:[615.15:614.254
Short communication

Pharmaceutical care for people with depression: experiences and challenges

Zahida Binakaj[1]*, Svetlana Stojkov[2], Bistra Angelovska[3]

[1]*Pharmaceutical Chamber of Federation of Bosnia and Herzegovina, Tuzlanska bb, Sarajevo 71000, Bosnia and Herzegovina*
[2]*Pharmaceutical Chamber of Serbia, Mutapova 25, Belgrade, Serbia,*
[3]*Pharmaceutical Chamber of Macedonia, 50 Divizija 34, 1000 Skopje, Macedonia*

Introduction

One of the most significant health issues are the disorders of mood because they have a significant effect on the quality of life of the patients, their families, their working and wider social surroundings. According to the World Health Organization (WHO, 1992; WHO, 2001), mood disorders are the fourth cause of morbidity and mortality with a tendency to move to the second place by the 2020. The prediction that there is going to be an increase in the number of ill in the future comes from the facts that the factors of risk, like stress, are in constant rise, that the demographic image (ageing of the population) is changing which has an effect on the rise of comorbidity of these illnesses with the chronic illnesses of the elderly population and that the incidence of genetically predisposed depressions increases etc.

Patients with depression are often not in a state to correctly apply the prescribed pharmacological therapy which leads to cessation/disruption of therapy, inadequate treatment of depression which as a consequence has frequent hospitalizations, incapability to work and are marginalised and stigmatised by the community. That is why a pharmacist through an interdisciplinary approach and appliance of pharmaceutical care can affect the proper selection of therapy and can also make a follow up of the patient, counsel him/her about the side effects and interactions, and finally improve the quality of life of the patient suffering from depression which is the final goal of including pharmacists and pharmaceutical care into a multidisciplinary team (Binakaj, 2015). Keeping track of compliance and concordance in use of the medication for the nervous system is of special importance. The development of pharmaceutical studies, increase of pharmaceutical industry as well as the changes in healthcare systems and policies sets additional challenges which include continuous monitoring of the needs of patients as well as the trade and consumption of medications for the sake of rational medication administration.

To define, describe and question the role of pharmacists in the treatment of patients suffering from depression, as well as measure the impact of pharmaceutical care on significant parameters in treatment of depression like the selection of appropriate therapy according to the guidelines, adherence of the patient, quality of life, work capability, anxiety, side-effects and interactions.

Materials and methods

The study is a randomised controlled clinical study in which a psychiatrist in General Hospital Tesanj will randomise patients according to the methods of random selection and the criteria of inclusion and exclusion defined in the protocol. The target population is adult patients 18 to 75 years old who suffer from diagnosed depressive episodes (according to the criteria set up for the diagnosis) and who are treated in General Hospital Tesanj (Bosnia and Herzegovina). A pharmacist will set up monthly appointments for the patients and educate them about depression and medications with a special review of side-effects and interactions of medications. During this study the pharmacist and the doctor will have two meetings where they will analyze observations of the pharmacist and evaluate the impact of pharmaceutical care with the intervention group.

* hida_b123@hotmail.com

The standard group will only fill in the questionnaire about quality of life during their regular visit to the doctor at the beginning of the study, after three months and after six months of the study. The gathered data will be analyzed with the SPSS software for statistical analysis of data and it will include qualitative and quantitative elements.

Results and discussion

Forty eight patients were monitored in this research, 11 men and 37 women, where we notice that the incidence of depression is more frequent in women, which is also visible in the research of WHO 2008.

During this research three tests were conducted using which we gathered information which showed us the significance of a pharmacist in the treatment of patients suffering from depression. Consideration of the anxiety test showed that the treatment with a team which includes a pharmacist compared to a team without a pharmacist shows progress in the treatment because there was a significant decrease in irritability and anxiety. 56% of the patients had these symptoms during the first visit while this figure in the third visit was 4.3%. When considering the questions from the life quality test we didn't get significant results which showed us progress in the patient's depression treatment with the presence of a pharmacist in an interdisciplinary team. Also, the patients' life test showed us the contribution of the pharmacists in the treatment of patients with depression.

These results showed us that the pharmacist should be included in the interdisciplinary team which takes care of the treatment of patients with depression and by further educating the pharmacist in the area of mental disorders, especially depression; even better results would be achieved. A research conducted in Belgium about the role of pharmacists in the care of patients with depression showed that pharmacists are not sufficiently involved in the treatment of patients with depression as well as the insufficient education of the pharmacists regarding mental disorders (Liekens et al., 2012). Pharmacists are the most devoted health professionals – patients visit pharmacists seven times more than they visit doctors. These data show us that pharmacists through pharmaceutical care can make the fastest and the cheapest contribution to a good outcome of this multidisciplinary approach in the treatment of patients with depression.

Conclusion

The high prevalence of depression and anxiety disorders and the trend of growth of the affected demand a detailed analysis of causes, treatment, cormobidity and interactions, as well as keeping track of the consumption of medications for the rehabilitation of symptoms caused by these disorders which in the future could facilitate improvement of adherence, the effect of therapy and improvement in quality of life.

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Macedonian pharmaceutical bulletin, **62** (suppl) 103 - 104 (2016)
ISSN 1409 - 8695
UDC: 618.19-006.6-085.277(496.5)
615.277:618.19-006.6(496.5)
Short communication

Adjuvant chemotherapy, with or without taxanes, among women with breast cancer in Albania

Erina Hilaj[1]*, VilmaPapajani[2], Alketa Ymeri[3]

[1]*National Centre of Continuing Education,Rr. AleksanderMoisiu, Nr.84 Tirana, Albania;*
[2]*Faculty of Pharmacy, University of Medicine, Rr e Dibres,371,1005, Tirana, Albania;*
[3]*University Hospital Center "Mother Teresa", Rr. e Dibres, 372, Tirana, Albania*

Introduction

In Albania, neoplasm is the second leading cause of death following circulatory system diseases. Within neoplasms, breast cancer is the second most frequent cancer after lung cancer (INSTAT, 2016). Breast cancer treatment generally involves multiple approaches including surgery, radiation and/or chemotherapy. Adjuvant treatment is the administration of additional therapy after primary surgery to kill or inhibit micrometastasis (Tarifa et al., 2007). Depending on the model of risk reduction, adjuvant therapy has been estimated to be responsible for 35-72% reduction in mortality rate (Newton and Grethlein, 2015).

The adjuvant chemotherapy of breast cancer has changed several times since the first effective chemotherapy regimen for breast cancer CMF, (cyclophosphamide; methotrexate; 5-fluorouracil) was developed (Bonadonna et al., 1976).

To date, there is no 'gold standard' regimen in adjuvant chemotherapy of breast cancer. The diversity of the disease depending on biomarkers, the proliferation process and diversity of trials, with different interpretations, has led to endless suppositions and discussion on the best treatment. However, there is general agreement that CMF-like regimens are 'better than nothing', that anthracycline-containing regimens are better than CMF, and that the taxanes further add to the benefit of anthracyclines (Peto, 2007).

The aim of this study was to evaluate the results of adjuvant chemotherapy, taxane-based regiment with anthracycline- based regiment in woman with breast cancer in Albania.

Materials and methods

A case-series study was conducted at the University Hospital Center "Mother Teresa" in Tirana including 181 women diagnosed with breast cancer and treated with adjuvant chemotherapy. Women included in this study had an age range 18-70 years, with stage I-III of breast cancer irrespective of nodal, hormonal, or HER2 status. Respectively, 35 women were treated with taxane-based adjuvant chemotherapy regimen and 146 women were treated with anthracycline-based adjuvant chemotherapy regimen after primary surgery. The clinical diagnosis was based on biopsy findings. A 5-year follow-up of took place where the relapse experience was recorded for all women included in this study.

Results and discussion

Taxane–based regimen has become the most popular adjuvant therapy for breast cancer in the last decade (Jones et al., 2009). Overall, in our study there were 35 women treated with taxane-based regimen. In the group of women treated with taxane-based (paclitaxel or docetaxel) regimen as adjuvant chemotherapy, 8 (22.9%) of them experienced relapse within four years compared with 27 (77.1%) women who did not manifest relapse after the fourth year of follow-up. The distribution of relapse time among women treated with 4AC-4T(paclitaxel or docetaxel) was as follows: there were 2 women (5.7%) who experienced a relapse within the first 12 months; 2 (5.7%) within 24 months; 1 (2.9%) within 36 months; and 3 (8.6%) women within 48 months. Conversely, 27 (77.1%) did not manifest relapses after the fourth year of follow-up.

Overall, in our study there were 142 women treated

* erinahilaj@yahoo.com

with anthracycline-based regimen. In the group of women treated with anthracycline-based regimen (CAF (N=142, or 97.3%) or AC-CMF (N=4, or 2.7%) of adjuvant chemotherapy, overall 34 (24.5%) of them experienced relapse within four years compared with 105 (75.5%) women who did not manifest relapses after the fourth year of follow-up. On the whole, 6 (4.3%) women experienced a relapse within the first 12 months; 13 (9.4%) within 24 months; 6 (4.3%) within 36 months; and 9 (6.5%) women within 48 months. On the other hand, 105 (75.5%) did not manifest relapses after the fourth year of follow-up.

After 5 years of follow-up, there was an improvement in relapse-free survival between taxane-based regiment and anthracycline based regiment (77.1% vs 75.5%). Benefits were observed irrespectively of nodal, hormonal or HER2 status.

The 2011 EBCTCG meta-analysis also included taxanes such as docetaxel and paclitaxel in its analysis of adjuvant therapy. Incorporation of taxanes into an anthracycline containing regimen resulted at 8 years in the reduction of the risk of recurrence, risk of breast cancer mortality, and overall mortality. This benefit was present irrespective of age, nodal status, tumor size, tumor grade or estrogen receptor (ER) status (Peto et al., 2012).

Our study obtained similar results, regardless of the small size sample. Hence, incorporation of taxanes such as docetaxel or paclitaxel into adjuvant chemotherapy regimens led to a reduction in disease recurrence. This benefit was evident independent of age, nodal status, tumor size, tumor grade, estrogen receptor (ER) and HER2 status.

Conclusion

After 5 years of follow-up, there was an improvement in relapse-free survival between taxane-based regimen and anthracycline-based regimen respectively 77.1% and 75.5% in Albanian women diagnosed with breast cancer and treated with adjuvant chemotherapy. Benefits were observed irrespectively of nodal, hormonal, or HER2 status. These findings have important implications for practitioners and policy-makers in Albania and beyond.

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Macedonian pharmaceutical bulletin, **62** (suppl) 105 - 106 (2016)
ISSN 1409 - 8695
UDC: 616.348/.35-006.6-085.277(497.7)
615.277:616.348/.35-006.6(497.7)
Short communication

Bevacizumab in addition to FOLFOX chemotherapy for metastatic colorectal cancer: A Macedonian–based cost-effectiveness/utility analysis

Aleksandra Kapedanovska-Nestorovska*, Zorica Naumovska, Aleksandra Grozdanova, Aleksandar Dimovski, Ljubica Suturkova, Zoran Sterjev

ISPOR Chapter Macedonia, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje, Republic of Macedonia

Introduction

Colorectal cancer (CRC), with an estimated 447,000 new cases and an estimated 215,000 deaths occurring in 2012, is the second most common cancer and also the second most common cause of cancer death in Europe (Graham et al., 2014). The incidence of CRC is strongly related to age (83% of cases occurring in patients who are aged 60 years or older). The ageing population and global effect of "westernization" are additional factors driving a rapid increase in new CRC cases worldwide. As a consequence, the already significant societal burden of CRC is likely to increase over time. Important economic components of this burden include direct medical care costs, direct nonmedical costs (such as patient time involved with receiving medical care), and productivity losses among patients and caregivers (Yabroff et al., 2013).

The most important CRC prognostic factor is the disease stage at the time of diagnosis (from a 90% of 5-year survival rate for cancers detected at the localized stage; 70% for regional; to 10% for people diagnosed for distant metastatic cancer). Approximately 25% of newly diagnosed patients have already developed metastases; almost 50% of all CRC patients will form metastases over time as the disease progresses. Metastatic colorectal cancer (mCRC) is characterised by a high mortality rate.

In the last decade, fluoropirimidine drugs (5-fluorouracil-FU and leucovorin -LV) combined with oxaliplatin or irinotecan chemotherapy regimens (FOLFOX and FOLFIRI), were best available mCRC treatment. In recent years, the significantly increased mCRC survival rates have resulted mainly from the introduction of monoclonal antibodies (mAbs) as additional first-line treatment to chemotherapy or in subsequent treatment lines (Lange et al., 2014).

According to the European Society for Medical Oncology (ESMO) clinical practice guidelines for mCRC, bevacizumab (mAb against the vascular endothelial growth factor -VEGF) and panitumumab and cetuximab (which bind the epidermal growth factor receptor - EGFR) in combination with chemotherapy can be considered as first line options for selected patients with mCRC. The ESMO recommendations are based on the improved outcomes reported for these biologics versus chemotherapy alone in clinical trials (Van Cutsem et al., 2010). Despite the established therapeutic efficacy and safety, this new mAbs based chemotherapy regimens have dramatically increased the cost of treatment.

Pharmacoeconomic assessments are a part of the decision process not only during reimbursement setting, but in clinical practice as well. Estimates of the costs associated with cancer care are essential both for assessing burden of disease at the population level and for conducting economic evaluations of interventions to prevent, detect, or treat cancer. Comparisons of cancer costs between health systems and across countries can improve understanding of the economic consequences of different health-care policies and programs.

The aim of this analysis was to evaluate the cost-effectiveness/ utility of bevacizumab plus FOLFOX vs. FOLFOX alone in 1st line treatment of patients with mCRC from the payer perspective in R. Macedonia

* alka@ff.ukim.edu.mk

Materials and methods

A Decision tree structure approach was selected to assess the cost-effectiveness of Bevacizumab plus FOLFOX relative to FOLFOX in the first line treatment of patients with mCRC. The model uses a 2-week cycle length and a time horizon of 2 years post treatment initiation. The entire patient population was composed of a simulated cohort of 100 mCRC patients/treatment branch. The patients progressed through the decision tree based on the risk of early therapy discontinuations because of severe toxicity (ST), disease progression (DP) and cancer associated death (CAD) during active treatment. After completion of the first 2 cycles of chemotherapy, patients without experienced ST from first-line therapy (FOLFOX ± bavacizumab) are continuing the treatment until DP. For patients with intolerable toxicity, first-line therapy is discontinued and second-line FOLFIRI is offered until DP. Upon progression, all patients receive best supportive care until death. The transition probabilities between the health states for bevacizumab plus FOLXOX and FOLFOX alone treatment were based on parametric survival curves estimated in a patient-level analysis of progression free survival (PFS) and overall survival (OS) from the NO16966 clinical trial (Saltz et al., 2008).

Only direct costs (bevacizumab and FOLFOX chemotherapy), were included in the analysis. Drug-acquisition costs were calculated utilizing official government and hospital pharmacy publicly available data. All costs are reported in 2016 Euros. The benefit and performance of medical treatments, measured as quality-adjusted life years (QALY) were extracted from the published data (Ramsey et al., 2000, 2002). The incremental cost per QALY gained was calculated as the difference in total costs divided by difference in QALY. All costs and outcomes (benefits) in the model were discounted using discount rate of 3.5% per annum.

A sensitivity analyses on the estimates of costs, utilities, and health state transitions were performed to evaluate the robustness of the model and address uncertainty of variables. In univariable sensitivity analyses, we varied the value of costs and utilities within ± 20% of their baseline values and examined the effect on the ICER. In probabilistic sensitivity analyses (PSAs), we ran the model 1000 times, each time randomly varying all parameters simultaneously. We used uniform distribution for cost parameters and gaussian distribution for the efficacy parametars and discount factor. The analysis was conducted from the perspective of both the Macedonian public and private health care systems.

Results and discussion

Base case analyses

The initial drug costs for bevacizumab +FOLFOX compared to FOLFOX were estimated at €2312.86 and €451.22, respectively, per 2-week cycle. In the primary analysis, the effectiveness and costs were compared in the first-line model for the FOLFOX and FOLFOX-plus-bevacizumab groups. FOLFOX provided 1.31 QALYs at a cost of €1963.86. FOLFOX plus bevacizumab provided 1.41 QALYs at a cost of €11021.899. The ICER for FOLFOX plus bevacizumab was €90580.374 per QALY.

Sensitivity analysis

According to the sensitivity analyses results, the base case model is robust to alternative parameters and assumptions. The parameters with the greatest influence on the ICERs were median OS and PFS for each regimen, drug cost of bevacizumab, and utility values for living with mCRC. The discount factor had a minor influence on the ICER in the first-line model. The PSA indicate that 82% of simulations performed showed bevacizumab plus FOLFOX to be more effective and more costly than FOLFOX chemotherapy alone.

Conclusion

Based on the model projections, the introduction of bevacizumab to the standard FOLFOX chemotherapy protocols for mCRC treatment resulted in longer survival and greater QALYs than FOLFOX alone at an incremental cost of € 90580.374 per quality adjusted life-year gained.

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Macedonian pharmaceutical bulletin, **62** (suppl) 107 - 108 (2016)
ISSN 1409 - 8695
UDC: 615.2:339.187(497.7)
Short communication

The advertising influence on pharmacist recommendations and consumer selection of over-the-counter drugs

Aleksandra Kapedanovska-Nestorovska*, Zorica Naumovska, Zoran Sterjev, Ljubica Suturkova, Aleksandra Grozdanova

Faculty of Pharmacy, University Ss. Cyril and Methodius, MajkaTereza 47, Skopje, Republic of Macedonia

Introduction

Over-the-counter (OTC) drugs are medicines that can be used safely and effectively by the general public without a prescription. Available for sale directly to a consumer, they are one of the most important and easily accessible public health aids for the treatment of common conditions or symptomatic relief (Håkonsen, et al., 2016; Halvorsen et al., 2016). In the last 10 years, many medicines that were originally prescription only have now become available over the counter (OTC), either from pharmacies or other general retail outlets (Bond et al., 2003). However, certain cases of misuse or overuse of OTC drug products resulting in adverse drug events have been recorded, leading to the conclusion that they may not be totally harmless. Although there are different means of providing the consumers with drug information, the advertising, used by the pharmaceutical companies, is one of the best and powerful methods for information broadcasting and introducing these products to the consumers (Hanna et al., 2010 Major, et al., 2010). Thus, trying to make appropriate recommendations and decisions regarding OTC medications can be tempting for the community pharmacists and daunting and confusing for patients (Cooper et al., 2013).

The aim of this study was to examine the advertising influence on pharmacist and consumers attitudes in selection of OTC medicines.

Materials and methods

The multicenter, observational cross-sectional study was conducted at 70 randomly selected community pharmacies in Skopje, R. Macedonia. Data were collected during one shift at each pharmacy. The instrument used for investigating the research questions in this the study is a previously validated survey available in the literature (Potnis, 2012). Pharmacists were provided with a printed copy of survey.

Variables measured in this study are the cumulative scores for pharmacists' perceptions of OTC drug advertising concerning their recommendations and consumer selection of over-the-counter drugs. The questionnaire included 27 items divided into 2 sections. Section I of the questionnaire was comprised of seven questions measuring the demographic variables (gender, years of practicing as a pharmacist, practice setting, location of the pharmacy, frequency of exposure on OTC drug advertisements, average weekly OTC prescription volume and medium of OTC drug advertisements) related to the pharmacists' practice.

Section II was comprised of 20 questions (divided in 4 subsections), measuring pharmacists experience regarding: 1) pharmacists' beliefs about the effects of over-the-counter drug advertising, 2) impact of OTC drug advertising on pharmacists' recommendations, 3) influence of OTC drug advertising on patients' choice of the drug products, 4) Effect of over-the-counter drug advertising on patient pharmacist interactions. The subsection 1 and 2 consisted of 4 questions, while sections 3 and 4 comprised of 6 questions. All questions from section II were measured on a 5-point Likert scale (items rated from one to five; '1' denoting 'Strongly Agree' and '5' denoting 'Strongly Disagree').

With each item having a maximum possible score of five, the highest score for the beliefs measure, consisting of four or six questions, was 20 or 30, respectively. Pharmacists, whose cumulative score for the above items was found to be less than or equal to 10 or 15 were considered to believe that over-the-counter drug advertising has a great effect on the measured variable. The obtained data were descriptively analyzed.

* alka@ff.ukim.edu.mk

Results and discussion

All of the participants were females (100%) working in large chain pharmacies, previously defined as pharmacies with more than or equal to 12 stores (61.1%). Approximately 33.3% of the pharmacist worked in small chain pharmacies (with 1-12 stores). Most of the pharmacists were working in pharmacies located in urban regions (81.5%) with most of them practicing for 1-10 years (70.6%). Approximately 59.3% of the surveyed pharmacists mentioned coming across over-the-counter drug advertisements/informations for 1- 5 times a week. Marketing representatives, as reported by 45% of the participants, were found to be the medium where the pharmacists gain most of the over-the-counter drug informations, followed by internet (33.3%). A large number of pharmacists (31.4%) were working at a pharmacy with a volume of prescriptions between 100-150 and more than 200 OTC drugs /week.

A majority of the surveyed pharmacists (61.5%) were found to have a cumulative score of 8 and thus believe that over-the-counter drug advertising has a great effect on patients. Approximately 77.7% of the pharmacists had a cumulative score of 12 and agreed that over-the-counter drug advertising does not impact on pharmacists' recommendations. A large number (81.4%) of pharmacists, based on a score of 14 agreed that the patients choice of the drug products was largely influenced by over-the-counter drug advertising. In all, 51.8% of the pharmacists, with a score of 12, were of the opinion that patient - pharmacist interactions were increased as a result of the over-the-counter drug advertising.

Conclusion

These findings serve as a starting point for additional research in the field of over-the-counter drug advertising in our country. Although pharmacists use marketing advertisement as a means of information about over-the-counter drugs they strongly believe that their role is to offer patients the best care at the least possible cost and to ensure that the recommendations regarding OTC drug provides enough revenue to maximize its value to the patient.

Considering that patients choice of the OTC drug products is greatly influenced by over-the-counter drug advertising in order to promote a rational usage of the OTC drug the advertisement has to contain all necessary patient information in a way that is clear and understandable and to encourage patients to carefully read the package leaflet or instructions. In view of the fact that in recent years, direct-to-consumer advertising of OTC drugs has increased all advertisements must give a clear message that the advertised product is a medicinal product.

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Macedonian pharmaceutical bulletin, **62** (suppl) 109 - 110 (2016)
ISSN 1409 - 8695
UDC: 615.1:340.13
Short communication

The relationship of law and pharmacy

Katerina Anchevska Netkovska*, Aleksandra Grozdanova

Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia

Background

Parallel with the development of pharmaceutical science and legal regulations (law on medicinal products and medical devices, law on health care), this area is gaining greater recognition in national and international level. Pharmacy, unlike chemistry and biology is not just science, rather, it is a profession that encompasses a broad range of academic and professional disciplines that are based on science, business, sociology and law. Pharmaceutical law as a discipline of recent date, although its foundation and academic profiling is an area that legally has be present in order to develop different approached to problems, and to cooperate with pharmaceutical science and practice (Appelbe and Wingfild, 1997). The rules in pharmaceutical laws may be universally agreed and respected or may be controversial and applied only in part or only by some of those concerned, so this article seeks to provide an outline of the law that affects the pharmacy.

Pharmacy law

Interdisciplinary is a kind of cooperation where experts from various academic disciplines work in order to achieve common outcomes. The past decade has witnessed an unprecedented interest and controversy surrounding in ethical and legal aspects of health, including pharmaceutical care. One reason for the establishment and development of such program as pharmaceutical law, is the conviction that traditionally established disciplines individually are unable to solve key problems. What is more there are many other areas of law and regulation affecting the industry, concerning, for example the pricing of medicines, the conduct of clinical studies, the health protection and health care insurance (Dukes, 2006). In some fields it is hardly impossible to maintain standards through regulation. So we can freely say that the manner and quality of the relationship on both sides (pharmacy and law) depends on boosting overall pharmaceutical activity and protection. This suggests that the pharmacy and law although at first glance are two mutually different areas, still they have numerous and important common ground. Considering that, interdisciplinary approach of pharmaceutical law is very complex and requires knowledge of the lawyers in the pharmaceuticalsbut also demands from pharmaceutical professionals better knowledge of the legal institutions whose application refers to them.

This pharmacy-law relationship puts the task of pharmacists not only in the implementation of regulations related to pharmaceutical law, but also needs the right approach to shape legal institutions to regulate rights and obligations, which requires a very serious approach by legal experts. The pharmacists work reaches out to entire community. Pharmaceutical law in an objective sense constitute a set of laws governing pharmaceutical activity, regulate the rights and obligations of health professionals and their relationship to the beneficiaries. It is especially important to emphasize that this discipline also called, "law for drugs" covers the legal definition and corresponding action to the pharmaceutical care of the individual in the health care system (Mujovik-Zornik, 2008). It covers parts of the regulation for legal issues relating to the manufacture, trade and use of drugs in the context of health care. For this reason pharmaceutical law is broadly viewed as an integral part of health law or legislation.

Pharmaceutical law as a discipline is recognizable recent, and appears separately in order to identify first of all regulatory aspects in the definition and implementation of health legislation. The need and the existence of these

* kaan@ff.ukim.edu.mk

two disciplines (medical and pharmaceutical law), on the one hand requires their integration and harmonization, and on the other hand allow consideration of legal problems from different aspects.For example, from the perspective of medical law separates the part of pharmaceutical regulation under which person (patient) consumed drugs comes into contact with them, but again portions over a health or medical procedures undertaken in connection therewith. This will also mean that aspects of pharmaceutical law, which relate to public or rather socially significant issues associated with the production of pharmaceuticals, intellectual property rights, and advertising may appear as second.

Unlike traditional branches of law, pharmaceutical law is not specifically codified but is defined by the field governed not uncommon liaison with ethical norms associated with this regulated profession, because it is a limited legal area, which refers to a profession and those who use its services. This arises and specificity of pharmaceutical law and its sources are simultaneously multiple of a different nature, legal and professional (intellectual property rights and norms associated with putting drugs on the market, quality and safety of medicines and other pharmaceutical products, health care insurance, competition, advertising, ethical codes, etc.).

Therefore, all legal norms and regulations of pharmaceutical law recognize and affect each other, because a decision to bring a specific legal issue entails application of the regulation and other areas of law (Junod, 2006). This indicates that the rules of pharmaceutical law have their own specifics, but when it comes to legally relevant complex cases, its use is public law and regulation, private and often even criminal character. Considering the constitutionally guaranteed right to health as a universal value, (The Constitution of the World Health Organization-1946 states that the "enjoyment of the highest attainable standard of health is one of the fundamental rights of every human being without distinction of race, religion, political belief, economic or social position ") pharmaceutical law as part of health activity has the same characteristics.

Although still in development in our country the national legislation related to these issues is in the process of harmonizing with the standards as in the other EU member states. Promotion of pharmaceutical law as a discipline and the ongoing debate on current issues of legal theory and practice is of great scientific and professional importance. Presence as a special study program at a growing number of faculties, slowly gaining status, along with medical law as a scientific discipline that is taught and continually exploring.

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Macedonian pharmaceutical bulletin, **62** (suppl) 111 - 112 (2016)
ISSN 1409 - 8695
UDC: 628.4.045/.046:615]:502.13(497.11)
Short communication

Pharmaceutical waste management: a necessity to position a pharmacist as a pillar of public awareness campaign

Nataša Jovanović Lješković*, Manda Dizdar, Branislava Rakić, Milan Ilić, Nikola Jojić, Marina Gavrančić, Slobodan Gigov

Faculty of Pharmacy, Novi Sad, University Business Academy in Novi Sad, TrgMladenaca 5, 21000 Novi Sad, Serbia

Introduction

Importance of the pharmaceuticals is well acknowledged and recognized for health and wellbeing of our society but on the other hands they have become chemicals of concern to the public because of their potential to reach drinking-water. Recent study in Serbia showed the presence of pharmaceuticals in water samples collected from 25 locations (in total 47 out of 81 investigated drugs were detected) (Petrović et al., 2014).

Although more research is needed to properly estimate the risk and the impact of measured concentrations of the pharmaceuticals on the environment and on human health, it is clear that more attention needs to be paid to this issue in coming years. This is especially because the production rate of pharmaceutical waste is constantly growing worldwide as a consequence of increased use of pharmaceuticals (Smith, 2002).

Pharmaceutical waste management is complex challenge requiring compliance with of health and environmental regulations as well as involvement of all parties included in providing and receiving of health service. To minimize the risk of uncontrolled pharmaceutical waste disposal preventive measures and systematic drug-take back programs are needed. However, equally important prerequisite for success of the whole process is enhanced communication to the public on proper pharmaceutical waste management. And for that purpose pharmacists can and should play key role. Focus of this work was to investigate to what extent raising of public awareness is needed and if there are any particular population groups (i.e. regarding education, age) that require additional attention.

Materials and methods

This research has been conducted among citizens of Serbia. The study included 100people. People in the pharmacies in the cities of Novi Sad, Subotica, Ruma, Arandjelovac and Vlasotince and their country sides, were approached randomly and invited to participate. The anonymous questionnaire consisted of 9 questions. Three questions were related to the socio-demographic characteristic of the population, and other six were related to medication storage and disposal. Participants could either complete the questionnaire themselves or have their responses marked by the investigators. Analysis was performed in Excel.

Results and discussion

Out of total sample size of 100 responders, 10% of responders were from municipality of Arandjelovac, 10% of responders were from Ruma, 9% of responders were from Subotica, 28% responders were from Vlasotince and 43% responders were from Novi Sad. The majority of the examinees were from the cities (83%) and 17% from the country sides. Fifty-four percent (54%) of responders had high school degree and forty-one (41%) had university degree. Out of all respondents, 18% were students, 52% were employed and 28% were retired.

More than 80% of responders said that they had kept the unused medicine at home.In the questionwhat do they do with unused medicine the examinees most often answered “I keep it in a case I may needed it for later” (60%), “I give to other people if they need it” (16%), “I dispose it in the household ban” (19%) and about 5% answered “I return unused medicine to a pharmacist or a doctor”.

Also, the examinees were asked about their disposal practice of primary packaging of medicines (prescrip-

* natasa.ljeskovic@faculty-pharmacy.com

tion bottles, blister packs, etc.) and 89% of them did this in an environmentally inappropriate way. Answers indicated that primary packaging of medicine was disposed as a house hold waste. Only 7% of the examinees were recycling and just 4% reported returning primary packaging to a pharmacy for disposal. This is in line with our previous study where some 87% out of 253 examinees answered to dispose primary packaging as household waste (Gigov et al., 2014).

Among the examinees who tried to return unused pharmaceuticals or primary packaging to a pharmacy, only 36% had founded pharmacy that accepted unused pharmaceuticals, 45% of them were referred to other institution and in a case of 19% of responders the pharmacy could not accepted pharmaceutical waste.

Obtained results show that the lack of public awareness of pharmaceutical waste management is unfortunately equally distributed throughout the population in Serbia. The majority of the examinees were not informed about proper pharmaceutical waste management. There are no differences between behavior of the responders related to the place where they were interviewed, neither to their education nor their employment/student status.

Conclusion

It is obvious that further education of the population is needed. Active involvement of the pharmacists is one of the most important steps. Pharmacists due to their education and their position to daily communicate with the patients about the medicines can offer the most updated and relevant info about pharmaceutical waste management. Raising public awareness with central role of pharmacists on one hand and introducing a systematic approach in pharmaceutical waste management with preventive measures should decrease a risk of unwanted and potential hazardous effect of pharmaceutical waste. This effort should be centrally promoted and supported by government and health sectors in Serbia that should enable a system for continuous education and regular update of the pharmacists with most recent regulations and trends in pharmaceutical waste management.

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Macedonian pharmaceutical bulletin, **62** (suppl) 113 - 114 (2016)
ISSN 1409 - 8695
UDC: 616.379-008.64-052-047.22(497.7)
Short communication

Awareness of the importance of self-management in Macedonian diabetes patients

Biljana Indova*, Zorica Naumovska, Aleksandra Kapedanovska Nestorovska, Aleksandra Grozdanova, Ljubica Suturkova, Zoran Sterjev

Faculty of Pharmacy, University Ss. Cyril and Methodius, MajkaTereza 47, Skopje, Republic of Macedonia

Introduction

Diabetes is a complex, chronic illness requiring continuous medical care with multifactorial risk-reduction strategies beyond glycemic control. Ongoing patient self-management education and support are critical to prevent acute complications and reduce the risk of long-term complications.

Hyperglycemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially heart, blood vessels, eyes, kidneys, and nerves. Type 1 diabetes is characterized by deficient insulin production and requires daily administration of insulin. Diabetes Type 2, results from the body's ineffective use of insulin and comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity. Until recently, this type of diabetes was seen only in adults but it is now also occurring in children. Gestational diabetes is hyperglycemia with blood glucose values above normal but below those diagnostic of diabetes, occurring during pregnancy. Impaired glucose tolerance and impaired fasting glycaemia are intermediate conditions in the transition between normality and diabetes.

About 347 million people worldwide have diabetes and this number is projected to reach 552 million in 2030 (Whiting et al. 2011). In 2014, 9% of adults (18 years and older) had diabetes. About 6% of Macedonian population has diabetes mellitus, either type 1 or 2. There is an emerging global epidemic of diabetes that can be traced back to rapid increases in overweight, including obesity and physical inactivity. In 2012 diabetes was the direct cause of 1.5 million deaths. Majority of diabetes deaths (80%) occur in low- and middle-income countries. The overall risk of dying among people with diabetes is at least double the risk of their peers without diabetes (Roglic et al. 2005). Early diagnosis can be accomplished through blood testing. Treatment of diabetes involves lowering blood glucose and the levels of other known risk factors that damage blood vessels. Nevertheless improvement in diabetes management in the last years, 33–49% of patients still do not meet targets for glycemic, blood pressure, or cholesterol control, and only 14% meet targets for all three measures and non-smoking status (Ali et al., 2013).

Patients with diabetes type 2, without cardio vascular disease (CVD) and mikroalbuminemija, are in the category of patients with very high 10-year risk (13.4±11.4%) of fatal CVD as a result of inadequate glycemic control with a mean HbA1c 9.5±2.0% (recommendations for adequate glycemic control HbA1c < 6.5%) (Smokovski I., 2009). In parallel to healthcare system further development and improvement, successful diabetes care requires a systematic approach to supporting patients' behavior change efforts, including: 1. Healthy lifestyle choices (physical activity, healthy eating, tobacco cessation, weight management, and effective coping), 2. Disease self-management (taking and managing medications and, when clinically appropriate, self-monitoring of glucose and blood pressure) and 3. Prevention of diabetes complications (self-monitoring of foot health; active participation in screening for eye, foot, and renal complications; and immunizations). High-quality diabetes self-management education (DSME) in US has been shown to improve patient self-management, satisfaction, and glucose control (Powers, 2015). To date, perception for the importance of self-management in diabetes patients has not been evaluated in Republic of Macedonia (RM).

The aim of this paper is to assess the awareness and level of perception of the self-management importance associated with glycemic control achievement in patients from RM.

* biljana.p.indova@gmail.com

Materials and methods

The study follows a descriptive design using a questionnaire-based survey. The questionnaire was created to assess perception for self-management in diabetes patients using the principles of Diabetes Self-Management Questionnaire developed at the Research Institute of the Diabetes Academy Mergentheim (Schmitt et al., 2013). First part of the questionnaire contains 6 demographic questions related to gender, age, urban or rural place of residence, education, history and type of diabetes. The second part contains 19 questions segmented in 4 subgroups related to: Glucose Management, Dietary Control, Physical Activity and Health-Care Use. The rating scale was designed as a four-point Likert scale, in a range 3-0 points, from the response options "applies to me very much" (3 points) to 'does not apply to me' (0 points). 300 diabetes patients have been assessed with this questionnaire in 11 different towns in R. Macedonia: Skopje, Bitola, Strumica, Kumanovo, Kicevo, Tetovo, Prilep, Veles, Ohrid, Struga, and Stip. Assessment has been performed in direct contact of pharmacist with patient, with previously gained patient's consent for participation. Statistical analysis was performed using SPSS 21.0.0. Group comparisons involved One-way Analysis of Variance, Student's t-test and Pearson's chi-squared test. In all analyses a P-value of < 0.05 (two-tailed test) was considered as criterion of statistical significance.

Results and discussion

300 diabetes patients were assessed, most at age >51years and older, 60% female and 40% male. Most of the patients come from urban environment with secondary education. 90% of the subjects have diabetes type 2 and a history of disease > 5 years. Regarding to our findings, diabetes patients from Republic of Macedonia mostly pay attention in regards to glucose management and regular treatment. In Dietary control questionnaire segment, most of the patients rated with 2 points (applies to me to a considerable degree). What is concerning is the low level of the perception for the need for physical activities and its impact on overall disease management.

Conclusion

The preliminary results suggest on concerning low level of perception for self-management in diabetes patients in R. Macedonia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 115 - 116 (2016)
ISSN 1409 - 8695
UDC: 615.035.7(497.731)"2013"
Short communication

Evaluation of rational / irrational drug use at orthopedic department in Clinical Hospital Stip in the period from January to April 2013

Biljana Lazarova[1]*, Aleksandra Kapedanovska Nestorovska[2], Zorica Naumovska[2], Aleksandra Grozdanova[2], Ljubica Suturkova[2], Zoran Sterjev[2]

[1]*Clinical Hospital - Stip, Ljuben Ivanov bb, Stip, Republic of Macedonia*
[2]*Faculty of Pharmacy, University Ss. Cyril and Methodius, MajkaTereza 47, Skopje, Republic of Macedonia*

Introduction

According to WHO, rational use of medicines is defined as process that allows prescribing of the right drug for a right patient in the proper dose, duration of therapy, with the lowest cost for the patient and the community. Worldwide, more than 50% of all medicines are prescribed, issued, or sold inappropriately. The most common reasons for the irrational drug use are: using too much medication per patient, improper use of antibiotics (inadequate intake for non-bacterial infection), use of the wrong medication for specific condition, drug use with questionable effectiveness, using a drug with uncertain security status, excessive use of parenteral formulations, where the use of oral formulations is more appropriate, inappropriate self-medication. Irrational drug use represents a major generator of progressively increasing costs in all health systems. (Holloway and Green, 2003).The aim of this study was to evaluate rational/irrational drug use at orthopedic department - Clinical HospitalStip.

Materials and methods

A retrospective evaluation for the period of 4 months concerning to consumption of analgesics (Tramadol and Ketoprofen), antibiotics (Ceftriaxon), unfractionated heparin and low molecular weight heparins was done. Total number of patients was 348 in evaluated period January - April 2013. The Evaluation of the therapy used in the study was according to the guidelines for evidence-based medicine issued by the Ministry of Health of RM for the treatment of diseases that are diagnosed in the test population. For the diagnoses for which there is not recommended guidelines, the European and American Associations for the treatment of given diseases guidelines were used. In order to detect discrepancies in the rational drug use for each of the evaluated drugs, DDD expressed per 100 hospital beds was calculated. The obtained data were statistically analyzed using SPSS.v.20 software licensed program.

Results and discussion

In Clinical hospital – Stip, annually expenditure for drugs is 500 000.00 euros and 7.1% belongs to the orthopedic department. From 348 hospitalized patients on this department in evaluated period, 218 patients (62.64%) were treated conservatively and 130 patients (37.36%) with surgery intervention. The average age of evaluated patients was 57.14 ± 20.3 years and average time of hospitalization per patient was 7.74 ± 10.4 days. In most of the hospitalized patients diagnose was bone fracture and almost all patients received more than 4 drugs as parenteral therapy. The most commonly used analgesic for the pain control treatment was Amp. Ketoprofen. According to the obtained data in February, 51 out of 75 patients (68%) received Ketoprofen of which 19 (38.59%) more than 5 days. In February 56.99 DDD/HHB Ketoprofen was used with extra costs of 27.35%. In March 66 patients (61%) received Ketoprofen of which 17 (25.76%) more than 5 days. In April 41 patients (43.16%) received Ketoprofen of which 16 (39.02%) received more than 5 days. The DDD/HHB for

* blazarova55@yahoo.com

Ketoprofen in April was 18.82. Ketoprofen was applied in almost all patients who were hospitalized at the orthopedic department at the Clinical Hospital Stip, much longer than recommendations of 5 days and parenteral form of the this drug was not replaced with an oral form.

The same situation is evident for the application of Tramadol. From total 160 inpatients for four months period who received Tramadol, 65 of them received more than 4 days. In January, 41 patients (58.57%) received Tramadol of which 22 (53.66%) received Tramadol more than 4 days. The DDD/HHB in January was 11.35. In February, 32 (42.67%) received Tramadol, 8 of which (25%) received more than 4 days. Relatively same situation we evaluated in March and April.

Prophylaxis of deep vein thrombosis (DVT) is indicated in patients who performed more surgical intervention. Evidence-based medicine shows that the prevalence of asymptomatic DVT detected by routine venography after larger orthopedic intervention is lower in hospitals that implement prophylaxis 10 days compared to hospitals that carry out prophylaxis only 5 days. This observation supports the current recommendations of the American College of Chest Physicians (ACCP) for a minimum 7-10 days prophylaxis in patients who undergo surgery at orthopedics. In January, total of 16 (22.86%) hospitalized patients received thromboprophylaxis with low-molecular-weight heparin (*LMWH*) and only 5 (31.25%) patients received prophylaxis in accordance with the recommendations (≥7days). The associated DDD/HHB value for this month was 20.07. In February, 3 patients (4%) received LMWH and only 1 (33.33%) received more than 7 days and the DDD/HHB was 1.52. In March 5 patients (4.63%) received LMWH with highest extra cost of 68.46 %. In April, 14 patients (14.74%) patients received LMWH 5 of which (35.71%) received more than 7 days. Low-Dose Unfractionated Heparin (LDUH) is used at the department in accordance with recommendations for thromboprophylaxis prevention and treatment of thrombosis for several decades. In January, 22 patients (31.43%) received thromboprophylaxis with LDUH of which 15 (68.18%) patients received prophylaxis as it was recommended (≥7days). In January utilization was 35.48 DDD/HHB with extra cost of 33.26%. In February, 32 patients (42.67%) received LDUH of which 15 (46.87%) received more than 7 days. In March, from 108 patients, 39 (36.11%) received LDUH of which 17 (43.59%) more than 7 days and the DDD/HHB value was 39.99. In April 34 (35.79%) received LDUH of which 23 (67.65%) received more than 7 days and DDD/HHB value was 34.95.

Regarding to the use of antibiotics for prophylaxis in orthopedic patients we found that ceftriaxone was mostly used antibiotic values for DDD/HHB from 26.24 to 42.74 and in all evaluated period the period of using of this antibiotic was more than 2 days.

Conclusion

Evaluation of the drug consumption at orthopedic department at Clinical Hospital Stip indicates irrational drug use in terms of all monitored indicators and recommendations for rational use of medicines. proper dose, duration of therapy, with the lowest cost for the patient and the community

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Macedonian pharmaceutical bulletin, **62** (suppl) 117 - 118 (2016)
ISSN 1409 - 8695
UDC: 615.2-027.512:347.77.038
Short communication

Intellectual property rights and patent litigation on biosimilar medicinal products

Aleksandra Grozdanova[1]*, Jadranka Dabovic Anastasovska[2], Katerina Ancevska Netkovska[1]

[1] *Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*
[2] *Faculty of Law "Iustinianus Primus", University "Ss Cyril and Methodius", GoceDelcev 9b, 1000 Skopje, Republic of Macedonia*

Biosimilar medicinal products (biosimilars) are defined as highly similar versions of reference biological products, which in the same time are offering new opportunity for innovation in the pharmaceutical industry. The complexity of the manufacturing process of those drugs, the character of the active substance and the nature of their activity, demands a stringent regulatory framework for these medicines. Biosimilar medicines, according to the World Health Organization (which refers to them as "similar biotherapeutic products"), are products which are manufactured following the expiration of the patents or the data protection for the first major group of the originator's biological products. There is a strong interest by healthcare stakeholders in measuring the biosimilar utilization and impact on the market entry. Regulatory issues, manufacturing, safety, pricing, and physician and patient acceptance have a big influence in the developing the biosimilar market. With regulatory approval pathways encouraging more competition among manufacturers of biologics, there follows the prospect of increased litigation and other threats to intellectual property rights. Biosimilar patent litigation continues to evolve as biosimilars enter new global markets. The European Union (EU) was the one of the first highly regulated areas to develop a legal and regulatory framework for the approval for highly similar versions of innovator biological products. Although formal approval pathway for biosimilar began in the EU very early in 2001 (European Medicines Agency, 2003), the European Commission (EC) first amended its market authorization forsimilar biological medicinal products in 2003 (Commission Directive 2003/63/EC, 2003), and issued its first general biosimilar guidance in 2005 (European Medicines Agency, 2005). The European Medicines Agency's (EMA) first approval under the new similar biological medicinal product pathway was in 2006 (Sandoz's somatropin called Omnitrope®) and since then 20 biosimilars are approved in EMA (European Medicines Agency list of approved biosimilars, 2016).While the EU's legal system is complicated by the lack of a unified patent litigation system that requires a multicountry patent litigation approach, the US's biosimilar patent litigation has been complicated by biosimilar applicants and the reference biological product applicants picking and choosing what portion of the default patent litigation exchange system to utilize. Despite the differences between the litigation strategies in the EU and the US, as biosimilar approvals become worldwide more globalized, and it will be increasingly important to appreciate these differences and consideration to how biosimilars patent litigation arguments and outcomes in one situation can benefit and be utilized in other situation, even if in different countries or legal systems.

Still there are some limitation on the patent issue of biosimilars like that the subject matter is not patentable, the invention was not explained clearly or completely enough or the subject matter extends beyond the content of the application filed. From the other side, there are also different possible outcomes such as rejection of patent, the patent is maintained in amended form with a new published specification, or the patent is revoked. Initial opposition decisions may be appealed within two months, and countries may have conflicting rules whether they stay or halt further legal process, national patent infringement actions while an opposition and any associated appeal is pending. The median time for an appeal is close to three years, which is the same approximate median time for an opposition for pat-

* agrozdanova@ff.ukim.edu.mk

ents concerning pharmaceutical and biotechnology products (Patient Protection and Affordable Care Act, 2010). Concurrently or following an opposition, biosimilars patent litigation may begin in member countries but still litigation in each county requires detailed knowledge of that country's national patent litigation procedures.

Due to the differences in patent litigation rules and biosimilars regulatory applications, there may be advantages to bring an initial biosimilars patent litigation in the one primary biosimilar markets, the place of business or manufacture of the biosimilar, or based on a received jurisdictional favoring the action. Because each country has its own law system where patent holders may enforce their patents, there is the possibility for conflicting patent enforcement decisions in different countries, where biosimilar manufacturers may pursue national patent litigations in member countries simultaneously or successively. Therefore there appears to be limited patent litigation challenges in the EU with a preference to patent issuance challenges and some limited country patent challenges. There are many unresolved issues that will affect European patent challenges, especially for bridging biosimilar products on the EU market.

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Macedonian pharmaceutical bulletin, **62** (suppl) 119 - 120 (2016)
ISSN 1409 - 8695
UDC: 615.276.035.7(497.6)
Short communication

Use NSAID drugs with prescpirtion of the doctor or without prescription in the one pharmacy in Bosnia and Hercegovina

Tea Mušić-Dreković[1]*, Vedina Čordalija[2], Maja Malenica[3]

[1]*Mobis pharm, Clinical pharmacist, Jovana Bijelića 8, 71000 Sarajevo BIH*
[2]*Psihiatric hospital, Clinical pharmacist, Panjina kula 73, 71000 Sarajevo BIH*
[3]*Pharmaceutical University in Sarajevo, Medical biohemistry, Branilaca Sarajeva 33, 71000 Sarajevo BIH*

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used medications in modern pharmacy. NSAIDs have anti-inflammatory, analgesic, antipyretic or antireumatic action and differ in activity and severity of adverse effects. These drugs have more or less serious side effects affecting the most gastrointestinal tract. NSAIDs should not be prescribed to patients with gastric or duodenal ulcer, because their use may lead to bleeding and even to a lethal outcome.

All NSAIDs can be structurally divided into several groups: derivatives of salicylic acid, p-aminophenol derivatives, propionic acid derivatives, indole derivatives, phenylacetic acid derivatives, oxicams, and fenamates naftilacetatne acid derivatives. NSAIDs are indicated in different treatments because of their anti-inflammatory, analgesic, antipyretic and antirheumatic effects. The NSAID anti-inflammatory action is due to inhibitory activity on prostaglandin G/H synthase (cyclooxygenase). This enzyme catalyzes the transformation of arachidonic acid to prostaglandins and thromboxanes. There are two forms of this enzyme: COX-1 and COX-2. COX-1 enzyme is normally present in the body, whiles the inducible COX-2 enzyme (cytokines, fibroblasts, epithelial cells) occurs only in inflammatory conditions. Both enzymes have very similar structure (COX-1 consists of 599 amino acids, a COX-2 of 604) (Varagić and Milošević, 1991).

The analgesic effect NSAIDs is achieved by reducing peripheral sensitization of polymodal nocireptor, while antipyretic effect is achieved by inhibition of prostaglandin E2. This prostaglandin is responsible for the activation of a special section in the hypothalamus, which regulates body temperature in the formation of an inflammatory condition. General side effects of NSAIDs are associated directly or indirectly with the irritation of the digestive system. NSAIDs have a twofold effect on the digestive system: acid molecules directly irritate the lining of the stomach, and the inhibition of cyclooxygenase-1 reduces the levels of protective prostaglandins.

Common side effects on the digestive system include, nausea, vomiting, dyspepsia, stomach ulcer/bleeding in the stomach, diarrhea. Higher doses treatment is associated with increased risk of ulceration. In efforts to reduce the adverse effects on the digestive system, it is recommended to use the lowest effective dose for the shortest possible period, which is not the case in practice (Walker and Edwards, 1998).

Materials and methods

In this study, data were collected at the pharmacy, where we monitored quantity of prescription for NSAIDs, and the utilization of these drugs issued at the request of the patient (without a prescription). Evaluation lasted one month and twenty days, in which we monitored the amount of received recipes and amount of patients who take this medication without consulting doctor or pharmacist. Also we asked doctor how many patients come and ask for a prescription for NSAIDs and how they used them.

Results and discussion

On average 200 patients come in the pharmacy and 130 out of them were looking for drugs that are used as

* tea_music@hotmail.com

analgesics or antipyretics, whereas approximately 90 patients used some NSAIDs. Only 15 out of 90 patients have a prescription for the drug. Patients were not familiar with the problems that are linked to use of this type of medication. According to results obtained from doctors approximately 85% of patients who use NSAIDs do not have a doctor's prescription. Patients used these drugs mainly for treatment of pain, and they chose them because are easily available.

Conclusion

NSAID medicines are of great importance and one of the most commonly used medicines in hospitals and outpatient conditions. They are relatively safe drugs but their complications sometimes can be severe and fatal. Patients with current ulcer disease are more eligible for treatment with proton pump inhibitors in therapeutic dose, while patients without current ulcer with a history of previous illness of bleeding for treatment with dose of maintenance. The use of selective COX-2 inhibitors is justified for patients at risk for gastrointestinal complications with previous consultation with doctor or pharmacist.

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Pharmaceutical analysis/ Quality assurance/ Regulatory affairs

Macedonian pharmaceutical bulletin, **62** (suppl) 123 - 124 (2016)
ISSN 1409 - 8695
UDC: 614.35:615.1
Short communication

QRM in the GMP environment - 10 years on since ICH Q9... Are medicines any safer now?

Kevin O'Donnell

Health Products Regulatory Authority, Kevin O'Malley House, Earlsfort Centre, Earlsfort Terrace, Dublin, Ireland

Background - ten years of QRM developments

Over the past ten years, since the finalisation of the ICH guideline titled Quality Risk Management, ICH Q9, there have been significant efforts by industry and regulators to drive risk-based approaches forward. This has included initiatives by regulators to update the GMPs and other official guidance documents to incorporate increased provisions relating to risk and its management (Campbell et al., 2016; European Commission, 2010; O'Donnell et al., 2012). There have also been many industry-led publications, guides and technical reports, offering practical guidance for how to implement the concepts of QRM in the GMP environment (Calnan et al., 2013; Vesper et al., 2006).

However, the question remains as to whether the management of risk in relation to the production of defective and sub-standard medicines has significantly improved in these last ten years, and whether, from a product quality perspective, medicines are any safer now than they were before the ICH Q9 guideline was introduced. While there are many different areas one could study when considering the question of medicines safety, such as pharmacovigilance, Marketing Authorisation withdrawals and drug shortages, to name but a few, the focus of this presentation is on GMP, and specifically on product quality considerations.

Looking for the added value for patients

The presentation discusses how, while the aforementioned QRM initiatives had product quality and patient safety as their overall focus, many of the product quality and compliance issues occurring before 2005 are still with us, and the evidence indicates that certain types of problems are increasing (Waldron et al., 2015). Some of these relate to:

- Serious quality defects and product recalls continuing to occur globally;
- Market Authorisation non-compliance issues sometimes resulting in the cessation of batch release;
- Product shortages as a result of serious GMP failures.

QRM and risk assessment inspection deficiencies

In relation to serious quality defect issues, the presentation explores that various factors that may have led to the level of those issues seen in the last ten years - and how formal research projects are currently underway in Ireland and at the European Medicines Agency to better understand this area. Of concern is the fact that most of these defective batches were manufactured using qualified equipment, trained staff, and validated manufacturing processes, and this leads one to question what is happening. The paper considers:

- Whether the manufacturing and control processes currently in place have been properly risk assessed and designed using QRM principles.
- Whether qualification and validation activities were actually based on risk, as required by Annex 15 of the EU GMPs. If so, why are defective batches of medicines still being produced, with the same kind of defects over and over – product contamination, mix-ups, labelling errors, etc.?
- What areas need most attention in the QRM activities at manufacturing sites?

* kevin.odonnell@hpra.ie

Improving QRM activities in the GMP environment

The presentation explains how, while GMP Inspectors have noted an increasing use of risk assessment and QRM activities at sites since ICH Q9 was finalised in 2005, and while some good practices have been seen in these areas (Fischhoff et al., 1993; Kahneman et al., 1972), significant concerns remain. These relate to the lack of good science, robustness and rigour in many of risk assessment and QRM-related activities taking place currently. The presentation presents a series of brief real-life case study examples showing some of the inspectional deficiencies that the HPRA has cited in recent years in these areas, and it also presents a number of suggestions and ideas for how to improve QRM activities in the GMP environment.

Notes

The views expressed in this paper are those of the author and do not necessarily represent the views of the HPRA. This presentation is based on a paper published in the Journal of Validation Technology in December 2015 (O'Donnell, 2015).

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Macedonian pharmaceutical bulletin, **62** (suppl) 125 - 126 (2016)
ISSN 1409 - 8695
UDC: 614.35
Short communication

The challenges of the qualified person in a complex pharmaceutical quality system

Miroslava Ilievska[1]*, Nina Ekart Oman[2], Jasmina Velevska Ivanovska[1], Verce Jovanovska Jankovska[1], Katerina Beldedovska Aleksievska[1], Maja Velinovska Chadinoska [1], Ksenija Brzilova[1], Ornela Kuzmanovska[1]

[1]*Alkaloid AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia*
[2]*Alkaloid INT Ljubljana, Šlandrova 4, 1231 Črnuče, Republic of Slovenia*

Over the last years the role and responsibilities of the Qualified Persons have been increasing considerably. As a key person in the company, the Qualified Person has to consider many issues and has to take up the challenges within its areas of responsibilities. The previous year 2015 was an eventful year. Again there were new developments in the Good Manufacturing Practice environment as well as announcements of changes that preoccupied the pharmaceutical industry. 2016 won't be less exciting, also because now many of the new requirements must be implemented:

- Excipients - Formalized Risk Assessment, EMA Guideline, "… on the formalized risk assessment for ascertaining the appropriate good manufacturing practice for excipients of medicinal products for human use", In operation from 21. March 2016 (EudraLex, 2015a; ICH, 2005).
- Qualified Person should be fully involved in the system, in the new concept of cleaning validation with Toxicological Evaluation - Shared Facilities, EMA/CHMP/ CVMP/ SWP/169430/2012 / Guideline on setting health based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities. Describes determination of Permitted Daily Exposure (PDE), on basis of all available pharmacological and toxicological data (clinical and non clinical) (CE, 2001; EMA, 2012; EudraLex, 2016).
- EU-GMP Guideline Part I, Chapter 3 "Premises and Equipment", EU-GMP Guideline Part I, Chapter 5 "Production", Prevention of Cross Contamination Chapter 5.17 - 5.22 (EudraLex, 2010).
- Revision Annex 15 – news in Qualification & Validation… includes elements of ICH Q8, ICH Q9 and ICH Q10 and advancing technologies, such as PAT and references the new chapters 3 & 5, coming into operation 1 October 2015 (EudraLex, 2010; EudraLex, 2015b).

A comprehensively designed Pharmaceutical Quality System (PQS) incorporating Good Manufacturing Practice (GMP) and Quality Risk Management (QRM), implemented, maintained and continuously improved, allows a consistent delivery of products with appropriate quality attributes. The level of GMP, is regularly checked by competent authorities, authorized with a GMP Certificate, and it is a basis for maintenance of Marketing Authorization (MA) and marketing of medicinal products within the EC/ EEA (CE, 2001; CE, 2003).

The results of product and processes monitoring are taken into account during batch certification, having into consideration deviations or OOS, OOT or OOE (if any) by implementing QRM. (EudraLex, 2016; ICH, 2005).

The Qualified Person is responsible for ensuring that each individual batch has been manufactured and checked in compliance with laws in force, in accordance with the requirements of the marketing authorisation (MA) and with Good Manufacturing Practice (GMP) (CE, 2001; EudraLex, 2016).

When the batch is intended to be sold in the EU, in the new annex is clear on this: "Samples may either be taken after arrival in the EU, or be taken at the manufacturing site in the third country in accordance with a technical-

* milievska@alkaloid.com.mk

ly justified approach which is documented within the company's quality system. Any samples taken outside the EU should be shipped under equivalent transport conditions as the batch that they represent (EudraLex, 2016).

Marketing authorizations require a QP declaration (issued by the Qualified Person) to confirm that the active substance (active pharmaceutical ingredient - API) has been manufactured in accordance with the EU-GMP Guide, Part II: Basic Requirements for Active Substances used as Starting Materials. The QP declaration template provides, in a format considered suitable for submission, a basis for demonstrating compliance of the active substance manufacture with GMP requirements, based on audit on site(s) and that the manufacturer has relevant knowledge of the supply chain (EMA, 2014).

Relying on assessment by third parties, e.g. audits, should be in accordance with Chapter 7 of the GMP Guide in order to appropriately define, agree and control any outsourced activity. Special focus should be given to the approval of the audit reports (CE, 2003).

A signed Quality or Technical Agreement(s) between all parties involved in the supply chain, have to be in place, including manufacturers of the starting materials, in bulk and finished products manufacturers, batch control testing laboratories, importation and batch certification and release site(s), transportation and distribution. The entire supply chain of the active substance and medicinal product up to the stage of certification is documented and available for the QP. This should include the manufacturing sites of the starting materials and packaging materials for the medicinal product and any other materials deemed critical through a risk assessment of the manufacturing process. The document should preferably be in the format of a comprehensive diagram, where each party, including subcontractors of critical steps are included (CE, 2003, EudraLex, 2016).

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Macedonian pharmaceutical bulletin, **62** (suppl) 127 - 128 (2016)
ISSN 1409 - 8695
UDC: 615.074
Short communication

Quality control of drug products: implementation of the new ICH Q3D guideline on elemental impurities

Gregory Lecornet

Agilent Technologies, 3 Avenue du Canada, Z.A. Courtaboeuf, 91970 Les Ulis, France

Introduction

The presence of impurities in pharmaceutical samples is a concern, not only because some contaminants are inherently toxic, but also because they may adversely affect drug stability and shelf-life, or may cause unwanted side-effects. As a result, inorganic elemental impurities (As, Pb, Cd, Hg,…) must be monitored and controlled in raw materials, including: water, used for drug manufacturing; intermediates; active pharmaceutical ingredients (APIs); excipients and in the final dosage form.

ICH, which includes the regulatory bodies from the US, Europe and Japan, as well as representatives from other countries and the pharmaceutical industry, has defined a Guideline for Elemental Impurities (Q3D) due to be implemented in December 2017 (Liba et al., 2011).

The new methods will introduce the use of closed vessel sample digestion and modern instrumental techniques (ICP-AES, ICP-MS) to ensure the accurate recovery and determination of individual analyte (McCurdy et al., 2004)

Material and methods

An Agilent 7800 ICP-MS with Octopole Reaction System (ORS4) fitted with a low-flow concentric quartz nebulizer was used for all measurements. The objective was to use the ICP-MS to determine a full range of elements. The ORS4 was operated in helium collision mode (He mode) only, which is effective at removing a wide range of plasma and matrix-based polyatomic species using kinetic energy discrimination (KED). Because He mode does not react with any analyte, and does not create any new interferences, elements that do not suffer from polyatomic interferences were also analyzed using He mode

Reagents and materials

HNO3 (BASF); ultrapure water (18.2 MΩ) produced by Milli-Q ultrapure water systems; 100 g/mL of multi element internal standard solution containing 6Li, Sc, Ge, Rh, In, Tb, Lu and Bi (Agilent Part # 5188-6525); multi-element calibration standard STD-2A solution (Agilent Part # 8500-6940)

Results and discussion

In this study, we investigated the use of collision cell technology using helium gas to eliminate interferences generated by the plasma and the sample matrix. Typical matrix-derived polyatomic interference ions arising from the analysis of gelatin capsules include 40Ar12C+, 51V1H, 35Cl16O1H and 40Ca12C, etc. on 52Cr and 40Ar13C, 37Cl16O and doubly-charged $^{106}Ru^{++}$ on 53Cr. The total dissolved solids (TDS) of the samples were less than 0.2% and any matrix effects were eliminated via the use of the internal standard calibration method.

Conclusion

Operating the Agilent 7700x with ORS3 in helium mode effectively removes polyatomic interferences via kinetic energy discrimination allowing the rapid, accurate, semi-quantitative screening of complex sample types, such as gelatin capsules. For each sample, semi-quantitative and full quantitative analysis can be performed simultaneously without the need to adjust the instrument.

* gregory.lecornet@agilent.com

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Macedonian pharmaceutical bulletin, **62** (suppl) 129 - 130 (2016)
ISSN 1409 - 8695
UDC: 633.888:615.212.7.074
Short communication

Chemometrics - powerful tool in tracking the origin of cannabis samples?

Slavica Ražić[1]*, Nataša Radosavljević-Stevanović[2]

[1] *Department of Analytical Chemistry, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia*
[2]*The National Crime - Technical Centre, Ministry of Interior, Republic of Serbia, Bulevar Dr Zorana Đinđića 104, Belgrade, Serbia*

Introduction

Illicit production and trade of Cannabis affects many societies. This herb is mostly produced for domestic or regional markets and its production in Europe is believed to be increasing in indoor settings, under the control of organized crime groups. Usually, the authorities are faced with the problem of a lack of information on where the cannabis was grown. This material is also widely consumed by the population in each country and therefore establishing of system for classifying the origin of this plant can be a challenge for the scientists and a great benefit for the police authorities.

When drugs are concerned and especially for the forensic purposes, the most significant compounds in Cannabis samples are cannabinoids, terpenophenolic compounds unique to cannabis. For the forensic practice, the most important cannabinoids are: Δ9-tetrahydrocannabinol (Δ9-THC), cannabinol (CBN) and cannabidiol (CBD). The first compound, Δ9-THC is psychoactive component, while the other two mentioned cannabinoids are not. On the other, hand, transition metals are important for plant growth and they are distributed in different cells, in certain concentrations due to the established homeostasis. Hemp has the ability to tolerate and accumulate heavy metals. Bearing in mind the lack of literature on possible correlations between content of cannabinoids in Cannabis plants and metals in both Cannabis samples and soil where the plants were cultivated, multivariate methods were applied aiming to assist in determination of origin of cannabis and its production.

Materials and methods

Young plants of Cannabis sativa species had been seized by the Police authorities as material planted by the criminal groups on different locations in Serbia. The plants were grown illegally under controlled indoor conditions by applying certain levels of temperature, humidity and intensity of light. The plants were randomly selected from the plantations in the early growing stage for the purpose of forensic analyses. The rhizosphere soil from the root zone at a depth of 5-10 cm was sampled. The plant samples were further separated into roots, stems and leaves. The roots parts were cleaned from traces of soil, washed with deionised water and dried. The Cannabis leaves were dried and grounded to a powder. The mass of 33.3 mg of dried leaves material was dissolved in 5 mL of methanol, shaken in an ultrasonic bath for 30 min, and filtered. The filtrate was heated for 12 min at 150 °C in order to run decarboxylation of the cannabinoid acids into cannabinoids. The evaporated extracts were reconstituted with 2 mL of methanol. The resulting solution of 1 µL, for each sample was applied on the TLC plate together with the volume of 1 µL of the dissolved reference materials: Δ9-THC, CBN and CBD in methanol in concentration of 0.05 mg/mL and 1 µL of pure methanol. Mobile phase was n-hexane: diethyl ether in ratio 4 : 1. Visualization was conducted by the dissolved Azoic Diazo Component in 0.1 M sodium hydroxide in water.

In the second step, 20 mg of each sample were dissolved in 5 mL methanol, shaken in an ultrasonic bath for 30 min, filtered and evaporated to dryness. The residue was reconstituted with 2 mL of methanol. 1 µL of the resulting solution for each sample was injected into the GC-FID system in order to analyse the content of cannabinoids.

* slavica.razic@pharmacy.bg.ac.rs

For the purpose of elemental analysis, the sampled soils, roots, stems and leaves were subjected to microwave-assisted acid digestion, according to a known procedure (Razic et al., 2006).

The determination of the cannabinoids by thin layer chromatography (TLC) with plates made of aluminium and covered with silica gel with fluorescence indicator of UV254, by conducting the semi quantitative analyses.

The determination of the cannabinoids was performed using an Agilent GC System, Model 7890A, fitted with a Flame Ionization Detector. The conditions were as follows: column HP-5 (30 m × 320 μm × 0.25 μm), injection temperature: 250 °C, splitless mode, oven program: initial temperature 150 °C for 0 min, heating rate 15 °C/min to 300 °C and held for 5 min, nitrogen flow rate: 46 mL/min.

The determination of the Cu, Zn, Mn, Fe, Ca and Mg was conducted on a Perkin-Elmer Model 5000 atomic absorption spectrophotometer, operated under optimized measurement (Razic et al., 2005). The determination of the Cr was accomplished using a Perkin-Elmer Model 5000 atomic absorption spectrophotometer with a graphite furnace HGA 400 Automatic Burner Control (Razic et al., 2006).

Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, IL) and Minitab 13.20 (Minitab Inc., State College, PA), for Windows software packages.

Results and discussion

Since the roots contain only trace amounts of these substances while the stems, branches and twigs have less than the leaf material, the cannabinoid content was measured only in the leaves. For the purpose of comparison of the cannabinoid content in the young plants and the mature one the semi quantitative method of the TLC was conducted and the difference in the cannabinoids content was detected.

The cannabinoids content in the leaves of the examined cannabis plants was determined by external calibration. The values varied from 0.03 to 1.47 mg/mL for THC and from 0.11 to 1.12 mg/mL for CBN. The lowest levels were from 0.003 to 0.06 mg/mL for CBN, what is reasonable since the level of this cannabinoid, as degradation product of THC, increases with time during storage of cannabis plants.

Quantification of metals was performed by external calibration. The accuracy of the methods was checked by analysis of a standard reference material, NIST SRM 1547 – Peach Leaves and NIST SRM 2711 - Montana II Soil, when satisfactory recoveries (90.06 - 115.35%) were obtained.

The objective of this study was to analyse the correlations of seven metals and three main cannabinoids using multivariate methods of analysis. After preliminary tests (Ryan-Joiner and Grubbs tests) the concentration data were subjected to principal component analysis (PCA) in order to highlight any relations between the elements. Four principal components (PCs) appeared to account for 89.42% of the variance of the data. These factors are related to the sources of the elements and cannabinoids in the studied samples. The first factor comprises Mn, THC and CBN with high loadings. A correlation like this indicates that they arise from the same sources. Fe and Cr show significant positive loadings in the second factor and are negatively correlated with Zn and CBD with high negative loading. The third factor is dominantly loaded by Cu as an essential element; a constituent of co-enzymes important for a plant cycle. It is also correlated with Mg, with a high but negative loading. These correlations led to the consideration of the biosynthesis of cannabinoids and the involvement of metals as cofactors in the enzymatic catalyzed synthesis cycles (De Meijer et al., 2002; Pate, 1994; Radosavljevic-Stevanovic et al., 2014; Sirikantaramas et al., 2005; Taura et al., 2007).

Conclusion

This work is conducted in order to determine whether the metals content in rhizosphere and cannabis can affect the levels of three important cannabinoids. The obtained results were subjected to chemometrics evaluation which gave an opportunity to get a better insight into role of metals in the biosynthesis of cannabinoids. Identified patterns could be helpful in tracking the origin of Cannabis plant and its product adding a value to the applied analytical and chemometrics methods in forensic sciences.

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Macedonian pharmaceutical bulletin, **62** (suppl) 131 - 132 (2016)
ISSN 1409 - 8695
UDC: 343.52:615.012]:614.35
Short communication

Pattern recognition techniques in preventing of API falsification

Jelena Acevska*, Katerina Brezovska, Natalija Nakov, Rumenka Petkovska, Aneta Dimitrovska

Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. Macedonia

Background

Falsification of active pharmaceutical ingredients (APIs) has been recognized as an important health issue since a series of health scares involving large numbers of casualties happened when altered APIs escaped detection in routine analytical testing (Dégardin et al., 2014).

API falsification usually means discrepancy between the information for declared manufacturer and the actual manufacturer of the APIs used for manufacturing of finished dosage forms (FDF). Any form of tampering the content or information about the content might affect the quality of an API and therefore constitutes a direct threat to the health of patients. Since the reason for any falsification is fast and easy money, there is no general rule what APIs can be subjected to falsification. For any substance that can bring economic benefits to the manufacturer / distributor there is a potential risk to be falsified.

The conventional analytical approach often fails to detect falsification since the quality and quantity of the API is within the standards. Instead, methods that allow for distinguishing API samples and determining the source of APIs suspected for falsification are needed. Alternative approach must include broader analytical window using specific techniques to reveal some characteristic analytical information, such as impurity profile, spectral data, physicochemical constants, etc., thus creating an 'API fingerprint' database. APIs can be characterized, compared and classified based on features revealed from such 'fingerprints', using chemometric methods, like different pattern recognition techniques (PRT) (Acevska et al., 2015; Petrusevski et al., 2015).

The aim of this research was to give an overview of analytical and chemometric methods used to differentiate the origin of the samples based on a 'fingerprint' database by the discriminatory matching of specific analytical features of the specific API.

API fingerprinting

API fingerprinting is the common name of a group of analytical applications for detection, identification and quantitative determination of related substances and other impurities. Impurity profiling (organic, inorganic impurities, residual solvents, enantiomeric purity), chemical characterization (polymorphism/solvatomorphism) and other contaminants (radioactivity) of the APIs requires advanced and robust fingerprint method. It usually comprise a set of several highly specific techniques, like: liquid and gas chromatography coupled with mass spectrometer (LC/MS, GC/MS), differential scanning calorimetry (DSC), & differential termogravimetric analysis (TG/DTG), Furrier-transformed infrared (FTIR), near infrared (NIR), Raman or nuclear magnetic resonance spectroscopy (NMR); inductively coupled plasma combined with optical emission (ICP-OES) or mass spectrometry (ICP/MS), X-ray diffraction (XRD) etc. The characteristic and reproducible chemical features revealed during the API fingerprinting are used for discriminating between samples. This data highlights the API fingerprinting as core activity in modern drug analysis and tool for getting reliable results for combating API falsification.

Pattern recognition of API fingerprints

Pattern recognition techniques that can be applied to API fingerprinting consist of two general areas; unsupervised or supervised. From the characterization of the samples of known origin, predictive models can be built and

* jelena@ff.ukim.edu.mk

tested with new samples of unknown composition. By determination of different origins of the APIs, their falsification can be detected.

In unsupervised pattern recognition (hierarchical cluster analysis (HCA), principal component analysis (PCA), generally viewed as exploratory data analysis methods, from which natural clusters in the data set can be identified, no knowledge concerning the origin of the samples is necessary. HCA gives no indication of the variables which contribute most to the classification of objects in the data set, unlike PCA, where the loadings indicate the variation contained within each variable. There may be a loss of information in the dendrogram generated by HCA, especially if clusters are not well resolved. However, HCA does present all the variation in the data set, in contrast to PCA where only a percentage of the variation is typically presented (Adams, 2004).

In supervised pattern recognition (classification and discriminant analysis (CDA), orthogonal partial least square regression (OPLS/O2PLS), soft independent modeling of class analogy (SIMCA), etc.), the groupings of samples must be known to allow predictions to take place. These methods require prior knowledge of the sample origin to develop models, which are subsequently used to assign unknown samples to a parent group. The OPLS-DA method is well suited for classification of data that have multi-collinear and noisy variables, which is common for many types of chemical data. Refined properties of the OPLS-DA methodology have illustrated how Y-orthogonal variation can be useful not only for evaluation, but also for classification, thus rendering a PLS-DA/SIMCA hybrid discriminatory model (Bylesjo et al., 2006).

Validation of the classification method

The outcome of the investigation on API falsification should be accurate and reliable. For sample authentication purposes, it is essential to ensure that their affiliation is properly defined (Gonzalez, 2007). In other words, detection of false negatives (excluding sample that belongs to the class) and false positive (includes a sample that does not belong to the class) results must be enabled. Ultimately a method should be found for which there are no false negatives at the cost of several more false positives. For illustration, obtaining a false positive result during the API fingerprinting, means further more detailed analytical testing of the substance suspected to be falsified. But the risk of obtaining a false negative result must be completely removed, as it is unacceptable to pass a falsified API to market.

Perspective

There are several milestones (sampling, choice of analytical and statistical methods, and dissemination of results) that need to be challenged.

Sampling is mainly based on a goodwill of the manufacturers/MAHs; the regulatory frame need to be strengthen for sampling can be possible on each step where tampering of substance or information of its origin might occur (manufacturing site, distribution, brokers if any, FDF manufacturer, APIs used for preparations in pharmacies).

Number of samples is often insufficient for robust statistical evaluation of results; wider involvement of OMCLs and regulatory authorities (including customs and police) is expected for more efficient sampling process.

Manufacturer's specification is not sufficient for API fingerprinting; an overall strategy against falsification including wide analytical window in combination with PRT should be implemented by competent authorities.

Dissemination of the results is sometimes burdened by intellectual or industrial property issues and therefore sometimes is only used by API manufacturer for protection from competition; in order for results to be used for better control of the APIs used for the medicines available on the market, proper agreement between industry and competent authorities should be in place.

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Macedonian pharmaceutical bulletin, **62** (suppl) 133 - 134 (2016)
ISSN 1409 - 8695
UDC: 615.2-027.512:615.11
Short communication

Orphan drugs - comparative review of FDA and EMA regulations

Zoran Nakov[1]*, Jasmina Tonic-Ribarska[2], Suzana Trajkovic Jolevska[2]

[1]Novo Nordisk Pharma DOOEL, blvd.Oktomvriska Revolucija 18, 1000 Skopje, Macedonia
[2]Faculty of Pharmacy, University "SS Cyril and Methodius", Mother Tereza 47, 1000 Skopje, Macedonia

Introduction

Orphan drug is a drug intended for treatment of rare (orphan) diseases. No single definition for an orphan disease exists worldwide, but it is generally a disease that affects a small portion of the population. Today, there are more than 6,000 to 7,000 known rare diseases, 80 percent of which have been identified as genetic in nature, with incidence of less than one in 2,000 people. Symptoms of some rare diseases may appear at birth or develop later in childhood or even during adult life. Other rare diseases are a result of infection (bacterial or viral), allergies or are caused by degenerative and proliferative conditions (Orphaned, 2012). Currently, the number of rare diseases for which there is no available treatment is estimated to be around 4,000 - 5,000 worldwide (Morin et al., 2013). The objective of this paper is to compare the regulation for orphan drugs defined by Food and Drug Agency (FDA) and European Medical Agency (EMA), in the United States and EU, respectively. These two regulatory agencies stimulate processes of a parallel application for orphan designation appropriate, when dealing with potential orphan drugs.

FDA regulations for orphan drugs

The first regulation for orphan drugs in USA was introduced in 1983. According to the FDA guidelines, orphan drug is a drug that is intended for treatment of disease or condition which affects less than 200,000 people in the USA or more than 200,000 in case when there is no reasonable expectation that the cost of developing and making available this drug in the USA will be recovered from it sales (Morin et al., 2013). Today, in USA there are three laws that apply to orphan drugs, as follows:

1. Orphan Drug Act (first regulation for orphan drugs) - legal act which aims to protect every orphan drug, produced and placed on the market in the USA by the pharmaceutical company, domestic or international. This regulation assumes that the drugs intended to treatment of rare diseases need patent protection, because basically this class of drugs is characterized by lower return of invested funds (Abramowicz, 2010).

2. Protection from generic competition - reference this legislation, when one drug is designated as "orphan" drug, FDA cannot give designation to a generic drug for the period of five to seven years. The main purpose of this legislation is to discourage the idea of development of non-patented drugs. This legislation allows better protection from generic competitors than the Orphan Drug Act, and the same applies for drugs intended for treatment of diseases that affect more than 200,000 people but the costs of research and development will not be cover by their projected sales (Abramowicz, 2010).

3. Hatch-Waxman Act is regulation for stimulating the generic competition. Under this act, a generic drug company that challenges the pioneer drug with patent protecting receives 180 days exclusivity period, meaning that no other generic drug manufacturer can enter the market during that time. The main goal is to accelerate the generic competition. The authorized generic manufacturer can charge considerably above marginal cost, allowing it to earn a profit and providing it an incentive to challenge pioneer patents (Abramowicz, 2010).

According to the FDA regulation, orphan drugs have market exclusivity for period of five to seven years, shortened procedure of registration; the company owner of orphan designation receives a tax incentives, protocol assistance as well as exemption from payment of certain fees (Morin et al., 2013).

* zona@novonordisk.com

EMA regulations for orphan drugs

The regulation for orphan drugs in EU legislation was first time adopted in 1999. According to EMA rules, orphan drug is designated drug which is intended for treatment of rare disease or condition which affects not more than 5 in 10,000 people in the EU. Most of these people suffer from diseases affecting less than 1 in 100,000 people (EMA, 2015). At the European Union granting of orphan designation can be made only by the European Commission. The application is evaluated by the EMA's Committee for Orphan Medicinal Products (COMP), which provides its opinion on whether or not the medicine qualifies as orphan medicines for the treatment, prevention or diagnosis of a rare disease. If the COMP issues a positive opinion, the European Commission may then grant the medicine orphan status (EMA, 2015). Rules for obtaining the orphan designation at EMA are defined by six regulations and two additional guidelines (EMA, Legal background).

To qualify for orphan designation, a medicine must meet a number of criteria: it must be indicated for the treatment, prevention or diagnosis of a disease that is life-threatening or chronically debilitating, the prevalence of the condition in the EU must not be more than 5 in 10,000 or it must be unlikely that marketing of the medicine would generate sufficient returns to justify the investment needed for development or there is no satisfactory method of diagnosis, prevention or treatment of the condition concerned can be authorized or if such a method exists, the new medicine must be of significant benefit to those affected by the condition. Drugs that meet the EMA's requirements for orphan designation received protocol assistance, access to centralized approval procedure for marketing authorization, a period of ten years market exclusivity in the EU with the possibility of additional two years market exclusivity for drugs that have valid and approved pediatric research plan (pediatric investigation plan). Additionally, all drugs with orphan designation by EMA received further compensate for micro, small and medium-sized companies, reduction of costs, grants and national compensate in the EU Member States (EMA, Activities after orphan designation).

Instead of conclusion

The comparative review of FDA / EMA regulations showed that FDA and EMA permit shortened registration procedure and both regulatory agencies allow exemption from payment of certain fees and provide protocol assistance. There are three main differences between FDA and EMA. The first difference is period of market exclusivity, noted that the period of market exclusivity in the USA could be from five to maximum seven years, apart from EU where market exclusivity for drugs with orphan designation is ten years, with additional two years for orphan drugs with valid and approved pediatric research plan. The second difference is in terms of tax incentives and exemptions, which are currently permitted in general in the all the USA countries by the FDA, as opposed to the EU, where EMA gives general tax incentives and exemptions, but each EU Member State has the possibility to provide additional national exemptions and incentives for some orphan drugs. And the third difference exists in terms of grants. In the USA they're getting from the part of the FDA Orphan products grants program. Unlike the FDA, EMA does not offer any additional grants from its budget for research and development of new orphan drugs. However EMA makes funds available through the European Commission such as Horizon 2020, The EU Framework Programme for Research and Innovation E-Rare, a transnational project for research programmes on rare diseases International Rare Diseases Consortium (IRDIRC).

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Macedonian pharmaceutical bulletin, **62** (suppl) 135 - 136 (2016)
ISSN 1409 - 8695
UDC: 615.014.4
Short communication

Development of cleaning validation master plan, including cleaning validation protocol

Nade Dimovska*, Nena Smiljanovska, Marina Petreska, Keti Shapkovska, Stojne Tanevska

Bionika Pharmaceuticals, Skupi 15, 1000 Skopje, Republic of Macedonia

Introduction

Development and validation of effective cleaning procedures are of a great importance to Quality Assurance System and these activities are described in the current GMP and FDA regulations.

In many cases, the same equipment may be used for manufacturing of different products. It is therefore essential to develop adequate cleaning procedures, in order to avoid cross contamination. Pharmaceutical products and active pharmaceutical ingredients (APIs) can be contaminated by other pharmaceutical products, by cleaning agents, microorganisms or by other material (i.e. airborne particles, dust, raw materials, products of degradation (EudraLex, 2014).

The cleaning validation is a very complex task, and the implementation requires a carefully elaborated plan. The Cleaning Validation Master Plan is a basic document according to which the validation is performed. It presents an overview of the scope of the project, establishes essential requirements, such as acceptance criteria, sampling procedures, methods of analysis and is used as a planning and monitoring instrument (EudraLex, 2015).

Establishing a cleaning validation program

Written cleaning procedures must be established for all equipment parts and surface which come into contact with the medicinal product. Responsible stuff has to be trained on the cleaning procedures. The critical parameters of the cleaning procedures can be determined by the risk-analysis. The risk-analysis should evaluate the influence of the product, equipment and process specific parameters on the cleaning objective (Hiob and Gomez, 2011).

Important phase during effective planning of cleaning validation study is selection of the worst-case product, manufactured in each equipment line. To assure effectiveness under worst-case conditions, the worst case must be defined in more exact terms. The first thing to be determined is whether all the products made using the equipment, can be cleaned according the same procedure, or several cleaning procedures are required due to different product properties. Within each group of products, for which an individual cleaning procedure is required, the product which is the most difficult to clean represents the worst case. If the cleaning procedure is designed in such a way that this product can be cleaned in a reliable manner, the effectiveness of the cleaning procedure is assured for all other products which are easier to clean and are in the same group as well (Haider and Asif, 2010a).

In the selection of worst-case product, the solubility of the ingredients, the tendency of the product towards crust formation and where relevant, residues left by colored ingredients should be reviewed as being critical factors to the success of the cleaning. The pharmacological characteristics and toxicity data of drugs have a significant impact on the selection of worst-case product also.

During the optimization phase the following information must be defined: maximum hold time for uncleaned equipment and clean hold time.

Analytical testing and reporting phase

Dirty hold time is defined as the time between the end of manufacturing process of the product batch and the beginning of the cleaning process. Clean hold time is the time between the completion of cleaning of the equipment and the initiation of the subsequent manufacturing operation, if in the production area the conditions are appropriate.

* nade.dimovska@bionikapharm.com

Acceptance criteria must be defined before the validation begins. A limit is an actual numerical value and is one of the requirements of the acceptance criteria of a cleaning validation protocol. Compliance with limits can be assured by direct (swab test) or indirect sampling (rinse test). Sampling (by swab) should be performed from the critical areas of the production equipment. These critical areas of the production equipment are usually difficult to access during cleaning and/or are very difficult to see during the final checks. These areas, as well as dirty and clean hold times, should be included in the cleaning Standard Operational Procedures (FDA, 2011).

Maximum Allowable Carryover (MACO) - maximum quantity of acceptable transferred residue from the active substance of the previous product is calculated by therapeutic dose criteria or by permitted daily exposure criteria.

The quantitative determination of residues of the substances, cleaning agent and microbiological contamination that should be carried out after sampling, will require validated analytical methods with sufficient specificity and sensitivity to be able to detect the results at or below the established acceptance limits.

Validation of analytical methods are carried out on the parameters: Limit of Detection (LOD), Limit of Quantification (LOQ), Specificity, Selectivity, Recovery, Precision, Range and Linearity (Borchert and Gomez, 2011a).

Validation of the cleaning procedure should be performed analytically after the approval of visual inspection (absence of stains or any materiel residue).

Selecting a method to detect cleaning agent be performed analytically after the approval of visual inspection (absence of stains or any materiel residue).overyraphy (HPLC), ion selective electrodes, flame photometry, derivative Ultraviolet (UV) spectroscopy, Thin Layer Chromatography, enzymatic detection, and titration.

It can also involve non-specific methods that detect the presence of a blend of ingredients such as: TOC, pH, and conductivity. The regulators prefer specific methods, but will accept non-specific methods with adequate rationales for their use. For investigations of failures or action levels, a specific method is usually preferable.

For the swab sampling method it is necessary to determine: the percent recovered with the swab extraction procedure; the effectiveness of the swab at recovering residues from equipment parts surface and the interference of swab materials in the analysis.

For the rinse sampling method it is necessary to determine: the percent recovered with the rinse solution extraction procedure; the effectiveness of the rinse solution at recovering residues from equipment parts surfaces and the interference of the rinse solution in the cleaning procedure and analysis; a correction for recovery efficiency in calculations for acceptable residue levels.

The percentage of recovery is important because it will be applied when evaluating the final concentration of residuals.

All equipment that comes in contact directly with raw material -intermediate as well as final product - must be included, because of its potential to act as a possible source for microbiological contamination. The microbial testing is performed on the selected surface of the equipment in order to determine the number of colony forming units (CFUs) present.

Analysts from Quality Control Department will perform the analysis on the swab and/or rinse samples. The results are reported in Quality Assurance Department and the Quality Assurance Manager will give final approval to the reviewed results by signing the final Report (Haider and Asif, 2010b).

Validated cleaning process must be subject to change control. Further, revalidation is necessary in order to maintain the validated status where a differentiation must be made between change-related and periodic revalidation. An efficient change control procedure must ensure that the effect of changes on the cleaning validation is identified and that any necessary follow-up action is initiated. Checks must be carried-out to verify whether revalidation is necessary following changes to the cleaning procedure, production equipment and products. A suitable time interval must be determined for the periodic revalidation. Implementation may be streamlined by linking this to the change control procedure and by reducing the scope of the validation based on a review of data and documents (Borchert and Gomez, 2011b).

Conclusion

Cleaning procedures should be suitable for their intended purpose. This should be confirmed by the successful execution of a cleaning validation study. The cleaning validation must ensure that the quality of the product produced using specified equipment is not influenced by cross-contamination from the previous product, cleaning agent's residues or microorganisms.

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Macedonian pharmaceutical bulletin, **62** (suppl) 137 - 138 (2016)
ISSN 1409 - 8695
UDC: 615.24:615.453.7.076.7
Short communication

Method suitability test for determination of microbiological purity of Gastoguard chewable tablets

Adrijana Nosacheva Trajkovska*, Maja Simjanovska Daskalova, Nadezhda Stojkova, Dragi Todorovikj, Hristina Babunovska

ALKALOID A. D., Blvd. A. Makedonski 12, 1000 Skopje, R. Macedonia

Introduction

Most people nowadays suffer from problems with the gastrointestinal tract and indigestion. Gastroguard chewable tablets are a pharmaceutical solid dosage form from the group of antacids, which contains 680 mg calcium carbonate and 80 mg magnesium carbonate. Its effect is to act locally and neutralize the gastric acid in the stomach, not depending on the systemic absorption. Therefore it alleviates the symptoms caused by indigestion and releases the feeling of bloating. The carbonate salts react with the gastric acid in the stomach developing water and mineral salts as products (http://www.alkaloid.com.mk/vademecum-step-2.nspx?LekTipId=150).

Every pharmaceutical product must be subjected to chemical and microbiological analysis before the batch release. Those analyses are under the jurisdiction of the Quality Control department. In addition to the microbiological analysis a working method for determination of the microbiological purity in this pharmaceutical solid form, which would be used in the routine, must be validated. For pharmaceutical solid forms the parameters that should be tested are Total Aerobic Microbial Count (TAMC), Total Yeasts and Molds Count (TYMC) and absence of *Escherichia coli* (Ph.Eur., 2013; USP, 2014).

The aim of this work is to validate the analytical method which would be used in the routine work for determination of the microbiological purity of Gastoguard chewable tablets as part of the quality control of the product before releasing of the batch. The method should be simple for manipulation and in compliance with the current European Pharmacopeia. The validation of the method was made by the microbiological team of the Quality Control department of Alkaloid AD.

Materials and method

Materials

During validation of the method standard laboratory equipment was used: Laminar flow Class II, Incubators Gallencamp on 20-25 ºC; 30-35 ºC and 42-44 ºC, Bunsen burner, Vortex, glass pipetes, glass tubes intended for microbiological use. In addition to validate the method 10g of Gastoguard chewable tablets were weighted using Sartorius analytical scale. Buffered sodium chloride peptone water pH 7 was utilized as medium for dissolving of the sample and for making the dilutions of the culture suspensions. Ready to use nutrient media from BioMérieux were used. 90 mm Petri dishes from Trypcase Soy agar, Sabouraud Dextrose agar and Mac Conkey agar and 100 mL Trypcase Soy broth and 100 mL Mac Conkey broth of the liquid media were used for manipulation.

The test microorganisms that were part of this validation were the standard microorganisms recommended by the current European Pharmacopeia for the method suitability test. Those are: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404 (Ph.Eur., 2013).

Method

For determination of the antimicrobial activity and validation of the test method challenge test with the above mentioned microorganisms were used and the microbiological method of choice was the surface-spread method and direct inoculation in the liquid media for the test of absence of *Escherichia coli.* The number of all microorganisms applied in the challenge tests was less than 100 cfu/mL. Each application was made in duplicate and the tem-

* anosacheva@alkaloid.com.mk

perature and the time of incubation depended of the type of the medium. Trypcase Soy agar was incubated for five days at temperature from 30-35 ˚C, Sabouraud Dextrose agar was incubated for seven days at temperature from 20-25 ˚C, Trypcase Soy broth was incubated for 24h at temperature from 30-35 ˚C, Mac Conkey broth was incubated for 24h at temperature from 42-44 ˚C and Mac Conkey agar was incubated for 24h at temperature from 30-35 ˚C.

After the incubation time the relation between the number of the each microorganism dissolved in buffered sodium chloride pepton water pH 7 and the number of the of the same microorganism in the presence of the product was calculated. When verifying the suitability of the plate-count method, a mean of any of the test organisms not differing by a factor greater than 2 must be obtained (Clontz, 2009; Ph.Eur., 2013; USP, 2014).

Results and discussion

The results from the determination of the antimicrobial activity of Gastoguard chewable tablets showed that it does not show antimicrobial effect against the used microorganisms. In addition to this the working dilution of the product would be 1:10 dilution.

The individual challenge test revealed the following results:

The number of cfu of the challenge microorganism in the test with *P. aeruginosa* was 93 cfu/mL and the average number of the microorganism in the presence of the product was 89 cfu/mL. The acceptability factor in this test is 1.04.

The number of cfu of the challenge microorganism in the test with *B. spizizenii* was 52 cfu/mL and the average number of the microorganism in the presence of the product was 50 cfu/mL. The acceptability factor in this test is 1.04.

The number of cfu of the challenge microorganism in the test with *E. coli* was 61cfu/mL and the average number of the microorganism in the presence of the product was 50 cfu/mL. The acceptability factor in this test is 1.22.

The number of cfu of the challenge microorganism in the test with *S. aureus* was 26 cfu/mL and the average number of the microorganism in the presence of the product was 20 cfu/mL. The acceptability factor in this test is 1.30.

The number of cfu of the challenge microorganism in the test with *C. albicans* was 31cfu/mL and the average number of the microorganism in the presence of the product was 37 cfu/mL. The acceptability factor in this test is 0.84.

The number of cfu of the challenge microorganism in the test with *A. brasiliensis* was 22cfu/mL and the average number of the microorganism in the presence of the product was 24 cfu/mL. The acceptability factor in this test is 0.92. The acceptability factors for all the used microorganisms were in the compatibility framework and they never exceed the value 2.

In the test for absence of *E. coli*, there was good visible growth in Trypcase Soy broth after 24h and in Mac Conkey broth the growth was followed by a change of color of the medium. There was also presence of red non-mucous colonies on Mac Conkey agar after 24h.

The growth promotion test of the used media showed that they are adequate for routine microbiological analysis.

Conclusion

Gastoguard chewable tablets do not have antimicrobial activity against the used test microorganisms.

The method suitability test of the product revealed that the routine microbiological analysis should be performed with surface-spread method from 1:10 dilution for TAMC and TYMC and the method of direct inoculation in the test for absence of *E. coli*. The acceptability factors were in the frame provided by the current European Pharmacopeia and never exceeded the value 2.

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Macedonian pharmaceutical bulletin, **62** (suppl) 139 - 140 (2016)
ISSN 1409 - 8695
UDC: 615.212.074:543.544.5.068.7
Short communication

High-performance liquid chromatography method for determination of caffeine from different matrices

Nevena Grujić-Letić, Branislava Rakić*, Emilia Šefer, Maja Milanović, Nataša Milić

University of Novi Sad, Faculty of Medicine, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

Caffeine, 1,3,7- trimethylxanthine, is odorless, bitter taste substance which can be naturally found in coffee, cocoa, tea leaves, and is intentionally added in food products. It is considered to be one of the most commonly consumed drugs with more than 80 percent of the world's population consuming caffeine daily. The Europeans are found to be the world's largest consumers of caffeine intake of approximately 4.6 kg/person/year (Norton et al., 2014). The presence of the pharmaceutical residues in the environment has become the subject of growing concern in the past decade. Due to the continuous input that leads to the long-term adverse effects on the aquatic and terrestrial organisms, the special attention is being paid to their concentration levels in the aquatic environment. Caffeine can be found in traces in surface water. The main paths for caffeine to enter wastewater stream are either in urine or when caffeine-containing products are discharged through household pipelines or sewers. Determination of caffeine in surface water is an important task for environmental researches and analysis because it is found to be a good indicator for human sewage because of its relatively high concentrations in surface water and its unambiguous anthropogenic origin. Other important usage of caffeine is as an analgesic adjuvant in drug formulations for the treatment of headache and pain related to postpartum, postoperative, and dental surgery and it is therapeutically applied for the treatment of migraine in combination with other drugs such as aspirin, paracetamol and tramadol. Caffeine is claimed to enhance the efficacy of these drugs (Madhusudhan, 2013). The goals of this study were to develop and validate high-performance liquid chromatography (HPLC) method for caffeine determination in food, beverages, surface water and drugs which is simple and easy to perform. This method could have a widespread application in food, environmental and pharmaceutical sciences.

Materials and methods

Samples for caffeine determination in food and beverages were purchased at the Serbian market, samples of surface water were collected in amber bottles from seven representative locations of the Danube River on the territory of Novi Sad, Serbia, and tablets of analgoantipyretics containing caffeine were purchased from pharmacies in the vicinity of Novi Sad, Serbia. Caffeine from all samples was separated by solid-phase extraction and analyzed by HPLC. The chromatography was using two-solvent isocratic elution. The HPLC-diode array detection (DAD) model Agilent HP 1100 system equipped with an autosampler (Waldbronn, Germany) was used. The analytical column was the Zorbax Eclipse XDB-C8 column (4.6 mm x 150 mm, i.d., 5 µm particle size). Mobile phase was water-THF (0.1% THF in water adjusted to pH 8 with 0.1 M NaOH) - acetonitrile (90:10) with a flow rate of 0.8 mL/min. The HPLC mobile phase was prepared fresh daily and filtered through a 0.45 µm nylon filter. Run time was 10 min, column temperature 25 °C and analytes were detected at 273 nm.

Results and discussion

The linearity between caffeine concentrations and the response was tested for concentration levels ranging from (0.010-100) mg/L. High value of coefficient of correlation r=1 showed an excellent correlation between concentrations and peak areas. Calculated limit of detection (LOD-3.3*σ/S) and quantification (LOQ-10*σ/S) were 0.007 mg/L and 0.012 mg/L, respectively. According to the re-

* branislava.rakic@faculty-pharmacy.com

covery values of (98.33-100.22)% the method showed acceptable accuracy. Repeatability of the method was tested by analyzing three different concentrations of caffeine standards in six repetitions. The relative standard deviations (RSD) ranged from 0.028% to 0.063% for retention time and from 0.015% to 0.65% for peak area showing excellent repeatability. There were no interferences in the HPLC results by impurities or matrix in tested samples, which indicate that developed method, had very good selectivity.

The results of caffeine content in food and beverages showed that it ranged 5.6-158 mg/100 g in food samples, 24.71-30.81 mg/100 ml in commercial tea samples, 1328-3594 mg/100 g in coffees and 9.69-30.79 mg/100 ml in energy drinks. Also, obtained values in this study were consistent with the available manufacturer data. Analyzing surface water samples for caffeine, significant concentrations were found at the sampling sites near the wastewater discharges. Mean caffeine concentrations for summer, fall, winter and spring periods were 24.78 ng/L, 26.83 ng/L, 24.61 ng/L, and 86.29 ng/L, respectively. The highest mean caffeine concentration was detected in samples collected in the spring period which is in an agreement with the literature (Fernandez et al., 2010). The caffeine content in combined non-narcotic analgoantipiretics varied from (96.48-100.74)% of the declared value. The requirements of the British Pharmacopoeia include (95-105)% of active drug content. According to obtained data it can be concluded that all brands did meet this specification in the HPLC assay method.

Conclusion

In this work a unique and simple to perform HPLC method was established for caffeine content determination in food, beverages, surface water and analgoantipiretics. Obtained data for accuracy, repeatability, selectivity and robustness confirmed that the proposed method could be used for routine control of caffeine content in different mixtures. The results of food and beverages analysis showed that caffeine content was consistent with the available manufacturer data and obtained data for combined commercial formulations of non-narcotic analgoantipiretics of all brands did meet specification of the British Pharmacopoeia for active drug content. Testing the surface water samples for caffeine showed that significant concentrations were found at the sampling sites near the wastewater discharges. The potential risk for chronic effects may occur in resident organisms in the long-term period so an appropriate treatment of wastewater would be essential in prevention of the potential pollution.

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Macedonian pharmaceutical bulletin, **62** (suppl) 141 - 142 (2016)
ISSN 1409 - 8695
UDC: 615.356:577.161.3]:543.544.5.068.7
Short communication

Determination of α-tocopheryl acetate in sunscreen lotion and cream by using the solid phase extraction and HPLC method

Milica Kostić* and Slavica Sunarić

Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar Zorana Đinđića 81, Niš, Serbia

Introduction

The α-tocopheryl acetate (TA) is the esterified form of vitamin E. Vitamin E is a fat-soluble vitamin that exists in eight different forms, among them, α-tocopherol is the most active form in humans. Vitamin E is very popular cosmetic ingredient. It acts as antioxidant to protect cells against free radicals and reduces oxidative stress. Ultraviolet light and environmental pollutants are known initiators of free radicals and skin damage. Topical antioxidants have the potential to diminish the reactive oxygen species generated from the UVA radiation. For that reason, there is an increasing interest in the incorporation of vitamins and antioxidants in skin care products (Lupo, 2001).

The stability of the vitamin E in the cosmetic formulations and when applied to the skin is low. When exposed to UV light, it forms α-tocopheroxyl radicals that consume other antioxidants for recycling. For better stability, vitamin E is commonly used as a biologically non-active esterified form, such as tocopheryl acetate (Shapiro and Saliou, 2001). Vitamin E esters act as a pro-drug since they are hydrolyzed to the active vitamin E (α-tocopherol) upon penetration into skin. However, there is conflicting evidence as to what extent this conversion actually takes place in the skin and many studies have shown that these substitutes have very low biological activity. While numerous topical skin care products claim to contain ‘‘vitamin E’’, these products may actually contain very different concentrations and forms including active vitamin E, its several esters and many other derivatives (Thiele and Ekanayake-Mudiyanselage, 2007).

The solid and emulsified cosmetic products must be carefully prepared for any of instrumental techniques which would be employed in the tocopherol analysis. The aim of this study was to develop an effective solid-phase extraction procedure for the HPLC determination of α-tocopheryl acetate, which could be important for assessment the content of this synthetic form of vitamin E in sunscreen lotions and creams.

Materials and methods

Materials

Methanol, ethanol and acetonitrile used in this work were of HPLC grade (J.T.Baker). α-tocopheryl acetate (TA) of analytical grade was obtained from Fluka (Fluka-Analytical BioChemika). Stock solution of 1.03 mg/mL was prepared by dissolving the appropriate amounts of the α-tocopheryl acetate in absolute ethanol. Two sets of calibration standards were prepared by diluting aliquots of the stock solution.

Cosmetic products

Commercial sunscreen products were purchased from a local cosmetic shop. Formulations under study were Producer 1-Sun Lotion SPF 30 and Producer 2-Sunscreen cream SPF 50. According to the product label, both formulations contained vitamin E only in the form of tocopheryl acetate and TA content was not exactly declared.

Sample preparation

A 0.5 g of the formulation was measured in 20 ml plastic volumetric container and 0.5 mL of 0.5% ascorbic acid was added. The emulsion was dissolved by vortex-mixing with 5 mL of ethanol. After centrifugation for 10 min, a 3 mL of the supernatant was pipetted and applied to the SPE column.

SPE conditions

Solid phase extraction was performed on Chromabond C18ec cartridges (1ml/100mg, Macherey–Nagel, GmbH, Düren, Germany), which was previously selected among

* milicakosticnipsa@gmail.com;
slavica.sunaric@medfak.ni.ac.rs

five cartridges with different sorbent type. Cartridges were conditioned with 1mL of deionized water, followed by 1mL of methanol and 1mL of acetonitrile. After the sample was aspirated through the cartridge, analyte elution was made by 2 x 1 mL of methanol.

HPLC conditions

The Agilent Technologies 1200 Series apparatus with DAD and FL detection was used for the analysis. Separation was performed on the Restek Ultra IBD C18 column (150 mm x 3 mm, 3 μm). UV detector was set at 220 nm and 295 nm. Based on our previous work, 100% acetonitrile was selected as the optimal mobile phase (Sunarić et al., 2012). The column temperature was maintained at 40°C and the flow rate was kept at 0.45 ml/min.

Results and discussion

Tocopheryl acetate in extracted samples of the sunscreen formulations was identified by comparing the retention time of the observed chromatographic peak with that of the standard. Recovery of the solid-phase extraction method was calculated by comparison of the TA peak area for extracted and non-extracted standard TA solution on five different cartridges. The obtained recovery value was higher than 85% for Chromabond C18ec, therefore this cartridge was selected for further analysis. Additionally, the cartridge selection was confirmed by extraction of the real samples supernatants obtained in sample preparation procedure for the examined cosmetics. Even in this case, the highest chromatographic peak area of TA was obtained on Chromabond C18ec cartridge.

The calibration curves for two concentration ranges, 0.2 μg/mL - 2.0 μg/mL and 0.1 mg/mL - 1.0 mg/mL with the correlation coefficients > 0.998 were constructed and applied for the determination of TA in the injected samples. Final tocopheryl acetate content found in the examined formulations was as follows: (200±15) mg/100g (0.2%) in Producer 1-Sun Lotion SPF 30 and (490±20) mg/100g (0.49%) in Producer 2-sunscreen cream SPF 50. These results are in agreement with those given by other authors, that TA content is from 0.1% to 1% (Shapiro and Saliou, 2001; Thiele and Ekanayake-Mudiyanselage, 2007). As was expected, much higher content of TA was found in the formulation with a higher SPF level.

Conclusion

Cosmetic formulations contain a high number of ingredients and the analysis often requires extensive pretreatments. A SPE method followed by HPLC determination has been developed for the analysis of tocopheryl acetate in cosmetics. For the present experimental conditions, it can be concluded that the proposed extraction method was appropriate for the determination of TA in sunscreen lotion and cream. The extraction procedure was efficient, showing good accuracy and precision. Proposed analytical procedure for TA determination can be useful tool to know the amount of this synthetic form of vitamin E in cosmetic products.

Acknowledgement

This research was supported by grant TR 31060 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 143 - 144 (2016)
ISSN 1409 - 8695
UDC: 615.212.076
Short communication

Validation of analytical method for determination of microbiological purity of active pharmaceutical ingredient in Caffetin cold tablets

Silvana Ilioska-Zlatanovikj*, Dragi Todorovikj, Elizabeta Popovska, Hristina Babunovska

ALKALOID A. D, Blvd. Aleksandar Makedonski 12, 1000 Skopje, R. Macedonia

Introduction

Non-sterile drugs must satisfy the appropriate microbiological purity criteria which are included in monographs in pharmacopoeias. Pharmacopoeial studies are prepared specifically to ensure that the medicinal product is therapeutically effective and safe for the patient.

Caffetin Cold is a combined medicinal product, containing several active ingredients: paracetamol (500 mg), pseudoephedrine hydrochloride (30 mg), dextromethorphan hydrobromide (15 mg) and ascorbic acid (60 mg). Paracetamol is a pain reliever and reduces body temperature. Pseudoephedrine hydrochloride decreases nasal congestion, allowing nasal and respiratory passages to open up. Dextromethorphan hydrobromide suppress cough due to minor throat and bronchial irritation. Ascorbic acid (Vitamin C) fortifies the immune system (www.alkaloid.com.mk/vademecum-step-2.nspx?LekTipId=64).

The importance of determination of microbiological purity of Active Pharmaceutical Ingredients (APIs) in solid forms comes from the fact that the final product must comply with the criteria for microbiological purity.

Objective: Validation of analytical method for determination of microbiological purity of paracetamol, pseudoephedrine hydrochloride, dextromethorphan hydrobromide and ascorbic acid in the composition of Caffetin cold tablets. This method further should be used for routine determination of microbiological purity of APIs in Caffetin cold tablets. The method should be simple for manipulation and in compliance with the current edition of European Pharmacopoeia (Ph.Eur., 2013).

Materials and methods

Materials

Standard laboratory equipment was used. Buffered sodium chloride peptone solution pH 7.0 was used as medium for dissolving of the sample and for making the dilutions of the culture suspensions.

Ready to use nutrient media from BioMérieux® (69280 Marcy-l'Etoile-France) 90 mm Petri dishes of Trypcase Soy agar and Sabouraud Dextrose agar were used.

Standard test microorganisms recommended by the current Ph.Eur. for the method suitability test: *Escherichia Coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404 were used for challenge test.

Methods

Surface-spread method and determination of antimicrobial activity of APIs in Caffetin cold tablets was used. The number of all test microorganisms applied in the challenge tests was less than 100 cfu/ml. Each application was made in duplicate and the temperature and time of incubation depended of the type of the medium. Trypcase Soy agar was incubated for five days at a temperature from 30-35 ˚C, Sabouraud Dextrose agar was incubated for 7 days at a temperature from 20-25 ˚C. After the incubation time, the relation between the number of the each test microorganism dissolved in buffered sodium chloride pepton solution pH 7.0, and the number of that test microorganism in the presence of the product was calculated.

When verifying the suitability of the plate-count meth-

* szlatanovic@alkaloid.com.mk

od, a mean of any of the test organisms not differing by a factor greater than 2 must be obtained (Clontz, 2009; Ph.Eur. 2013; USP, 2014).

Results and discussion

Microbiological purity was determined with direct application on the nutrient medium with surface-spread method.

Results for paracetamol (1:10) showed that acceptability factor is 0.93 when *Escherichia coli* was used, 1.35 for *Pseudomonas aeruginosa*, 0.97 for *Staphylococcus aureus* and 1.05 for *Bacillus spizizenii*. For *Candida albicans* the acceptability factor ranges from 0.93 to 1.07 and for *Aspergillus brasiliensis* from 1.06 to 1.13.

Paracetamol does not have antimicrobial activity on the used test micro-organisms. Acceptability factor ranged within the compatibility framework and it never exceeded the value of 2.

Results for pseudoephedrine hydrochloride (1:10) showed that there is no increase of colonies of *Escherichia coli*. Acceptability factor when *Pseudomonas aeruginosa* was used is 29.33, for *Staphylococcus aureus* is 1.61, for *Bacillus spizizenii* is 6.00, for *Candida albicans* is 2.15 and for *Aspergillus brasiliensis* ranges from 1.30 to 2.93.

Pseudoephedrine hydrochloride has an antimicrobial activity on the used test micro-organisms.

Inactivation of antimicrobial activity of pseudoephedrine hydrochloride was done using the dilution of 1:100 in buffered sodium chloride peptone solution pH 7.0.

Results for pseudoephedrine hydrochloride (1:100) showed that acceptability factor when *Escherichia coli* was used is 1.00, for *Pseudomonas aeruginosa* is 1.20, for *Staphylococcus aureus* is 1.06, for *Bacillus spizizenii* is 1.20, for *Candida albicans* ranges between 0.96 to 1.00 and for *Aspergillus brasiliensis* ranges from 0.92 to 1.00.

Acceptability factor ranged within the compatibility framework and it never exceeded the value of 2.

Results for dextromethorphan hydrobromide (1:10) showed that there is no increase of colonies of the test micro-organisms. Dextromethorphan hydrobromide has an antimicrobial activity on the used test micro-organisms.

Inactivation of antimicrobial activity of dextromethorphan hydrobromide was done using the dilution of 1:100 in buffered sodium chloride peptone solution pH 7.0.

Results for dextromethorphan hydrobromide (1:100) showed that acceptability factor when *Escherichia coli* was used is 1.23, for *Pseudomonas aeruginosa* is 1.44, for *Staphylococcus aureus* is 1.06, for *Bacillus spizizenii* is 1.28, for *Candida albicans* ranges between 1.03 to 1.12 and for *Aspergillus brasiliensis* ranges from 0.90 to 0.93.

Acceptability factor ranged within the compatibility framework and it never exceeded the value 2.

Results for ascorbic acid (1:10) showed that there is no increase of colonies of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus spizizenii*. Acceptability factor when *Escherichia coli* was used is 7.00, for *Candida albicans* ranges from 1.18 to 1.38 and for *Aspergillus brasiliensis* ranges from 0.94 to 1.21.

Ascorbic acid has an antimicrobial activity on the used bacterial test micro-organisms. Inactivation of antimicrobial activity of ascorbic acid was done using the dilution 1:100 in buffered sodium chloride peptone solution pH 7.0.

Results for ascorbic acid (1:100) showed that acceptability factor when *Escherichia coli* was used is 1.23, *Pseudomonas aeruginosa* is 0.79, *Staphylococcus aureus* is 0.79, *Bacillus spizizenii*is 0.80, *Candida albicans* ranges from 1.05 to 1.70 and for *Aspergillus spizenii* ranges from 0.89 to 1.00.

Acceptability factor ranged within the compatibility framework and never exceeded the value of 2.

Conclusions

Paracetamol does not have antimicrobial activity against used test micro-organisms. Direct application method with the dilution of 1:10 is suitable for further routine testing.

Pseudoephedrine hydrochloride, dextromethorphan hydrobromide and ascorbic acid in the dilution of 1:10 have an antimicrobial activity against used test micro-organisms. Inactivation of antimicrobial activity was done using the dilution of 1:100 in buffered sodium chloride peptone solution. Direct application method from the dilution 1:100 was suitable for further routine testing.

Validation of analytical method showed that routine microbiological analysis could be done with the surface-spread method.

All results showed that the acceptability factor ranged within the compatibility framework and it never exceeded the value of 2.

The test micro-organisms and culture media were in compliance with current pharmacopoeial requirements.

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Macedonian pharmaceutical bulletin, **62** (suppl) 145 - 146 (2016)
ISSN 1409 - 8695
UDC: 615.07
Short communication

Contemporary approach in LC-MS/MS bioanalytical method development

Natalija Nakov*, Zoran Kavrakovski, Rumenka Petkovska, Aneta Dimitrovska

Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University SS Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

The development of reliable LC-MS/MS bioanalytical method is a challenging process due to the large number of variables and pitfalls that can compromise the quality of the data. The reliability of the method is remarkably dependent upon the timely recognition of all factors that may generate erroneus resuts once the validated method is applied in practice. Therefore the scientific approach in course of LC-MS/MS bioanalytical method development should involve risk analysis. The risk analysis denotes assessment of all critical steps (factors) that may affect the accuracy and ruggedness of the method. Matrix effect, metabolite interference, stability of the analyte and the selection of an adequate regression model are identified as most common sources of method imprecision (Mulvana, 2010).

Assessment of critical steps in bioanalytical method development

Matrix effect (ME) is directly related to the sample clean-up procedure, thus selecting the optimal extraction procedure plays a crucial role for ensuring data quality in LC-MS/MS bioanalysis (Chambers et al., 2007). Different types of extraction procedures, depending on the analyte, exhibit different extraction recoveries and ME. Therefore testing out various extraction procedures should become inevitable part of the contemporary bioanalytical method development. The implementation of Quality by design, as a new trend, enables shortening the time needed for the optimization of the sample preparation procedure. Until recently, the optimal extraction procedure was chosen only upon the extraction recovery. However, considering the LC-MS/MS analysis, the cleanliness of the extract, expressed through ME, obtained with different extraction procedures gains paramount importance. The EMA validation guideline (EMA, 2011) recommends evaluation of the variability of the internal standard (IS)-normalized ME. However, it is more rational to assesses the degree of the absolute ME itself and not just the variability of the IS-normalized ME. This derives from the fact that the IS-normalized ME indicates that ME is consistent and not affected by the different lots of biological material. On the other hand, the absolute ME points out to the cleanliness of the extracts (Nakov et al., 2015). Hence, it becomes evident that the selection of the sample preparation procedure should be based on the absolute ME and the extraction recovery should be put in second place, especially when problems related to the sensitivity of the method are not expected.

The quantitative acceptance criteria applied in bioanalytical validation guidelines (EMA, 2011; FDA, 2001) rely on data generated using quality control (QC) samples. However the QC samples do not contain phase I and II metabolites of the analyte of interest and therefore may be unreliable model for real study samples. These metabolites are usually not stable and they can convert back to their parent drug in the mass spectrometer (in source back-conversion) and/or during sample preparation (in vitro back-conversion), jeopardizing the accuracy of the bioanalytical method (Wenkui et al., 2011). Therefore the assessment of the possibility of metabolite back-conversion prior the method validation becomes a crucial part of risk analysis. The first step includes assessment of the in source back-conversion through the verification of the chromatographic separation between the analyte and its metabolite. This is especially important for high throughput LC-MS/MS analysis, where chromatographic separation between the analyte and the metabolite can be compromised, resulting in overestimated measurements. In case of this type of inter-

* natalijan@ff.ukim.edu.mk

ference, optimization/modification of the ion source parameters and/or the chromatographic conditions should be applied. The metabolite back-conversion could also occur during the different steps of the sample preparation process, so second step involves investigation whether the proposed extraction procedure leads to metabolite back-conversion. For that purpose the optimized extraction procedure should be applied on samples containing only the metabolite standard and the amount of analyte generated by metabolite back-conversion is then computed. In case of unavailability of metabolite standards, stability experiments under different storage conditions using real samples could be applied as well (Jemal et al., 2010).

The investigation of the sample stability in course of method development helps to avoid stability related problems that may latter appear during the validation and application of the method. Although various sources may affect the sample stability, the focus within this paper is placed on chiral interconversion. All chiral compounds may undergo in vitro R/S conversion occurring at physiological pH and temperature or under extreme pH and elevated temperature (Briscoe and Hage, 2009). Therefore the assessment of the possibility of R/S interconversion during the overall analytical procedure should be an integral part for every enantioselective method development, thus allowing quantification of the extent of in vivo interconversion without bias.

The selection of an adequate regression model is the basis for accurate and reproducible quantification over the whole concentration range (Rozet et al., 2011). Most bioanalytical methods usually have wide concentration range, so heteroscedasticity of the data may be expected. In the above instance weighted linear or quadratic regression models should be applied. The recommendation of the regulatory agencies (EMA, 2011; FDA 2001) is that the simplest model that adequately describes data should be used and the selection of more complex model should be justified. Therefore the use of statistical approach based on non-parametric statistical tests (Nakov et al., 2014; Singtoroj et al., 2006) during the selection of the regression model will provide justification of the choice of the adequate regression model, and will enable accurate quantification in whole calibration range.

Summary

The implementation of risk analysis during the LC-MS/MS bioanalytical method development is longer lasting process compared to conventional method development. However the timely perceiving and assessment of the critical steps generates highly reliable bioanalytical methods and lowers the possibility of late discovery of unexpected problems than often lead to method re-optimization and revalidation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 147 - 148 (2016)
ISSN 1409 - 8695
UDC: 615.272.4.075
Short communication

Implementation of design of experiments for optimization of forced degradation of simvastatin

Maja Hadzieva Gigovska[1]*, Marija Grozdanoska[1], Ana Petkovska[1], Jelena Acevska[2], Biljana Sapkarova[1], Irena Brašnarska[1], Sonja Ugarkovic[1], Aneta Dimitrovska[2]

[1] *Research & Development, ALKALOID AD, Blvd. Aleksandar Makedonski 12, 1000 Skopje, R. Macedonia*
[2] *Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, R. Macedonia*

Introduction

Forced degradation studies provide data to support identification of possible degradants; degradation pathways and intrinsic stability of the drug molecule and validation of stability indicating analytical procedures (Singh and Bakshi, 2002). Although most of the literature defines the concept of forced degradation, they do not provide detailed information about a forced degradation strategy (Klick et al., 2005) and the experimental conditions to conduct forced degradation are described in a general way (Singh and Bakshi, 2000) without description of the exact stress conditions to be applied.

Generally, a trial and error approach is adopted to select the strength, temperature and time of exposure to achieve loss of active substance from 10–30% (ICH, 2003; Singh and Bakshi, 2000). Such approach involves considerable cost, time consumption and scientific expertise and there is high incidence of random results. Therefore a need for more systematic approach is recognized (Dolan, 2002).

A contemporary approach in the field of forced degradation is to evaluate correlation of degradation parameters by applying the concept of design of experiment (DoE). In turn, this can help studying the combination of conditions where optimal enrichment of degradation products is obtained.

The basic concept of factorial design is performing an experiment in which all the possible combinations of factors and levels are investigated (Lundstedt et al., 1988).

Literature review reveals the implementation of DoE and factorial design concepts for optimization of forced degradation conditions (Kurmi et al., 2014; Singh et al., 2013; Sonawane and Gide., 2011), but no report exists on application of DoE for simvastatin (SIM) as model drug.

The aim of this study was to develop stability-indicating LC-MS method for quantification of SIM and its impurities, and also, an attempt was made to simplify forced degradation studies by adopting DoE concept.

Materials and methods

Simvastatin (SIM) samples with Certificate of Suitability to the monographs of the European Pharmacopoia (CEP) were used. Simvastatin CRS and Simvastatin for peak identification were provided by the EDQM (Strasbourg, France). All reagents used were of analytical grade.

Experiments were made on Dionex UltiMate™ 3000 (Thermo Scientific), interfaced with mass spectrometer with an electrosparay-ionization source, operated in negative mode. Mass parameters were optimized as follows: Ion source heater temperature was set at 350 °C; capillary temperature at 300 °C; capillary voltage was 10 V with collision energy 35 eV. The separation was performed on Poroshell 120 EC C18 (Agilent Technologies, USA), 50 x 3.0 mm, 2.7 µm, using buffer (10 mM ammonium format, pH 4.0) and acetonitrile as a mobile phase in a gradient mode. The column temperature was 40 °C. Flow rate was 0.5 mL/min. Injection volume was 5 µL. UV detection was performed at 238 nm.

SIM was subjected to stress under acidic, alkaline, oxidative, thermal and photolytic conditions. Variables, chosen from previously conducted initial experiments were: time of exposure, temperature and stressor strength. The forced degradation experiments set-up on the basis of 2^n full factorial design were performed and the obtained results from LC-MS samples were analyzed by Design Ex-

* Hadzievam@yahoo.com

pert Software (Stat-Ease Inc., Minneapolis, MN, USA).

Acid and alkali degradation was performed using 2^3 factorial design (three variables considered at two levels: 0.01M and 0.1M HCl/ NaOH heated at 25 °C and 60 °C for 60 and 120 min) was conducted to set up eight experiments for each stressor.

For oxidative degradation two variables were considered at two levels (the high level for H_2O_2 and time was 30% and 24 h, and the low level was 3% and 2 h, respectively), a 2^2 factorial design was conducted to set up four experiments.

2^2 factorial designs was conducted to set up thermal degradation where the high level values were 105°C and 5 h and the low level were 80 °C and 3 h, respectively.

Photo degradation study was performed by exposing the drug powder, spread as a thin film in a covered petri plates and exposed to direct sunlight for 3, 5 and 12 h. Additionally, control study in dark was run simultaneously. All stress studies were performed at an initial drug concentration of 1 mg/mL in amber color glassware in order to protect the solutions from light degradation.

Poster presentations

Results and discussion

Under the proposed chromatographic conditions, satisfactory separation (Rs >1.5) of SIM and all seven impurities stated in the European pharmacopeia was achieved proving the stability-indicating power of the method. According to the results obtained from the degradation study SIM is susceptible to degradation under acid, alkali, oxidative and photolytic conditions. The applied factorial design, determine significant factors responsible for degradation and provides prediction of the optimal conditions.

The relationship between each factor and their interaction were evaluated by creating Pareto chart. These analysis indicated that under acidic condition, strength of HCl and temperature were most significant factors and for alkaline condition, temperature and time of exposure. Furthermore, desirability plots were generated for acid and alkali conditions, providing prediction of conditions for optimum degradation.

It was suggested that for acidic stress, 10% degradation would be achieved by treating with 0.1 M HCl at 25 °C for 60 min. When these conditions were adopted in practice, the resulting degradation was 10.78%.

Also, for alkaline degradation 10% degradation would be achieved by using 0.1M NaOH heated for 10 min. at 25 °C. The actual degradation, when these conditions were adopted in practice was 11.12%.

For oxidative degradation, it has been observed that 18.75% degradation can be achieved with 30% H_2O_2 at 25 °C for 60 min. For photolytic conditions about 3.9% degradation has been obtained after exposure of UV light for 12h.

The desirability plots obtained from thermal degradation study demonstrated that an increase in temperature from 80 °C to 105 °C favored the degradation significantly. The targeted drug degradation was obtained heating the solution at 80 °C for 5 h.

Thus, factorial design approach was successfully used to achieve optimum degradation conditions for SIM and simple, specific, stability-indicating LC/MS method was developed.

Conclusion

This study highlights significant utility of DoE in optimization of forced degradation conditions. Using 2^n full factorial design degradation conditions were optimized to obtain the targeted level of degradation. The use of DoE to identify theoretical values of variables for optimum degradation was successful, because when these parameters were put in practice, the % degradation obtained matched the predicted degradation. This suggests that factorial design approach can replace the trial and error approach used to achieve optimum degradation in forced degradation studies.

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Macedonian pharmaceutical bulletin, **62** (suppl) 149 - 150 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:544.351-145.82]:615.074
Short communication

Residual solvent profiling in active pharmaceutical ingredients; approaches in sample preparation and method optimization

Ana Poceva Panovska*, Jelena Acevska, Katerina Brezovska, Rumenka Petkovska, Aneta Dimitrovska

Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Organic solvents are widely used in the manufacturing process of pharmaceutical products. Their application in the synthesis of active pharmaceutical ingredients (APIs) is of exceptional importance as the use of appropriate solvents may enhance the yield or determine characteristics such as crystal form, purity and solubility (Grodowska and Parczewski, 2010). The European Pharmacopoeia (Ph.Eur., 2015) limits the amount of residual solvents (RS) in pharmaceuticals, considering the ICH guideline for RS (Q3C). Beyond the influence on safety and quality of the active pharmaceutical ingredients, the residual solvent profile indicates their manufacturing history. This core parameter for API fingerprinting allows for authentication and identification of the API origin (Poceva-Panovska et al., 2016).

Methodology

The main technique employed in RS analysis is gas chromatography (GC) because of its suitability for analysis of volatile samples and substantial separating capability of capillary columns. European Pharmacopoeia (Ph. Eur.) in the general chapter recommend GC coupled with headspace (HS) sampling system for qualitative and quantitative determinations of residual solvent. This technique is robust, convenient and readily automated and it is the method that is generally used for the control of residual solvents. As reported in 80% of the literature citations for GC procedures on RS testing, flame ionization detector (FID) is most commonly used for the detection of resolved compounds because of its low detection limits, wide linear dynamic range, robustness, ease of operation, and general reliability and utility, especially for trace organic compounds (Camarasu et al., 2006).

However profiling of the RSs in practice using the GC-FID method, usually require additional identification tool because of the co-elution and possible misinterpretation of the RS peaks. In order to confirm identifications and solve co-elutions Ph. Eur. proposes conformation analysis to be performed on a second chromatographic column coated with different stationary phase.

Another more convenient approach to solve possible misinterpretation of the RS peaks is the use of GC-FID/MS, since the mass spectrometer adds an additional spectrometric dimension to the identification. Mass spectrometry also excels for determination of co-eluting peaks through use of extracted ion or SIM ion chromatograms, thereby eliminating the need of additional analysis on different columns (Poceva-Panovska et al., 2016).

Sample preparation

Although pharmacopoeias propose their methods for control of residual solvents, practically and through the literature they are found to be disadvantageous due to their long equilibration and analysis time, relatively complex sample preparation and quantification problems. In order to solve these issues, different aspects of method development and sample preparation have to be considered. Optimized sample preparation conditions, including HS conditions and dilution media, are crucial for the reliability of the residual profiling results.

* anpo@ff.ukim.edu.mk

Internal standard

The proposed Ph. Eur. methodology for RS analysis does not utilize internal standard (IS) for quantification. The use of IS in RS analysis can be beneficial since it accounts for routine variation in the response of the GC system and can considerably improve the precision of quantitative analysis. When choosing IS for HS-GC analysis, beside the chromatographic and physico-chemical characteristics, it is mandatory to consider the stability and reactivity of the compound candidate for the IS. Many of the organic compounds have functionalities that may be reactive at elevated temperatures and different pH values during the equilibration time. IS decomposition or formation of a new product can lead to misinterpretation of the results and poor quality of the analysis (Poceva-Panovska et al. 2016).

Optimization of headspace parametars

Vial equilibration temperature and time are major HS parameters that can be used to improve the transfer of volatile analytes from the sample into the HS of the vial.

The equilibration temperature has a profound effect on the sensitivity of the method because temperature has a direct impact on the equilibrium concentration of the RS in the HS of the sample vial. However, effect of the temperature is specific for the RS tested. The increase of the temperature enhances the headspace sensitivity for the polar compounds with high partition coefficients (K) only, while the non-polar compounds with low K were practically not affected, since temperature affects the fraction of the analyte (RS) in the condense fraction only.

Sufficient time is also crucial in order to achieve constant state of equilibrium. Equilibration times should be set to optimal as they can considerably shortened overall method analysis time.

According to our recent study (Poceva Panovska et al., 2016) the increase of equilibration temperature and equilibration time generally resulted in better sensitivity, although the effect of equilibration time was not as profound as temperature. For equilibration times longer than 20 min, no significant increase in the chromatographic responses of RS was observed. This observation has considerably shortened the overall time for RS analysis when compared to the official Ph.Eur. method.

Dilution media

Another approach to increase the sensitivity in the RS analysis is by selecting suitable dilution media as it has direct influence on partition coefficient. Organic solvents used as dilution media (DMSO, DMF etc.) offer higher partition coefficient values for most of the RSs when compared with water and consequently lower the concentration of the RS in the gaseous (HS) phase. According to the Ph.Eur., the choice of sample preparation technique is dependent on sample solubility in the dilution solvent. For water soluble substances water is used and for water insoluble substances an alternative sample preparation is considered, using DMF as solvent.

The use of single sample preparation for water soluble and water insoluble substances is highly practical and beneficial. A mixture that includes DMSO, as a solvent with exceptional properties for organic and inorganic chemicals and low toxicity (class III), and water that is crucial for better sensitivity, in a suitable ratio usually offers satisfactory results as dilution media. Additionally the sensitivity of the analysis can be increased with the decrease of the percentage of organic dilution media (DMSO). A mixture of DMSO/water in ratio 1:4 (V/V) can be used as a dilution media regardless of the sample solubility (Poceva-Panovska et al. 2016).

Chemometric approach in optimization of analytical methods

Development and optimization of methods for RS profiling require a well-designed strategy for efficient data processing in an economical and timesaving manner. For exact determination of the relationship between the sample preparation conditions and the chromatographic response of the analytes, design of experiments (DoE) can be effectively employed since it enables the maximum utilization of data from previously planned experiments. Furthermore, by conducting the optimization of the method in very rational manner using DoE, a great deal of excessive and unproductive laboratory research work can be avoided. Response surface methodology (RSM) can be used to optimize experimental values for important experimental factors (Lundstedt et al., 1998).

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Macedonian pharmaceutical bulletin, **62** (suppl) 151 - 152 (2016)
ISSN 1409 - 8695
UDC: 615.28:614.2(497.774)
Short communication

Detecting the weakness in the hygiene - a mean for prevention of the health care-associated infections and improvement of the patients' health care in the Clinical hospital Bitola

Tatjana Dimitrovska Manojlovikj[1]*, Dona Trombeva[1], Lenche Najdovska[1], Magdalena Vrchkovska[2], Angjela Delova[3], Marta Ivanovska[3], Ljupcho Anastasovski[4]

[1]*Clinical Hospital "D-r Trifun Panovski", ul. Partizanska bb, 7000 Bitola, R. Macedonia*
[2]*"Karta Medika" Pharmacy, ul. Partizanska bb, 7000 Bitola, R. Macedonia*
[3]*Centar for Public Health, Bitola, ul. Partizanska bb, 7000 Bitola, R. Macedonia*
[4]*Alkaloid AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, R. Macedonia*

Introduction

Health care-associated infections or nosocomial infections can be defined as those occurring within 48 hours of hospital admission, 3 days of discharge or 30 days of an operation. Hospital-acquired infection is an infection that is contracted from the environment or staff of a healthcare facility. It can be spread in the hospital environment, nursing home environment, rehabilitation facility, clinic or other clinical settings. Infection is spread to the susceptible patient in the clinical setting by a number of means (Inweregbu et al., 2005; WHO, 2007). Nosocomial infections are huge problem all over the world. They are a major problem in health care facilities, resulting in extended durations of care, substantial morbidity and mortality, and excess costs. Effective hand hygiene of the health care workers remains the single most important and economical means of controlling the nosocomial infections. Every health care worker is responsible for maintaining high personal standards of hygiene and hand cleanliness (WHO, 2007). The etiology of nosocomial infections, the frequency of contaminated hands with the different nosocomial pathogens, and the role of health care workers' hands suggest that special attention should be paid on selection of hand hygiene preparations as well on the compliance to the hand disinfection protocols. From the times of I. P. Semelweiss, we know that clean hands are the most important single factor that can decrease the extent of the health-care associated infections. Handwashing frequently is called the single most important and the cheapest measure to reduce the risks of transmitting skin microorganisms from one person to another or from one site to another on the same patient (Borges et al., 2012). Since alcohol-based hand sanitizers combine high immediate antimicrobial efficacy with ease of use (Christiaens et al., 2006) they are the main disinfectants in our hospital as they are in almost every hospital worldwide. The pharmacists from the hospital pharmacy in our hospital are obviously members in the tender commission for selections of the disinfectants and the inevitable link in the supplying of disinfectants as well, so a complaint from the intensive care ward nurse, for inappropriate selection of the disinfectants was directly addressed to us. Considering that we decided to conduct microbiological tests of antimicrobial efficacy of the two rapid alcohol-based hand disinfectants used by caregivers in the hospital.

Materials and methods

We selected "fingerpad method"-appropriate samples collected from the subject's fingers, as a suitable model for testing the in vivo efficacy of hand-washing agents (Ansari et al., 1989), for our purpose-to test microbiological efficacy of two rapid alcohol-based hand disinfectants. The examination consisted of two parts on the randomly selected health care workers including nurses, physicians and subsidiary hospital staff. In the first part of the examination these seven clinical wards were included: Maxilo-facial surgery, Ophthalmology with ophthalmo-surgery, Ot-

* tatjanadm@yahoo.com

to-Rhino-Laringology, Gynecology and Obstetrics, Anaesthesia Reanimation and Intensive Care, General surgery and Orthopedy. The selection of the clinical wards was planed according to grade of risk for developing nosocomial infection. One of the selected wards was the one that the complaint for the inappropriate selection and supply of disinfectants came. The observers and facilitators of the test procedures were three pharmacists and one nurse. Each pharmacist had different task in the examination with intention of minimizing the bias. Total number of included respondents (health care givers) was 54; 34 in the first part and 20 in the second part of the examination. 54 (Biomerieux) COUNT-TACT AGAR for aerobic mesophyllic bacteria and 54 (Biomerieux) VRBG AGAR for Gram negative/enterobacteria determination were used, for each part of the examination proportionally to the respondents. Ecosal® Forte an ethanol/2-propanol based disinfectant and Ecosal® Ultra a 1-propanol, 2-propanol+benzalconium chloride disinfectant were used. Incubation, cultivation and determination of the bacterial growth were done in the Centre for public health at the Department for sanitary microbiology (accredited according to ISO 17025).

Results and discussion

Fingerpad/touch method with not disinfected hands showed abundant bacterial growth on both type of agar. There was only one VRBG AGAR for Gram negative/enterobacteria determination, from the undisinfected hands, without any bacterial growth, that represented 2.940% of total VRBG AGAR in the first part of examination. There were 9 cases (of total 34) of aerobic mesophyllic bacterial growth on disinfected hands. That number represented 26.471% of which, 2 plates were for Ecosal® Forte and 7 plates for Ecosal® Ultra. Those were from the hands of subsidiary hospital workers that were not very familiar with the hand disinfection protocol and from staff not applied sufficient volume of disinfectant or/and didn›t let sufficient time (30 sec.) for antimicrobial action. Both, facilitators and observers of the examination, noticed and reported various irregularities in the hand disinfection protocol during first part of examination (Alkaloid-AD Skopje, 2014). Trend of recontamination of the hands of the health care workers included in the study, with touching other objects or part/s of the face, after the hand disinfection was reported. Those irregularities were decisive for performing the second examination part-repetition of the test on the clinical departments where microbial growth appeared after disinfection of the hands, on the same workers as in the first part, but after a lesson of appropriate education. In the first part of examination there was no case of Gram negative/entherobacteria growth. In the second part of the examination, i.e. the repeated tests with both disinfectants, there was no case of any bacterial growth, nor aerobic mesophylic or entherobacteria neither on Ecosal® Forte nor on Ecosal® Ultra. The presence of aerobic mesophyllic bacterial growth, in the first part of the examination, after the disinfection of the hands appeared from the air or from the weaknesses stated above.

Conclusion

This examination highlighted the importance of efficient disinfection of health care workers' hands. Absence of any bacterial growth on the repeated controls implied of efficient antimicrobial activity of both disinfectant. Selected rapid hand disinfectants were effective. Non-adherence to the hand hygiene protocol was reason for bacteriological contaminated smears occurrence. That confirmed that the hand hygiene on the medical staff is a quality indicator for nosocomial infections prevention. In the Commission for prevention of nosocomial infection in our hospital, a pharmacist should be incorporated for education of the medical and subsidiary staff for the importance of hand disinfectants selection and of the adherence to the hand hygiene protocols. That will contribute for reducing of the nosocomial infections and improvement of the patients' care, outcomes and safety.

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Macedonian pharmaceutical bulletin, **62** (suppl) 153 - 154 (2016)
ISSN 1409 - 8695
UDC: 615.076
Short communication

Bacterial endotoxin test: a microbiological challenge

Eva Troja

Profarma SH.A. Pharmaceutical industry, Microbiology laboratory, Tirana, Albania

Background

Sterile drugs and medical devices must be tested with the bacterial endotoxin test (BET) and must be shown to contain less than Endotoxin Limit. BET testing is a category IV test as listed in United States Pharmacopeia <1225> and requires specificity testing. Procedure should be verified to establish its suitability for use, such as their accuracy and absence of interference when used for a new product or raw material. In common with many other Quality Control (QC) tests, validation is a key element in preparing for testing and product release.

Bacterial endotoxin test

The three essentials for BET include endotoxin limits for pharmaceutical and medical devices, procedures for validation in laboratory, and procedures for conducting routine testing. Different pharmacopoeias, LAL manufacturer's documentation, and US Food and Drug Administration (FDA) document are available to the user when undertaking validation. Finished preparation release checks are still required according to United States Pharmacopeia (USP) Chapter <797> (USP, 2006). The QC laboratory facilitates these release checks by utilizing its staff's thorough understanding of the regulatory requirements and employing analytical techniques that ensure preparation safety. Even with this knowledge, challenges arise in microbiological testing of some preparations. The key challenges are the development of new and more sensitive methods and the interpretations of some results (Troja, 2010). On june 22, 2011, FDA withdrew the LAL test Guideline, which was issued in 1987 (Cooper, 2011). Elements of Guideline continued to impact current methods because they can be found in other references relied on by the industry. Following European, Japanese, and US Pharmacopeia harmonization, the tests methods are identical and these tests are described in the Bacterial Endotoxins Test chapter in the USP (Chapter <85>) and in the equivalent chapters in the European pharmacopeia (Chapter 2.6.14) and the Japanese Pharmacopoeia (General Tests, No. 4.01). Endotoxin testing presents many challenges, yet troubleshooting is generally more effective because of the information generated from each test (USP, 2006). It is critical that the user know the concentration of active substance, excipients, final pH and maximum human dose per hour, and whether the preparation contains a suspending agent such as carboxymethyl cellulose. The analyst must determine the conditions under which the product does not interfere with BET. The dilution of product is the best choice to overcome inhibition or enhancement. This information is helpful in performing calculations to find the maximum valid concentration, and in determining the endotoxin limit when not published. The maximum human dose is especially important in establishing an endotoxin limit for combination medicines such as multivitamins. On the basis of this information, the laboratory can test the preparation and obtain the accurate endotoxin data. By incorporating the patient's body weight (EU/kg) into a suitable equation, the allowable endotoxin limit (EL) can be calculated for the preparation class (e.g., intrathecal, subcutaneously, or radiopharmaceuticals). The potency of an endotoxin to cause pyrogenic reaction is variable according to different factors, such as the nature of the toxin, the weight of the subject and the maximum human dose. Therefore, the endotoxin concentrations are expressed as Endotoxin Units (EU) for result comparisons. The endotoxin limit for pharmaceuticals products is calculated using the formula: K/M, where K is the threshold human pyrogenic dose of endotoxin per kilogram (kg) of body weight per hour, and M is the maximum recommended human dose/kg of body weight that would be administered in a single one-hour pe-

* eva_troja@yahoo.com

riod. The creation of the general endotoxin limit of 5 EU/kg/hour enabled the industry to develop dose-related limits for individual and classes of parenteral drugs and devices. However the EL for intrathecal administration was assigned arbitrarily and was not science-based (Munson, 2010). Williams (2007) has recently shown endotoxin tolerance or allowable limit by type of parenteral products. FDA proposed to the USP Micro committee an endotoxin limit of 2.0 EU/dose/eye for intraocular administration, consistent with ISO 11979-9:2006 *Ophthalmic Implants, Intraocular Lenses*. An endotoxin limit for topical ophthalmic administration was not proposed. FDA suggests to applicants that EU for topical eye drops is 0.5 EU/ml, but BET interference is high because of additives. A majority of the substances tested for endotoxin interfere with the LAL test to some degree (the obvious exception being water samples). An understanding of the chemistry of the test sample and its possible effects upon endotoxin and/or LAL, can aid in overcoming interference problems (Dawson, 2005). It is often possible to overcome interference by diluting the sample at which the endotoxin limit concentration is still detectable or choosing ultrafiltration method. The greatest dilution at which the endotoxin limit can be detected is the maximum valid dilution (MVD). Dilution is always the technique of choice and should be attempted first. The LAL Guideline described ways to develop test parameters by use of the MVD and Minimum Valid Concentration (MVC) to prepare samples for endotoxin testing, independent of the method. MVD and MVC are calculated as below: MVD= EL/λ ; MVC= λ/EL, where λ is sensitivity of lysate. MVD is used for products with EL in EU/mL. MVC is used for products with EL in EU/mg or some other unit. Once calculations of EL, MVD, MVC are done, the analyst can start the interference/validation testing. The user should prepare several sample dilutions between undiluted and the MDV or MVC. The other steps of product validation are Control Standard Curve, Positive Product Control (PPC) and Positive and Negative Sample and Water Controls. The critical components are a test material (diluted to an extent that was validated) and a positive control, which must be recovered within a prescribed range. Recovery of the PPC allows the analyst to have confidence in test results. Several researchers have found that even the selection of lysate reagent source can contribute to variability in test results. Therefore, if any change in reagent source is made, the test must be re-validated.

Conclusions

After the determination of EL, MVD, and MVC, the analyst should undertake validation to confirm performance and to assure reproducibility. Product validation steps include reagent and sample preparation, selection of sample preparation, and calculation of endotoxin value in product. Final choice to set up efficient BET testing method is up to the budget, application (water, raw material, in-process sample or final products), material and volume to be tested, endotoxin limit and degree of compliance.

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Macedonian pharmaceutical bulletin, **62** (suppl) 155 - 156 (2016)
ISSN 1409 - 8695
UDC: 615.28:614.2(497.715)
Short communication

Quantity of disinfectants and antiseptics used in general hospital in Gevgelija in relation to appearance of intra-hospital infections

Biljana Gjorgjeska*, Sofija Petkovska

Department of Pharmacy, Faculty of Medical Sciences, Goce Delcev University, str. "Krste Misirkov" no.10-A, 2000 Stip, Republic of Macedonia

Introduction

A number of chemical preparations in recent years are used as disinfectants and antiseptics. Disinfectants are chemicals that act as microbicides or microbiostatics of objects and the environment. They are present on the market as finished industrial products obtained in different concentrations and forms depending of its purpose. In the composition of disinfectants enter a wide variety of active chemical agents (biocides). According to their chemical composition they represent alcohols, aldehydes, anilides, biguanides, diamines, halogen release agents, silver compounds, peroxides, phenols, bis-phenols, halophenols, quaternary ammonium compounds, volatile compounds for sterilization. Biocide is a general term describing a chemical agent, usually with a broad spectrum of activity that inactivates microorganisms. The biocide activity is within the antimicrobial activity, but depending on the circumstances may have "static" activity directed to agents that inhibit it growth (bacteriostatic, fungistatic and sporostatic), and also "cidal" activity directed to agents that completely destroy all microorganisms present (bactericidal, sporocidal, fungicidal). The antiseptics are chemicals that are safely used for disinfection of skin and mucous membrane contact (McDonnell and Russell, 1999).

Intra-hospital or nosocomial infections are caused by organisms acquired during hospitalization of the patients and clinically manifest from 48 to 72 hours after their administration (Rutala et al., 2008).

Mechanical cleaning before applying the disinfectant is essential. The mechanism of action of disinfectants is summarized in a number of papers published and available to the entire scientific community. For example, alcohols show quick action, and broad spectrum antimicrobial activity through denaturation of proteins, but do not act sporocidal. Aldehydes bind with amino groups of proteins, RNA and DNA. Oxidizing agents such as peroxides and halogen elements oxidize the protein thiol groups, and surface active agents act mainly on the cytoplasmic membrane of the bacterial cell. Phenols generally destroy the cell membrane (Block, 2001).

It is well known that the type and quantity of used disinfectant and the disinfection procedures is directly related to the effects of their use (Ducel et al., 2002).

The aim of this study was to review of the use of antiseptics and disinfectants in general hospital in Gevgelija in Republic of Macedonia over five years period, to make analysis of the amount of antiseptics and disinfectants consumed annually on each department in hospitals, to analyze of the total amount of consumed antiseptics and disinfectants in selected hospitals for five years and to compare the results to the data obtained from the microbiological evaluations conducted periodically in each department in selected hospitals for five years.

Materials and methods

The data were collected from the general hospital in Gevgelija over five years, form 2010 till 2014. The data from the annual reports for disinfectants and antiseptics (Bactosal, Ecosal 10%, Ecosal ultra, Dezintal, Betadine 10%, Betadine 7,5%, Hydrogen peroxide 30%, Formaldehyde 33%, Ethanol concentrate, Gigazyme, Deconex 36 Intensiv, Gigasept forte AF, Gigasept FF, Gigasept PAA, Deconex 54 Sporocide, Microzid AF liquid, Arcana alca combi, Plivasept, Arcana san, Arcana anti fat) used on the selected departments (gynecology, surgery, transfusion, treatment of infections, internal medicine etc.) were collected. The results of microbiological testing conducted by the public health center in Gevgelija over five years were

* biljana.gorgeska@ugd.edu.mk

collected and used. Routine testing period for microbiological controls in hospitals was 15 days.

Results and discussion

The obtained data are getting an overview of the disinfectants and antiseptics used in the departments each year. It can be concluded that the highest consumption is in the operating room. The reason for this is that there are used all cleaning tools for disinfection of surfaces and instruments and antiseptics. Reduction of use of disinfectants and antiseptics in surgery room would cause much higher risk for intra-hospital infections. The highest amount (227 l) in the operating room is wasted in 2012. The lowest consumed amount of antiseptics and disinfectants was in mental health center and center for addicts.

To determine the relationship between the amount of used antiseptics and disinfectants with the emergence of intra-hospital infections, in addition the data obtained by the microbiological analysis in public health center in Gevgelija were processed. In 2012 a total of 27 inspections were conducted, 156 swabs and sediments were taken, 147 were sterile and 9 conditionally pathogenic.

The reason for the reduced number of isolated pathogens was increased amount of antiseptics and disinfectants used during 2012. From children's department and neonatology, all taken swabs were sterile. From surgical operation room, 2 pathogen samples were detected. In the rest of the surgical department, 2 pathogenic bacteria are isolated: *Staphylococcus epidermidis*, and *Staphylococcus albus*, but they have been isolated from patient beds and hands which is normal findings. Comparing to 2011 when they were isolated 6 conditionally pathogenic samples at the gynecology department; in 2012 the number of conditionally pathogenic samples was reduced to 2. At dialysis, a total number of swabs taken was 16 and 3 of them were conditionally pathogenic.

It is detected that the number of conditionally pathogenic bacteria is reduced starting from 2012. The reduction of the quantity of disinfectant used is also noted from 2012. By appointment of Ministry of health in 2012 each hospital had been established the intra-hospital infection times. The results indicate a significant reduction of contamination with conditionally pathogenic bacteria when disinfection is conducted according to the standardized procedures controlled by the intra-hospital infection times.

Conclusion

In general the disinfectants and antiseptics are used optimally and correctly according to the needs of the hospital investigated. The amount of disinfectants and antiseptics consumed comparing with the microbiological data indicates their rational utilization starting from 2012. Use of disinfectants according to the standardized procedures established by the intra-hospital infection time allows current daily care for patients and staff in the hospital investigated. The processed data from public health center confirm the above and point out the precautions to be taken when conditionally pathogenic bacteria have been detected.

It is pointed out the role of intra-hospital infection times in the hospitals as well as the role of hospital pharmacists. We would like to suggest the implementation of disinfection process validation as standardization measure as well as more often routine microbiological controls in the hospitals.

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Macedonian pharmaceutical bulletin, **62** (suppl) 157 - 158 (2016)
ISSN 1409 - 8695
UDC: 615.262:547.857.4]:543.544.5.068.7
Short communication

HPLC determination of caffeine in anti-cellulite gels after the solid phase extraction

Kristina Mladenov* and Slavica Sunarić

Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar Zorana Đinđića 81, Niš, Serbia

Introduction

Caffeine, an organic nitrogenous base is a naturally occurring methylxanthine alkaloid, which is being increasingly used in cosmetics due to its high biological activity and ability to penetrate the skin barrier. As for a cosmetic purpose, caffeine is used as an active compound in anti-cellulite products. Cellulite deposition is one of the most common problems associated with a large population male and female person throughout the world. Caffeine prevents an excessive accumulation of fats and promotes the lipolysis process. It inhibits phosphodiesterases, which leads to lipogenesis reduction. Caffeine also stimulates the draining lymph systems in fatty tissue by removing accumulated fat and toxins, which can improve the microcirculation of blood vessels (Herman and Herman, 2013). The commercially available topical anti-cellulite formulations usually contain 1-2% of caffeine, although some products may contain up to 3% of caffeine (Hexsel and Soirefmann, 2011).

There are number of papers about caffeine determination in different food products, but limited works which describe caffeine determination in cosmetics (Injac et al., 2008; Marchei et al., 2013). Cosmetics are complex formulations and for their analysis different and lengthy sample treatments are required. Sample preparation and clean-up are crucial, especially for the HPLC analysis, where the sample solutions are directly injected to HPLC column. Also, anti-cellulite gels and creams contain a wide variety of synthetic and natural (plant-derived) substances which may interfere with the caffeine determination. Solid phase extraction (SPE) is useful for sample preparation and clean-up in the analysis of pharmaceutical creams. It is more rapid and efficient than liquid/liquid extraction, yields quantitative extractions that are easy to perform without using of high volumes of toxic organic solvents.

In this study, a solid phase-extraction method followed by HPLC with ultraviolet-diode array detection to determine of caffeine content in anti-cellulite gels was developed.

Materials and methods

Caffeine (1,3,7-trimethylxanthine) of analytical grade was obtained from Sigma-Aldrich (Germany). The solvent used, methanol, was of HPLC grade (J.T.Baker). For the sample preparation ethanol (95-96%, Ph.Eur.V, Zorka-Pharma, Serbia) was used. Stock solution of caffeine concentration of 1.0 mg/mL was prepared in deionized water. This solution was stored for up to one week at 4 °C.Working solutions were prepared by diluting and mixing the stock with deionized water.

Cellulite reduction cosmetics were obtained from a local perfumery. Two products were analyzed: Producer 1-Aqua Destock and Anticelulit gel-Producer 2. Both products contain caffeine in significant amount, and many synthetic substances and plant extracts also. The exact content of caffeine was not declared.

Solid-phase extraction Chromabond®HR-X cartridges (100 mg, 1 mL volume, Macherey-Nagel, Germany) were selected as optimal for the clean-up and extraction of caffeine from anti-cellulite gels. SPE cartridges were conditioned with 3 mL of methanol and 3 mL of deionized water. One milliliter of the prepared sample was loaded on the cartridge at the flow rate of 1 mL/min. Desorption of caffeine was achieved with 4 x 1 mL of methanol.

HPLC analyses were performed on the Agilent Technologies 1200 Series apparatus with DAD and FL detection. Separation was carried out on the Restek Ultra IBD

* kristina_mladenov@yahoo.com;
slavica.sunaric@medfak.ni.ac.rs

C18 column (150 mm x 3 mm, 3 μm) at 30 °C. The mobile phase consisted of a methanol-water solution (40:60, isocratic) at a flow rate of 0.4 mL/min. A 2 μL of the final eluate was injected in the HPLC column. UV detection at 274 nm was used as the optimal wavelength for caffeine determination.

Sample preparation

A 0.5 g of the gel was accurately weighed and dissolved in 10 mL of 95-96% ethanol. NH_4OH was added to obtain pH 7-8. Insoluble excipients were separated by centrifugation for 10 min. Then, 1 mL of the supernatant was loaded on the cartridge and SPE procedure was performed.

Results and discussion

Optimization of SPE procedure included cartridge selection, solvent selection and recovery evaluation. To evaluate the accuracy of the extraction method, recovery studies were carried out. A known amount of caffeine standard solution, at three different concentration levels was extracted on different cartridges and recovery was calculated as the ratio of the loaded and eluted caffeine. The percentage extraction recovery was found to be 82-90% for the Chromabond®HR-X cartridge, while for the other types of cartridges this value was significantly lower. After the procedure of gel preparation, the obtained supernatants were also loaded on different SPE cartridges. The highest chromatographic peak purity, peak area and concentration levels of caffeine were also obtained for Chromabond®HR-X cartridge. For that reason, this type of cartridge was selected for the extraction and clean-up of the examined anti-cellulite gels.

The caffeine content in the examined samples was determined by using the calibration curve in the range of 0.01-0.2 mg/mL. The caffeine concentration found in Producer 1-Aqua Destock was 1.70 g/100 g (1.70%), while the value for Anticelulit gel-Producer 2 was 0.69 g/100 g (0.69%). The found content of caffeine in investigated cellulite reduction cosmetics were in agreement with those reported by other authors (Injac et al., 2008; Marchei et al., 2013). and with the expected values.

Conclusion

The most effective and marketed products for cellulite reduction contain caffeine as the active ingredient, but its exact content in these products is not strictly declared. The extraction step is necessary for the analysis of caffeine in anti-cellulite gels by chromatographic methods. Because of the fact that caffeine is contained in the complex cosmetic matrix, its extraction is difficult and depends on the nature of the sample. A purification of the cosmetics and extraction of the analyte can be achieved by using SPE technique.

The present results show the applicability of the proposed SPE procedure for the caffeine extraction from anti-cellulite gels and its HPLC determination, which could be used for the routine analysis of these commercial products.

Acknowledgement

This research was supported by grant TR 31060 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 159 - 160 (2016)
ISSN 1409 - 8695
UDC: 615.076
Short communication

Validation and quantification of bacterial endotoxins with turbidimetric kinetic method for benzyl alcohol

Elizabeta Popovska[1]*, Silvana Ilioska-Zlatanovic[1], Hristina Babunovska[1], Biserka Simonovska[2]

[1]*Alkaloid A. D, Blvd. A. Makedonski 12, 1000 Skopje, R.Macedonia*
[2]*Pharmachem Diagnostic Laboratory, Str. 4th July No 2/3, 1000 Skopje, Macedonia*

Introduction

Bacterial endotoxins are contaminants from gram-negative bacteria and are the most common cause of pyrogenicity in pharmaceutical products. Any preparation administered parenterally should be sterile and comply with the test for bacterial endotoxins (BET) as described in the Ph. Eur. (Ph. Eur., 2014). Finished products often contain ingredients in addition to the active drug substance. Excipients serve as a solvents, solubilizing, suspending, thickening, and chelating agents; antioxidants, antimicrobial preservatives, buffers, pH adjusting agents, bulking agents, and special additives (British Pharmacopoeia, 2014; CHMP, 2013; ICH, 2009; Ph Eur., 2014).

Benzyl alcohol is an aromatic alcohol with the formula C_7H_8O. In the body, benzyl alcohol is metabolised into benzoic acid. It is used as an excipient for its preservative properties or as a solubilising agent. Benzyl alcohol is also used as an active ingredient in local antiseptics and local anesthetic products. It is also present in medical products administered intravenously (anti-cancer drugs, heparins, cardiovascular drugs) and as a preservative in many topical preparations, such as antifungal and anti-inflammatory products (Ph.Eur., 2014). However benzyl alcohol is mainly used as an excipient in medicinal products that are administered intramuscularly, such as antibiotics, anti-inflammatory or neuroleptic medicines where its anesthetic properties reduce pain at the injection site.

Objective: Validation and quantifications of Bacterial endotoxins with Turbidimetric kinetic method, for benzyl alcohol.

Materials and methods

Benzyl alcohol (Merck Certified; Merck KGaA, Darmstadt, Germany); Reagents for Kinetic turbidrimetric method: PYROGENT™- 5000, LAL Reagent Water, and Pyrosperse™ Dispersing Agent, one of a group of metallo-modified polyanionic dispersants which has proven useful as a sample modifying agent for certain types of products showing inhibition in the BET assay from Lonza (Lonza Group Ltd, Walkersville, MD21793), along with Microplate reader ELx808 from BIO-TEK® (BIO-TEK Instruments, INC.,P.O.BOX998, Hghlsand Park, Winooski, Vermont 05404 USA). The results were calculated and stored with WinKQCL 4.0.2 software™ (Lonza Group Ltd, WalkersvilleThe Kinetic turbidrimetric method is based on measuring the turbidity (optical density) of an LAL/sample mixture at regular interval troughout the test. The time required before the appearance of turbidity (Reaction time) is inversely proportional to the amount of endotoxin present.

In accordance with the Ph. Eur. Commission at its 149th Session, June 2014: Substances to be used in parenteral preparations must comply with the BET, whatever their origin, since:

- contamination by bacterial endotoxins can take place prior to or during the manufacturing process;
- bacterial endotoxins cannot easily be removed by the manufacturing process;
- bacterial endotoxins should be detected as early as possible in the manufacturing process.

It is to be noted that the ICH Guideline Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products provides the following recommendation under section Drug Substance Specification: “Pharmacopoeial tests (e.g., endotoxin detection)

* epopovska@alkaloid.com.mk

should be performed on the drug substance, where appropriate".

Benzyl alcohol, as an excipient in parenteral preparations, has strongly inhibitory effect on Bacterial endotoxins test (Kinetic Turbidimetric method) when diluted with LAL reagent water (LRW). It is necessary to overcome the inhibition and for that purpose 2% solution of Pyrosperse™ Dispersing Agent from Lonza was used in combination with laboratory techniques: shake 30min, vortex 30sec. and centrifuge 3min. (3000rpm/min), (British Pharmacopoeia, 2014; EMA, 2013; FDA, 2012; Ph.Eur., 2014; Thomas et al., 1984)

Results and discussion

Validation was performed by beginning with screening (Inhibition / Enhancement) test. For that purpose PYROGENT™- 5000, Lonza's reagents were used; lysate sensitivity λ = 0,01 EU/mL; maximum valid dilution (MVD) = ELC/λ = 2.5 / 0.01 = 250. Most products interfere with the performance of the BET. The easiest way to overcome interference is to dilute the product in LRW. Dilutions used for preliminary, Inhibition / Enhancement test were as follows: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and the MVD 1:250. All dilutions were made with LRW (FDA, 2012; Ph.Eur., 2014; McCullough, 2011; Williams, 2009). Benzyl alcohol shows strong inhibition including MVD. To overcome the inhibition new dilutions were made. For the first dilution 1:10, as a solvent 2% solution of Pyrosperse™ Dispersing Agent, from Lonza was used, shaked for 30 min, vortexend for 30 sec and centrifuged 3 min (3000 rpm/min). Tested dilutions: 1:50, 1:100 and 1:200 were made from the aqueous part of the first dilution. Aqueous part was used for analysis. There was an inhibition only at 1:50 dilution, PPC recovery: not applicable (N/A). The inhibition was overcomed at 1:100 and 1:200 dilution which gave PPC recovery 73% and 115% respectively (Lonza Group Ltd, Walkersville; Ph.Eur., 2014).

Upon received results the validation further was done on three consecutive batches of benzyl alcohol with dilution 1:200 (considering that recovery must be close to 100%). The endotoxin concentration reported for the three consecutive batches was less than 2.00 EU/ml with PPC 115%, 149% and 151% respectively. All three batches of the tested benzyl alcohol showed endotoxin recovery between 50 and 200%.

Conclusion

Validation of the Bacterial endotoxins test on Benzyl alcohol was successfully done. The inhibition was overcome. Further, routine testing of Benzyl alcohol should be done with dilution 1:200 using Pyrosperse™ Dispersing Agent from Lonza, shaking for 30 min, vortexing for 30 sec and centrifuge 3 min (3000 rpm/min) in accordance with the method developed.

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Macedonian pharmaceutical bulletin, **62** (suppl) 161 - 162 (2016)
ISSN 1409 - 8695
UDC: 615.214.24.074:543.544.5.068.7
Short communication

A rapid and validated reverse phase liquid chromatographic method for *in vitro* dissolution test for determination of bromazepam in tablet formulations

Irena Brchina[1], Biljana Gjorgjeska[2]*

[1]*AD Jaka 80,Prvomajska 75A, Macedonia*
[2] *Department of Pharmacy, Faculty of Medical Sciences, Goce Delcev University, str. "Krste Misirkov" no.10-A, 2000 Stip, Republic of Macedonia*

Introduction

Disorders that involve anxiety are the most common mental disturbances. Many of the anti-anxiety medicines also cause some sedation, so the same medicine is often functioning clinically as both, anxiolytic and hypnotic agent. Benzodiazepines are the most widely used anxiolytic medicines. They have largely replaced barbiturates in the treatment of anxiety, because the benzodiazepines are safer and more effective. Anxiety is an unpleasant state of tension, apprehension, a fear that seems to arise from a sometimes unknown source. The physical symptoms of severe anxiety are similar to those of fear (such as tachycardia, sweating, trembling, and palpitations) and involve sympathetic activation. Episodes of mild anxiety are common life experiences and do not warrant treatment. However, the symptoms of severe, chronic, debilitating anxiety may be treated with antianxiety medicines (sometimes called anxiolytic or minor tranquilizers) and/or some form of behavioral or psychotherapy.

Bromazepam is a benzodiazepine (BZD) generally used for a number of medical reasons, it is an intermidiate-acting tranquiliser (Ashton, 2005), prescribed for the treatment of moderate to severe anxiety and panic attacks for the short-term treatment of insomnia. It has been widely used in psychiatry disorders for four decades, with selective anxiolytic, anticonvulsant, myorelaxant and hypnotic actions. It acts on the central neural system as an inhibitor of the neurotransmitter gamma aminobutyric acid (GABA) (Nascimento et al., 2012).

Bromazepam is an active substance that belongs to class of 1,4-benzodiazepine and chemically corresponds to 7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepine-2-one, $C_{14}H_{10}BrN_3O$ (Ph.Eur., 2010). It is a controlled psychotropic substance-B1 class according to the National Agency of Sanitary Vigilance in Brazil (ANVISA), with the DCB identification numbers: 01366, DCI: 2692 and CAS: 1812-20-2. The solid dosage form (tablet) is the widespread used and prescribed in clinical practice. The solid dosage form presents problems associated to the bioavailability (FDA, 2003). The absorption of active substance from the solid dosage form for oral application depends on the solubility and dissolution in physiologic liquids and its permeability through the gastrointestinal tract, factors that influence directly its bioavailability and subsequent pharmacological effects. The biotransformation from solid into absorbable form depends on its dissolution in organic liquids; therefore, dissolution tests became an essential parameter to determine the properties of biopharmaceutical formulations in order to predict their quality. The quality of pharmaceutical formulations is important in financial and ethical terms because it is directly associated with the patient's health. Thus, there is a real need for the development of dissolution tests able to predict *in vivo* physiological conditions.

Materials and methods

Dissolution test is a standardized method for measuring the rate of active substance release from a dosage form (FDA, 1997). The test was performed on ERWEKA DT 700, apparatus 2 (paddle), using 0.1 M HCl as a dissolu-

* biljana.gorgeska@ugd.edu.mk

tion medium, in volume of 500 ml, at 37°C, with 75 rpm for 45 minutes.

The quantity of the dissolved bromazepam was determined using analytical method based on High Performance Liquid Chromatography (HPLC). Validation of the method was performed on Shimadzu Nexera HPLC system.

To optimize chromatographic parameters several mobile phase compositions were tested in this method. A satisfactory separation, good peak symmetry and optimal retention time was obtained using mobile phase consisting of a mixture of methanol, acetonitrile and potassium dihydrogen phosphate buffer (11.33 g/l of KH_2PO_4, pH 7.0 adjusted with KOH, 0.5 M) in ratio of 45:5:50 (v/v/v), at flow rate of 1.0 ml/min. A LiChrospher RP Select B column (125 × 4.0 mm, 5µm) was used as stationary phase with temperature of column oven, 50 °C. The elution was monitored at 239 nm.

Results and discussion

A simple reverse phase HPLC method for *in vitro* dissolution test was developed and validated for the determination of bromazepam and its release from pharmaceutical dosage form. Several high-performance liquid chromatographic (HPLC) methods have also been reported for the determination of bromazepam and other BZDs (Sruthi et al., 2013). Chromatogram showed a peak of bromazepam (BZP) at retention time of 3.50 ± 0.1 min. The most suitable mobile phase was selected on the basis of time required for the analysis and the sensitivity of the method. The method was validated according to ICH Q2 guideline with respect to specificity, linearity (with correlation coefficient of 0.999), accuracy, precision, robustness, solution stability and filter paper compatibility. All results of the validation parameters were within the limits of ICH guidelines (ICH, 2005). The benefits of the proposed method include simple preparation of the solutions for the analysis and usage of readily available solvents.

Conclusion

The proposed method is simple, rapid, accurate, precise and specific without interference of excipients. The short chromatographic run time allows the analysis of a large number of samples in short period of time. Therefore, it is suitable for the routine analysis of bromazepam in pharmaceutical dosage forms and it could be used for the rapid and reliable determination of dissolved bromazepam in tablet formulations. The present study was focused on minimizing method limitations and developing a simple and economic method for determination of dissolved bromazepam from tablet dosage form.

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Macedonian pharmaceutical bulletin, **62** (suppl) 163 - 164 (2016)
ISSN 1409 - 8695
UDC: 615.213.074
Short communication

New generation antiepileptic drugs: affordable bioanalytical method for therapeutic monitoring

Arlinda Haxhiu Zajmi[1]*, Jasmina Tonic Ribarska[2], Emilija Cvetkovska[3], Rumenka Petkovska[2], Suzana Trajkovic Jolevska[2]

[1]*Department of Pharmacy, Faculty of Medical Sciences, State University of Tetova, Ilinden bb, 1200 Tetovo, R. Macedonia*
[2]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia*
[3]*Clinic of Neurology, Faculty of Medicine, University "Ss Cyril and Methodius", Vodnjanska 18, 1000 Skopje, R. Macedonia*

Introduction

The goal of antiepileptic drugs (AED) therapy for people with epilepsy is seizure freedom without side effects (Vajda, 2007). In the last decade, several new AEDs have been licensed around the world (Krasowski, 2010). Considering the variable nature of epilepsy, patient's pathophysiological features, co-medications, AED therapy must be carefully optimized for each patient by the aid of therapeutic monitoring (TDM), concerning both therapeutic and toxicological profiles. In many cases TDM facilitates drug treatment by increasing clinical effectiveness and safety while minimizing adverse effects, as well as reducing treatment costs. The availability of a simple, validated and inexpensive analytical method for reliable measurements of drug concentrations in biological fluids is pivotal for its successful utilization in pharmacokinetic and bioequivalence studies, and for therapeutic drug monitoring in different clinical situations. Various analytical methods have been reported for measurement of AEDs in biological fluids, including immunoassays and chromatographic methods (gas chromatography and high-performance liquid chromatography). Immunoanalysis techniques are specifically designed to analyze a single drug but the chromatographic methods can allow for the simultaneous separation and quantitation of many compounds in a single run which is important when is need the simultaneously monitoring the AEDs in polytherapy, which is given very common to patients (Aldaz et al., 2011)

The aim of this study was to develop a sensitive and fast bioanalytical method for simultaneous determine the plasma concentration of recent old and new AEDs used in the current clinic practice: lamotrigine (LTG), levetiracetame (LEV), carbamazepine (CBZ), the active metabolite carbamazepine -10,11-epoxide (CBZ-EP) and valproic acid (VPA).

Materials and methods

Chemicals and reagents

LTG, LEV, CBZ, CBZ-EP, VPA and nitrazepam (internal standard,IS), were purchased from Sigma Aldrich, USA. Methanol and acetonitrile, HPLC grade, were purchased from Merck, Germany. Potassium dihydrogen phosphate and phosphoric acid for buffer preparation were analytical grade and were also obtained from Merck, Germany. For all analyses HPLC grade water purified with a TKA_LAB Reinstwasser system (Niederelbert, Germany) was used. OASIS HLB cartridges (30 mg/1 ml) used for sample preparation were supplied by Waters, USA.

Human plasma sampling

Plasma was collected from healthy volunteers (drug-free plasma for the method validation) and from the epileptic patients undergoing chronic AEDs therapy. Blood samples were collected in the morning, just before the first daily drug administration, into heparinized tubes and centrifuged at 3000 rpm for 15 min. The supernatant plasma was transferred into test tubes and frozen at -20 °C until analysis. The Ethics Committee at the Faculty of Pharmacy and the Faculty of Medicine, Ss. Cyril and Methodius University - Skopje, approved the research protocol for this study

* arlindahaxhiu@gmail.com

and all volunteers signed the Study Informed Consent form.

Chromatographic conditions

The assay was performed on Shimadzu LC-30 Nexera, using Zorbax Eclipse XDB C-18 (150 x 4.6 mm; 5 µm) column, using gradient elution with acetonitrile and phosphate buffer (pH 3.0), as a mobile phase. The analyses were run at a flow rate of 1 mL/min, volume of injection 50 µL, temperature 25 ºC and UV detection was set at 210 nm.

Experimental design (Fractional factorial 34) was used for the optimization of chromatographic conditions. The influence of four experimental factors (pH of buffer solution in the mobile phase, initial and final content of acetonitrile in mobile phase during gradient elution and the gradient time) was investigated at three factor levels. All remaining factors such as the flow rate of mobile phase, wavelength of UV detection, temperature of the column and volume of injection were kept at constant level. The method was validated according to the EMA Guideline on validation of bioanalytical method (EMA, 2011).

Preparation of standards and quality control standards

Stock solution of analytes (500 µg mL LTG, 500 µg/mL LEV, 500 µg/mL CBZ, 500 µg/mL VPA, 200 µg/mL CBZ-EP) and the IS (500 µg/mL) were prepared by dissolving appropriate amounts of each compound in methanol. Working solutions were prepared daily from stock solutions by dilution with purified water. Calibration standards were made by spiking the blank plasma aliquots with appropriate volume of working solutions of LTG, LEV, CBZ, VPA and CBZ-EP at 7 different concentrations containing the IS at constant concentration 10 µg/ml. The resulting plasma concentration ranges were: 1.0 - 25.0 µg/mL (LTG); 3.0 -70.0 µg/mL (LEV); 1.0 - 25.0 µg/mL (CBZ); 0.5 -15.0 µg/mL (CBZ-EP); 25.0 - 250 µg/mL (VPA). Four levels of quality control (QC) samples were prepared at the concentration range of: 1.0; 5.0; 10.0; 20.0 µg/ml for LTG; 3.0; 7.5; 30.0; 60.0 µg/ml for LEV; 1.0; 5.0; 10.0; 20.0 µg/ml for CBZ; 0.5; 2.5; 5.0; 10.0 µg/ml for CBZ-EP and 25.0; 100; 150; 200 µg/ml for VPA, in same way as describe above and stored at - 20 ºC until analysis.

Sample preparation

The sample preparation step is an important component of bioanalytical method i.e determination of plasma concentration of AEDs. To find the most efficient method, sample pre-tretment methodology was initially investigated by protein precipitation (PP) and than a solid- phase extraction (SPE) using Oasis ® HLB cartridges.

Results and discussion

Under the proposed chromatographic conditions, no interfering peaks were observed in the retention times of analytes. Calibration curves were constructed by means of the least-squares method, obtained by plotting the analyte - IS peak area ratios versus the respective analyte concentrations. The correlation coefficients were 0.9989 for LTG, 0.9979 for LEV, 0.9986 for CBZ, 0.9978 for CBZ-EP and 0.9978 for VPA, respectively. The results for within-run and between-run accuracy and precision were within recommended limits. The pretreatment of plasma samples by protein precipitation, gave no profitable results because the processed samples were relatively unclean with low recovery. Since SPE procedures are usually associated to high and reliable extraction of AEDs from human plasma, several SPE conditions were tested, including washing steps and eluting solvents. The use of SPE procedure (loaded a 200 µL of sample, washing with 1ml water and 1 ml 5% methanol and eluting with methanol) demonstrated to be the best option as it allowed a more effective elimination of interfering substances with good recoveries. The absolute mean recoveries for determined AEDS and metabolite ranged from 85.2% to 99.6% while mean recovery of IS was 98.9% for SPE. The stability studies analytes indicate that stock solutions and plasma samples were stable under different storage conditions and that no stability related problems would be expected during the routine plasma sample analysis.

The proposed method gives satisfactory results when was applied to plasma samples collected from epileptic patients simultaneously treated with carbamazepine and levetiracetame; lamotrigine and valproate; or in combination levetiracetame, lamotrigine and carbamazepine.

Conclusion

A simple, sensitive, precise and accurate HPLC method has been developed for simultaneous determination of LEV, LTG, CBZ, CBZ-EP and VPA in human plasma samples. Time of analysis and resolution were simultaneously optimized by applying Fractional factorial 34 design. Total chromatographic analysis time per sample was approximately 15 min. The proposed method can be applied successfully in routine analysis for the estimation of therapeutic concentration of LEV, LTG, CBZ, CBZ-EP and VPA in human plasma.

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Macedonian pharmaceutical bulletin, **62** (suppl) 165 - 166 (2016)
ISSN 1409 - 8695
UDC: 615.212.7.014.4
Short communication

Trend analysis in stability data for Caffetin Cold film coated tablets

Sanja Despotovska*, Milena Dobrkovic Shotarovska, Mena Ivanoska, Ana Aleksandric, Marina Mandzukovska, Dragana Kafedziska, Vasilka Dubrova Koceva, Hristina Babunovska

ALKALOID AD-Skopje, Pharmaceutical, Chemical and Cosmetics Company, Aleksandar Makedonski 12, Skopje, Macedonia

Introduction

High-quality stability data is crucial to pharmaceutical industries around the world. These data form the basis for justifying specification limits (Huynh-Ba, 2008). The stability data, combined with the specification limits, are used for setting and/or extending product shelf life and for establishing product storage conditions. Stability studies are initiated annually and may be used to support product or process modifications (ICH, 2003). These studies are vital for ensuring the continuous quality of production batches. In order to identify potential issues and to ensure data quality, it is often advantageous to use objective and statistical methods for trend analysis of stability data.

The presented paper describes trending of stability results for pharmaceutical product Caffetin Cold film coated tablets. This paper will provide an overview of one statistical approach for trending stability results. According to ICH Q1E guideline (ICH, 2003), evaluation of the stability data, after the assessment of the shelf life, should progress through the trends and variability of the long-term stability data (Yoshioka and Stella, 2000). The evaluation of stability data (not necessary statistical) is done in order to identify the trends and their impact on the stability of the product (Yoshioka and Stella, 2000).

Materials and methods

In order to evaluate stability data and find the pattern of data that indicates change over time, stability studies for Caffetin Cold film coated tablets were subjected to trend analysis. The trending analysis was performed to evaluate whether the data demonstrates increasing or decreasing trend (change of mean) for the stability indicating parameters over time or the data indicates no discernible change at all. In order to evaluate trends and variability of the long-term stability data, we evaluated three stability indicating parameters: assay, dissolution rate and related and degradation products. In order to assess the trend of the parameters Paracetamol assay, Paracetamol dissolution rate, Dextromethorphan assay, Dextromethorphan dissolution rate, Pseudoephedrine assay, Pseudoephedrine dissolution rate, Ascorbic acid assay, Ascorbic acid dissolution rate and related and degradation products, we collected all the stability data obtained for this parameters through the years and graphically presented them. The results are presented both as a table and as a graph.

Results and discussion

Caffetin Cold film coated tablets is a pharmaceutical product with long manufacturing history with the same manufacturing route, on the same manufacturing site and following described manufacturing procedure in Alkaloid AD, Skopje and with well-established stability. Ongoing stability studies in Alkaloid AD, Skopje are performed on one batch annually for long term stability testing, including the batches from years of 2010, 2011, 2012, 2013, 2014 and 2015.

Evaluation was conducted using one common statistical graph (scatter plotting of the results) in order to compare the obtained results. The obtained results were

* sdespotovska@alkaloid.com.mk

incorporated into respective trends. The suitable trends for all stability indicated parameters were defined from the collected stability data. The evaluation of stability data was conducted in non-statistical way in order to identify the trends.

Parameters such as Paracetamol assay, Paracetamol dissolution rate, Dextromethorphan dissolution rate, Pseudoephedrine dissolution rate and Ascorbic acid dissolution rate indicate no discernible change. All the results retain almost constant around the initial result value. Results for this investigated parameter remain within the shelf-life requirements.

Parameters Dextromethorphan assay and Ascorbic acid assay indicate mild decreasing trend, but results remain within the limits for shelf life specification. Parameter Pseudoephedrine assay indicate a trend in which a single result within a batch is resulting in atypical trend compared to the other batches. This situation shows a single atypical result within a study that cannot yet form a trend and needs further investigation in the following frequencies of testing (in order to avoid false alarm). For all other collected data we find that they indicate mild decreasing trend, but results remain within the limits for shelf life specification. Parameters 4-aminophenol, unknown impurity and total impurities indicate no discernible change.

Parameter Paracetamol assay indicates no discernible change. All results retain almost constant around the initial result value. Results for investigated parameter Paracetamol assay remain within the shelf-life requirements. Parameter Paracetamol dissolution rate indicates no discernible change. All the results retain almost constant around the initial result value. Results for investigated parameter Paracetamol dissolution rate remain within the shelf-life requirements.

Parameter Dextromethorphan assay indicates mild decreasing trend, but the results remain within the limits for shelf life specification. Parameter Dextromethorphan dissolution rate indicates no discernible change. All the results retain almost constant around the initial result value. Results for investigated parameter Dextromethorphan dissolution rate remain within the shelf-life requirements.

Parameter Pseudoephedrine dissolution rate indicates no discernible change. All the results retain almost constant around the initial result value. Results for investigated parameter Pseudoephedrine dissolution rate remain within the shelf-life requirements. Parameter Pseudoephedrine assay indicate a trend in which a single result within a batch is resulting in atypical trend compared to the other batches. This situation shows a single atypical result within a study that cannot yet form a trend and needs further investigation in the following frequencies of testing (in order to avoid false alarm). For all other collected data we find that they indicate mild decreasing trend, but results remain within the limits for shelf life specification.

Parameter Ascorbic acid assay indicates mild decreasing trend, but results remain within the limits for shelf life specification. Parameter Ascorbic acid dissolution rate indicate no discernible change. All the results retain almost constant around the initial result value. Results for investigated parameter Ascorbic acid dissolution rate remain within the shelf-life requirements. Parameters 4-aminophenol, unknown impurity and total impurities indicate no discernible change.

Conclusion

From the presented results it could be observed that the parameters assay, dissolution rate and related and degradation products, considered as critical quality attributes, reveal very similar profile for all batches tested at the same conditions. The results of all batches follow a trend suggesting that there is no statistically significant difference in parameters trends.

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Macedonian pharmaceutical bulletin, **62** (suppl) 167 - 168 (2016)
ISSN 1409 - 8695
UDC: 615.322:659.1(497.7)
615.322:659.1(497.11)
Short communication

Comparative analysis of advertising and promotion of traditional herbal medicine and food supplement at different markets - case study

Marjan Dzeparoski[1]*, Suzana Trajkovic-Jolevska[2]

[1]*Bionika Pharmaceuticals, Skupi 15, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia*

Introduction

Macedonia and Serbia fully follow EU regulation of medicines. Herbal medicines in Macedonia are regulated by the Law on Medicines and Medical Devices (2007) and the Regulation on the registration of traditional herbal medicinal products from 2008. Serbia also follows EU legislation. Laws on Medicines in Macedonia and Serbia are harmonized with the EU regulation of medicines.

Macedonia follows EU legislation for food supplements. The products are regulated by the special requirements for safety of food supplements from 2012 (Official Gazzette of Republic of Serbia no. 45/10, 27/11; 50/12), and the Rulebook amending the Rulebook on special requirements for food supplements from 2013 and 2015 (Official Gazzette of Republic of Serbia, no. 41/13), and in compliance with the Croatian Rulebook on food supplements in terms of their classification from 2013. Serbia also follows EU legislation, but has not implemented and do not follow the Regulation on nutrition and health claims, novel food regulation procedures and the law on using symbols on packaging and packaging waste. For food supplements, indications and properties that are not possessed should not be attributed (Dzeparoski, 2013; Kotler, 1989; Kotler and Keler, 2009).

The main goal of this paper was to make comparative analysis of advertising and promotion of the product Sentis by the company Bionika Pharmaceuticals in Macedonia and Serbia. The marketing campaigns, TV spots and internet advertisement have been analyzed. The choice of the countries is based upon different product categorization in which Sentis is classified.

Materials and methods

Comparative method for analyzing the regulation, advertising and promotion of the food supplements and traditional herbal medicines was used.

Results and discussion

Case study

Sentis is a natural sedative in the form of soft gelatin capsules containing 200 mg of extract of valerian root (*Valeriana Officinalis*). In Macedonia Sentis is registered in 2012 as traditional herbal medicine (THM), while in Serbia is registered as a food supplement in 2013. Valerian root has been used since ancient times before the existence of synthetic sedatives. The action is due to valerenic acid; experimental pharmacological studies have shown that it reduces motility, causing hypnotic activity, offsetting the effect of caffeine and improves coordination. In both countries registered indications are temporary relieve of the symptoms of nervous tension, stress and insomnia. Benefits are unlike synthetic sedatives, it does not cause drowsiness and dependence (Bionika, 2013; PDR, 2007; WHO, 1999).

The differences regarding dosing and indications, resulting from the different classification of the same product are as follows:

- dosing registered as THM in Macedonia is 4 x 200 mg, while as food supplement in Serbia maximum daily dose is decreased to 2 x 200 mg;
- -indications are the same in both countries, except that in Macedonia additionally is registered the sedative effect.

* marjan.dzeparoski@bionikapharm.com

These differences concern advertising and promotional activities.

In Macedonia and Serbia same package in green and stylized cubist design of flowers and brain with minimalistic look is used. The text on the package and the patient leaflets are different in accordance with the categorization, in correlation with the specific regulation. For THM it is written clearly that it is medicine without prescription, with specific 6 line content structure of the patient leaflet and indications based on traditional use. For the food supplement is clearly stated that the product is food supplement and also ingredients, nutrition and energetic values must be printed on the package, whereas the leaflet and claims are not obligatory. In Serbia the food supplement Sentis is sold with leaflet which contains claims.

Internet is universal and powerful digital marketing tool in promotion of company products, including company web-sites. The content of websites also varies in different countries.

Promotional materials are with similar text content and same design in both countries, except the statement that it is THM or food supplement. The other difference for the THM is also that "the patient leaflet should be read, for more information you should consult your pharmacist or doctor". The last is valid for all forms of advertisement.

TV spot for Sentis as THM has been approved for advertising in electronic media to the general public by the Ministry of Health. The message which should remain in the subconscious of the audience is that when you use Sentis the life will be without anxiety, stress, carefree, without worries and the consumer will continue on with life. TV spot for Sentis as food supplement in Serbia is from informative character. It is not necessary approval for advertising, because is based on generally accepted scientific data and they are comprehensible to the average consumer. In Serbia should be submitted for approval only health claims for reducing the risk of disease. The message which should remain in the subconscious of the audience is that it is a natural product for healthy people, which if used will help reduce certain symptoms.

Except promoting the product, with both advertisements is also promoted the new pharmaceutical company. TV advertising in Macedonia with a strong and distinctive message, along with the promotion activities, helped the company to achieve significant market share in this segment. Marketing of products by companies should be in accordance with the current country regulation for specific product category.

Conclusion

There are major differences in the marketing of THM/OTC and food supplements. All promotional materials for THM to the general public are subject to approval by regulatory bodies, based on the approved summary of product characteristics and patient leaflet. The marketing of food supplements is more liberal in advertising and in Serbia is not subject to approval, if based on generally accepted scientific data. Macedonia follows the EU Regulation on nutrition and health claims for food supplements. The results of the case study can also be used for preparation of marketing materials for other products on other markets of interest with similar regulation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 169 - 170 (2016)
ISSN 1409 - 8695
UDC: 615.276.074:543.422.3-74
Short communication

Validation of NIR methods for identification of ibuprofen lysine

Biljana Bujaroska*, Marija Spasevska, Bisera J. Trajkovska, Maja Ilijoska, Andrea Alagjozovska, Hristina Tomovska, Nada Stojanoska, Hristina Babunovska

ALKALOID AD-Skopje, Pharmaceutical, Chemical and Cosmetics Company, Aleksandar Makedonski 12, Skopje, Macedonia

Introduction

Near InfraRed - Fourier transform infrared spectroscopy (FT-NIR) in recent years has gained much interest due to its simple maintenance and short measuring time. This technique differs from the conventional techniques such as HPLC and GC, because the essential component is the sampling interface. That allows for fast, non-destructive measurement of materials with little or no sample preparation.

Near Infrared Spectroscopy (NIRS) is used for identification on every container that has been delivered. The principle is based on the comparison of the spectral data of the substance with the spectral data of several samples, which are present in a spectral reference library. These samples have positive chemical/physical and microbiological test analysis, tested in accordance with producer Certificate of Analysis and producer Drug Master File. Positive identification of these samples originally is confirmed with IR Spectrophotometry or other specific identification method. Besides others parameters, these analyses include testing of loss on drying, content of water, optical rotation, related substances and assay that may cause of variables in the NIR spectra.

According to the "Guideline on the use of NIR spectroscopy by the pharmaceutical industry and the data requirements for new submission and variations" issued by the European Medicine Agency, when NIR procedures are used for qualitative analysis should be validated for a minimum specificity and robustness (EMA, 2009). For the specificity, independent set of samples is created of different batches, which are not included in the creating of the reference library. All of the samples should be approved correctly (pass). The reference library should be also challenged with relevant structural analogues, which should be rejected (fail). As for robustness, the effects of relevant variables should be understood, tested and documented. Some of the variables include: environmental temperature, humidity, different probe depth, different packaging material, changes in the instrumental method, for example the number of scans. Also instrumental variation can be consider, e.g. changing lamps or reflectance material.

Materials and methods

The NIRS analyses were performed on Thermo Antaris II FT-NIR Analyzer. It is dedicated FT-NIR analyzer designed specifically for use in the industrial environments of the pharmaceutical, food and beverages, chemical and polymer industries (Hirsch, 2008). Some of the characteristics of the instrument are: the detection is performed on high - sensitive, high - stable matched InGaAs; the light source is long - life, high intensity halogen NIR source; the interferometer is frictionless stable, long - life Michelson. The sampling method used is Fiber Optic. NIRS sampling by fiber optics allows rapid point-of-use QC for raw material identification. The Antaris II Fiber Optic module can be used with the SabIR hand - held diffuse reflectance probe which can analyze samples directly or indirectly through packaging materials. The spectral range of SabIR Probe is 12000 – 4000 cm^{-1}. The probe shaft is made of stainless steel, and the window material of the shaft is made is made of high quality, chemical resistance sapphire according to the instrument specification (Antaris II FT-NIR Analyzer).

The method that is used in Alkaloid is with SabIR Fiber Optic module. The spectral range in which the collecting is performed is 10000 - 4000 cm^{-1}. The number of scans in 30, and the resolution is 8 cm^{-1}.

In the first step of creating the reference library, NIR

* bbujarovska@alkaloid.com.mk

spectrum of Ibuprofen Lysine Reference standard was collected. Due to lack of certified reference standard from pharmacopoeia, in – house standard sent from the suitable manufacturer was used. Further the library was continually updated with samples of commercial batches of Ibuprofen lysine received from the manufacturer.

The spectral reference library is composed of spectra collected with direct scanning of the substance and indirect scanning (through the polyethylene bag). In routine, indirect scanning is preferred, by reason of faster scanning and not requiring opening every package.

The NIR method for identification of Ibuprofen lysine was validated in terms of Specificity and Robustness, according to the Guideline on the use of Near Infrared Spectroscopy (NIRS) by the pharmaceutical industry and the data requirement for the new submissions and variations, issued by the European Medicine Agency.

Results and discussion

Verification workflows are created for performance the following tests of specificity and robustness. These workflows are chosen for verification of the production workflow for Ibuprofen lysine.

Verification workflow for performing specificity

One verification workflow that contains independent set of three samples, which are batches not included in the reference library are included is created. The results for these samples are PASS. The other verification workflow has three different substances that have similar structure with Ibuprofen Lysine, and therefore is expected to have similar NIR spectrum. That is Ibuprofen, the difference is with the Lysine group; Ketoprofen, belongs to the same group of Nonsteroidal anti-inflammatory drug; Paracetamol, together with Ketoprofen, they have similar structure. The result for Ibuprofen Lysine is PASS, and for the other three substances is FAIL.

Verification workflow for robustness

Robustness is performed for testing the effect of minor changes to normal operating conditions on the analysis (Tang, 2012). Verification workflows are created for performance these tests. The parameters for validation robustness of the method, which can affect the method in our operating environment, are the following: change in the number of scans (performed only with indirectly scanning), change in probe depth (performed with direct scanning) and change in thickness of the packaging material (performed with indirect scanning). The results for these three workflow are PASS, which brings the conclusion that certain changes in the method does not affect the test results.

Conclusion

The obtained results confirm that the NIR method for identification of Ibuprofen lysine is verified and appropriate for it use.

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Macedonian pharmaceutical bulletin, **62** (suppl) 171 - 172 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:544.351-145.82]:615.074:543.544.3
Short communication

Verification of method for determining methanol in sodium citrate with gas chromatography

Marija Spasevska*, Biljana Bujaroska, Gordana Mitrovska, Miona Manasova, Andrea Alagjozovska, Dafinka Damcevska, Bisera J. Trajkovska, Hristina Babunovska

Alkaloid AD - Skopje, Pharmaceutical, chemical and Cosmetics Company, Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Residual solvents in pharmaceuticals are defined as organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products. The solvents are not completely removed by the manufacturing techniques and therefore it is required appropriate selection of the solvent for the synthesis. The solvent used can enhance the yield, or even determine some of the characteristic of the substances, such as crystal form, purity and solubility (CHMP, 2015). Considering that residual solvents do not provide any therapeutically benefits, they should be removed, to the extent possible, to meet ingredient and product specifications and good manufacturing practices.

The residual solvents, based on their toxicity, are divided into three groups. The solvents that are known to cause unacceptable toxicities are class 1. In this group belongs: benzene, carbon tetrachloride; 1,2-dichlorethane; 1,1 - dichlorethene and 1,1,1-trichlorethane. These solvents are proven human carcinogens and environmental hazardous. Considering the unacceptable toxicity, this class of solvents should be avoided during the manufacturing process, unless their use can be strongly justified in a risk-benefit assessment. The Class 2 solvents are associated with less severe toxicity. Some of the solvents that belong to this group are: acetonitrile; chloroform; 1,2-dichlorethene; N,N-dimethylacetamide; N,N-dimethylformamide; 1,4-dioxane; ethylene glycol; formamide; methanol; hexane; pyridine; toluene. The use of these solvents should be limited in order to protect the patients from potential adverse effects.

Ideally, less toxic solvents should be used where is possible. Those are solvents that belong to Class 3. They are counted as less toxic and of lower risk to human health then Class 1 and Class 2 residual solvents. The solvents in this class are not known as a human health hazard at level normally accepted in pharmaceuticals. However there are no long-term toxicity or carcinogenicity studies for many of the residual solvents in Class 3. Available data so far, indicate that they are less toxic in acute or short term studies and negative in genotoxicity studies. Some of the solvents that belong to this group are: acetic acid; acetone; 1-butanol; 2-butanol; dimethyl sulfoxide; ethanol; ethyl acetate; ethyl ether; formic acid (USP, 2015).

Residual solvent that was used in the synthesis of sodium citrate is methanol. Since methanol belongs to class 2 residual solvents, quantification should be performed.

Materials and methods

The experiments were performed on Shimadzu 2010 Plus GC instrument. The pressure was set to 9.8 Psi, column flow was 2.08 mL/min and total flow was 15.5 mL/min. Helium was used as the carrier gas. Other gases that were used are Hydrogen and Air. The injection volume was 1000 µL and the split was 1:5. The column used is DB-624, 30 m x 0.32 mm, 1.8 µm (Agilent). Methanol standard was supplied from Merck Darmstad (Shimazdu, 2013).

The methanol standard solution was prepared in final concertation of 150 ppm. 1 mL of this solution was put in headspace vial of 20 mL and 5 mL water was added. The sample was prepared with weighting 50 mg of Sodium citrate in a headspace vial and 6 mL water was added. 6 mL in a headspace vial was used as blank.

* mspasevska@alkaloid.com.mk

Results and discussion

Several modifications of the general method for determining water soluble residual solvents Class 1 and 2, were made (USP, 2015). The general method is: chromatography system, composed of gas chromatograph equipped with a flame-ionization detector, 0.32 mm x 30 m fused-silica column coated with a 1.8 µm layer of phase G43 (6% cyanopropylphenyl 94% dimethylpolysiloxane). The temperature of the column is maintained at 40 °C for 20 minutes, then raised at a rate of 10 °C per minute to 240 °C, and maintained at 240 °C for 20 minutes. The headspace conditions are: incubation at 80 °C for 60 minutes and syringe temperature of 90 °C (USP, 2015).

This method has undergone several modifications and adaptations during the verification process. The adaptation was made in the temperature program of the column. The peak of methanol was at ~2.2 min, and since the diluent was water, there are no other peaks than methanol; consequently it was not necessary to run the full temperature program. Therefore finally, the method was as follows: injector temperature 140 °C, detector temperature 250 °C, temperature program for the column: 40 °C / hold 20 min, then raised to 120 °C, at a rate of 30 °C per minute. In addition, adaptation in the headspace condition was made, precisely the incubation time was shorted to 30 minutes, and because it has been proven that there are no extra peaks, other than methanol.

Tests that were performed during the verification were: specificity, repeatability, accuracy, detection limit (LOD) and quantification limit (LOQ).

The specificity of the method was validated with demonstrating that there is no other peak from the diluent that interfere with the peak of methanol.

The repeatability of the chromatographic system was determined with ten replicate injections of the standard and determining the Relative Standard Deviation (RSD). This RSD value for organic volatile solvents should be below 15.

The accuracy of the method was determined by interaction studies for constant amount of sample, but varying concentration of methanol. The concentrations are: 50 ppm, 150 ppm, 300 ppm, 500 ppm, 2000 ppm and 3500 ppm. On each concentration level, three samples were injected and calculations were made. The limits for the recovery are 80 - 120% or RSD below 15%.

For determination of the LOQ and LOD, criteria of signal-to-noise (S/N) ratio of about 10:1 and 3:1, respectively was employed. In the first step S/N ratio was determined on the standard of 150 ppm and that standard is used as a stock solution. Consequently several dilutions were prepared and S/N ratio was determined. S/N ratio of about 10:1 was obtained at concentration of 10 µg/ml, and that concentration was taken as LOQ. S/N ratio of about 3:1 was obtained at concentration of 3 µg/ml, and that concentration was taken as LOD.

Conclusion

The verified method was proven to be suitable for determination of residual solvent methanol in Sodium Citrate.

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Macedonian pharmaceutical bulletin, **62** (suppl) 173 - 174 (2016)
ISSN 1409 - 8695
UDC: 615.076
Short communication

Validation and quantification of bacterial endotoxins with turbidimetric kinetic method for (*S*)-Lactic acid

Silvana Ilioska-Zlatanovikj*, Elizabeta Popovska , Hristina Babunovska

ALKALOID A. D, Blvd. A. Makedonski 12, 1000 Skopje, R.Macedonia

Introduction

Endotoxins are high-molecular-weight complexes associated with the outer membrane of Gram-negative bacteria, and are the most significant pyrogen for the pharmaceutical industry. The importance of endotoxin contamination in sterile manufacturing becomes apparent when confronted with four aspects of its existence: its ubiquity in nature, potent toxicity, stability or ability to retain after being subjected to extreme conditions and relative likelihood of its occurrence in pharmaceutical products. Any preparation administered parenterally should be sterile and comply with the test for bacterial endotoxins, BET (Ph. Eur., 2014).

Lactic acid is an organic compound with the formula $CH_3CH(OH)CO_2H$. It is a white, water-soluble solid or clear liquid that is produced both naturally and synthetically. Lactic acid is chiral, consisting of two optical isomers. One is known as L-(+)-lactic acid or (*S*)-lactic acid and the other, its mirror image, is D-(-)-lactic acid or (*R*)-lactic acid. In humans, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. In industry, lactic acid fermentation is performed by lactic acid bacteria, which convert simple carbohydrates such as glucose, sucrose, or galactose to lactic acid.

Lactic acid is used as an excipient in medicinal products that are for intravenous administration. In medicine, lactate is one of the main components of antibiotics and infusion solutions. These intravenous fluids consist of sodium and potassium cations along with lactate and chloride anions in solution with distilled water, generally in concentrations isotonic with human blood.

Objective: Validation and quantification of BET (Bacterial endotoxins test) with Turbidimetric kinetic method for Lactic acid.

Materials and methods

Materials

(S)-Lactic acid; LAL reagent Water; Sodium Hydroxide 0.1 N for pH adjustment of sample; reagents for Kinetic turbidimetric method; PYROGENT-5000™, LAL Reagent Water, disposable endotoxin-free glass dilution tubes; disposable sterile microplates; automatic hand - held pipettes with sterile tips; timer; vortex mixer; Microplate reader Elx808cse from BIO-TEK® (BIO-TEK Instruments, INC.,P.O.BOX998, Highlsand Park, Winooski, Vermont 05404 USA).

The results are calculated and stored with Win QCL 4.0.2 software™ (Lonza® Walkersville, MD21793).

Methods

The Kinetic turbidimetric method is based on measuring the turbidity (optical density) of LAL/sample mixture at regular interval troughout the test. The time required before the appearance of turbidity (reaction time) is inversely proportional to the amount of endotoxin present.

In accordance with the Ph.Eur., 2014 and British pharmacopoeia commission, 2014, substances to be used in parenteral preparations must comply with BET, whatever their origin, since:

- contamination by bacterial endotoxins can take place prior to or during the manufacturing process;
- bacterial endotoxins cannot easily be removed by the manufacturing process;

* szlatanovic@alkaloid.com.mk

- bacterial endotoxins should be detected as early as possible in the manufacturing process.

It is to be noted that the ICH Guideline Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products provides the following recommendation under section 4.1 Drug Substance Specification: "Pharmacopoeial tests (e.g., endotoxin detection) should be performed on the drug substance, where appropriate".

Lactic acid, as an excipient in parenteral preparations, has strongly inhibitory effect on Bacterial endotoxins test (Kinetic Turbidimetric method) because of its pH value and it is necessary to overcome this problem. Problems with pH value are the most significant biochemical cause of LAL-test inhibition. When dealing with acid or base solutions, correction of pH should be made in optimum rang 6-8. Lactic acid has pH value of 1.2 and the correction should be done by diluting in with 0.1N NaOH (Zink McCullough, 2011; Williams, 2009).

Results and discussion

Validation was performed begining with screening (Inhibition / Enhancement) test (FDA, 2012). The validation was performed on three batches. For that purpose Lonza® reagents were used; lysate sensitivity λ = 0,01 EU/ml; MVD = ELCxPP/λ = 0.005x10/0.01 = 5. Dilutions used for preliminary, Inhibition/ Enhancement test were as follows: 1:2, 1:4 and the MVD (maximum valid dilution) = 1:5 with LRW.

To overcome the inhibition of lactic acid, the first dilution was made with 0.1N NaOH from Lonza® and vortex for 1 minute. Test solutions (at dilutions: 1:2, 1:4 and 1:5) were obtained by diluting with LRW™ from Lonza®. Lactic acid is not inhibitory in 1:2 dilution (pH=6.85-6.92), showing PPC recovery 120%. Test solutions (at dilutions 1:4 and 1:5, MVD) gave PPC recovery of 105% and 118% respectively. Upon received results the validation was done on three consecutive batches of lactic acid with dilution 1:2 (considering that recovery must be close to 100%). The endotoxin concentration reported for the three consecutive batches is less than 0.00200 EU/mg with PPC 78%, 94% and 91% respectively. All three batches of the tested lactic acid showed endotoxin recovery between 50 and 200%.

Conclusion

Validation of the Bacterial endotoxins test on (*S*)-Lactic acid was successfully done. The inhibition was overcome. Routine testing of (*S*)-Lactic acid should be done with dilution 1:2 (conc. 5 mg/ml) using LRW from Lonza®. According to the results, the bacterial endotoxins test - Turbidimetric kinetic method for (*S*)-lactic acid was validated and can be used for routine testing.

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Macedonian pharmaceutical bulletin, **62** (suppl) 175 - 176 (2016)
ISSN 1409 - 8695
UDC: 636.09:[615.2:347.77.038(497.7)
Short communication

Marketing authorization of veterinary medicinal products in Macedonia

Todor Šapov[1]*, Suzana Trajković-Jolevska[1], Romel Velev[2], Jasmina Tonic-Ribarska[1], Nataša Krleska-Veleva[3], Biljana Shapova[4]

[1]*University "Ss. Cyril and Methodius", Faculty of Pharmacy, Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*University "Ss. Cyril and Methodius", Faculty of Veterinary Medicine, Lazar Pop Trajkov 5-7, 1000 Skopje, R. Macedonia*
[3]*Replek Farm, Kozle 188, Skopje, R. Macedonia*
[4]*St.Naum Ohridski City Surgical Clinic-11Oktomvri bb,Skopje, R.Macedonia*

Introduction

The regulatory approval process of the new veterinary medicinal products is intricate and rigorous, since its primary purpose is to have a drug on the market that can guarantee quality, effectiveness and safety to both, animals and humans. This is part of the marketing authorization procedures, strictly stipulated and regulated by law. Macedonian Law on Veterinarian medicinal products (2010) is harmonized with the standards defined within the EU legislation.

The main aim of this study is to present the current situation regarding the marketing authorization of veterinary medicinal products in Macedonia and to identify strengths and weaknesses in the process of registration.

Materials and methods

Research presented in the study was completed within the period September-November 2015, through direct contact with the distributors of veterinary medicinal products from all over Macedonia. Main research method was a questionnaire composed of 20 questions with option of answers. In order to reduce subjectivity the questionnaire offered a binary model with yes, no and not familiar as possible answers. All questions in the questionnaire were concrete and specific, targeting issues related with registration and selection of veterinary medicinal product, distribution practice, pharmacovigilance etc. All answers were carefully examined and the results of the research have indicated the current situation of the marketing authorization process of the veterinary medicinal products in the Macedonia. To all contacted companies anonymity in responding as well as data confidentiality was guaranteed.

Results and discussion

All new veterinary medicinal products need to be approved by the competent authority. After positive scientific evaluation of the submitted documentation, competent authority issues marketing authorization that allows the veterinary medicinal products to be placed on the market. National procedure is a type of authorization for veterinary medicinal products in Macedonia. The applicants need to submit an application to the Food and Veterinary Agency as a competent authority. The registration process of the veterinary medicinal products in Macedonia is governing by the Law on Veterinary Medicinal Products (2010), but its implementation is closely defined by the rulebooks, where it is clearly described troughs of the entire procedure.

The report of this research contains qualitative information of the structural indicators and perceptions which were subsequently used for obtaining the qualitative parameter with which reflect the present situation of knowledge of registration process for veterinary medicinal products.

In the Republic of Macedonia there are 23 veterinary medicinal products wholesalers which are registered by the Food and Veterinary Agency, and authorized to distribute veterinary medicinal products. Only 16 of them are active

* tshapov@gmail.com

in the field. During the preparation of this study, in order to receive more accurate information, all of them were contacted, but only 9 of them responded positively to our request.

The results of this research showed that veterinary medicinal products wholesalers in some points are familiar with the process of marketing authorization and post-marketing authorization and the Law on Veterinary medicinal products (2010), but there in certain areas of the eponymous Law are not familiarized. On the basis of their answers, it can be concluded that most companies are familiar with the basics of the process, such as, the procedure for marketing authorization for veterinary medicinal product and legal obligations, the existence of a register of veterinary medicinal products, good distribution practice, good storage practice and procedure for withdrawal of veterinary medicinal products. On the questions such as: information of public interest, the existence of national reference laboratories, the existence of a national list of essential veterinary medicinal products, pharmacovigilance system, some of respondents did not know the exact answer, indicating a poorly knowledge of the marketing authorization and post-marketing authorization of veterinary medicinal product on these specific issues.

The results also show that the Good distribution practice and Good storage practice are implemented and that the cooperation between companies and Food and Veterinary Agency is on satisfactory level. On the website of the Food and Veterinary Agency there are clearly set of information of public interest for implementation of the marketing authorization process for the interested parties, but some of them had trouble in their availability

This research indicated satisfactory level of the implementation of the law in practice, identified, as follows: the existence of publicly available written information about the procedure of marketing authorization of veterinary medicinal products, formally established commission for review of applications for registration, the existence of a formal system for submitting complaints commission feature. There are some weaknesses in the process of marketing authorization, such as: an official national essential list for veterinary medicinal products is not amended by 2012, absence of timed deadline for providing official information on the status of application for approval for marketing, absence of publicly available pharmacovigilance system, and additionally, not always satisfactory level of applicable knowledge on the process and issues connected with the process by the veterinary medicinal products wholesalers' representatives.

Conclusion

In this paper all aspect of the issue of marketing authorization of veterinary medicinal products in Macedonia were studied and critically analyzed. On the basis of the collected data, the existing legislation, as well as, administrative procedures of marketing authorization and post-marketing authorization of veterinary medicinal products can be revised and adjusted providing better efficiency and transparency. Furthermore, although the cooperation between the wholesalers and Agency of Food and Veterinary was found to be satisfactory, improvement of communication could be the added value.

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Macedonian pharmaceutical bulletin, **62** (suppl) 177 - 178 (2016)
ISSN 1409 - 8695
UDC: 615.273.074
Short communication

Transfer of analytical procedures for quality control of Cilostazol 100 mg tablets

Cveta Dolikjoska Trajkova*, Nikola Pavleski, Ana Giceva - Pepovska, Blagica Samarova Stoev, Silvija Saveska, Maja Stojkovska, Hristina Babunovska

ALKALOID AD-Skopje, Pharmaceutical, Chemical and Cosmetics Company, blvd. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

The transfer of analytical procedures (TAP), also referred to as method transfer, is the documented process that qualifies a laboratory (the receiving unit, RU) to use an analytical test procedure for quality control, that is fully validated in another laboratory (the transferring unit, TU) (USP, 2012). Usually, the most common need for this procedure is transferring methods developed and validated in the research and development department to the quality control unit in the same or different site. In the last years, with widening the business strategies of the pharmaceutical companies, necessity for different types of transfer occurs. Transfer activities are required between Quality control laboratories of the manufacturers and different batch release laboratories: laboratories of the contactor suppliers, contract laboratories and European Union batch release testing sites.

The presented paper describes the process of Analytical method transfer for Cilostazol 100 mg tablets in which Alkaloid QC laboratory is involved as receiving laboratory.

Materials and methods

Analytical method transfer protocol is created, reviewed and approved by both laboratories, prior to experimental performing of the methods (Eudralex, 2014). According the protocol, assessing the sort of test and the experience of the receiving laboratory, the methods to be transferred have been determined:

- The method for Dissolution - UV Spectrophotometry method;
- The method for Assay of cilostazol - gradient HPLC method with UV detection;
- The method for Identification of cilostazol - assessed in Assay;
- The method for Related and degradation substances - gradient HPLC method with UV detection

Methods for all the rest specification parameters: appearance, uniformity of mass, subdivision, water content, Uniformity of dosage units by mass variation don`t need to be transferred. Microbial examination method should be verified but it is not part of this protocol.

One batch of Cilostazol tablets 100 mg was tested in both laboratories.

Results and discussion

In the analytical procedure for dissolution test, following system suitability acceptance criteria are set: no significant blank absorbance is noticed, RSD% of 6 replicate measurements of cilostazol standard 1 should be $\leq$ 2.0%, cilostazol standard 2 recovery should be between 98.0 - 102.0%. System suitability criteria are met in both laboratories. The analysis for dissolution was performed on six samples in the TU and on 12 samples in the RU involving different days, instruments and analysts. The results were compared at the specification time limit of 60 minutes. The obtained results for inter-laboratory repeatability: mean values of 96.33% and 91.81% are within the limits of not less than 85% (Q=80) in 60 minutes. Absolute difference between 6 samples per each laboratory is 1.80% and 3.80% respectively, absolute difference between the laboratories is 4.52%. All differences are below 5% which is set

* cdolikoska@alkaloid.com.mk

as acceptance criteria (Medsafe, 2007; WHO, 2011). The obtained results for intra-laboratory repeatability (reproducibility in RU): absolute difference between each analysis of 6 samples is 3.80% and 1.80% respectively, absolute difference between 12 samples is 1.24%. All differences are below 5% which is set as acceptance criteria. The linearity of the method in the concentration range for cilostazol from 20 % to 120 % of the theoretical sample solution concentration is proved in the RU. Acceptance criteria for Linearity are accomplished: correlation coefficient ≥ 0.999, Y-intercept confidence interval includes zero, residual plot presents no particular trend, RSD (%) of the response factor ≤ 2.0%.

In the analytical procedure for assay following system suitability acceptance criteria are set: no blank interference is noticed at the cilostazol elution time, RSD of the areas of cilostazol peak of 5 consecutive injections should be ≤ 2.0%, symmetry factor (A_s) of cilostazol peak should be ≤ 2.0, cilostazol standard 2 recovery should be between 98.0 - 102.0%. System suitability criteria are met in both laboratories. The analysis for assay was performed on six sample preparations in the TU and on 12 sample preparations in the RU involving different days, instruments and analysts. The obtained results for inter-laboratory repeatability: mean values of 100.18% and 99.59% are within the limits for cilostazol content of 95.0 - 105.0%. Absolute difference between 6 samples per each laboratory is 1.20% and 0.80% respectively, absolute difference between the laboratories is 1.50%. All differences are below 3% which is set as acceptance criteria (Medsafe, 2007; WHO, 2011). The obtained results for intra-laboratory repeatability (reproducibility in RU): absolute difference between each analysis of 6 samples is 0.8% and 0.5% respectively, absolute difference between 12 samples is 1.8%. All differences are below 3% which is set as acceptance criteria. Identification is confirmed by obtaining similar retention times for cilostazol in sample and standard solution. The linearity of the method in the concentration range between 80 % of the theoretical sample solution concentration to 120% is proved in the RU. Acceptance criteria for linearity are accomplished: correlation coefficient ≥ 0.999, Y-intercept confidence interval includes zero, residual plot presents no particular trend, RSD (%) of the response factor ≤ 2.0%.

In the analytical procedure for Related substances following system suitability acceptance criteria are set: resolution between peaks of impurity B and cilostazol > 1.5, no blank interference is noticed during the cilostazol elution time, RSD of the areas of Cilostazol peak of 5 consecutive injections ≤ 2.0%, Standard 2 recovery should be between 95.0 – 105.0%, A_s of Cilostazol peak should be ≤ 2.0. System suitability criteria are met in both laboratories. Limit of quantification (QL) is determined and confirmed in the RU. 0.01% cilostazol standard solution gives S:N ratio of 14 which is ≥ 10:1. The obtained RSD at QL level is 7.83% which is lower than the acceptance criteria ≤ 15.0% (ICH, 2005). The analysis for related substances was performed on six sample preparations in the TU and on 12 sample preparations in the RU involving different days, instruments and analysts. Acceptance criteria for inter and intra laboratory repeatability are set: the % impurity should comply with the release specification Impurity B ≤ 0.2%, unknown impurities ≤ 0.2%, total impurities ≤ 0.5%. Observing chromatographic profiles and results obtained by both laboratories, it can be concluded that they are qualitatively and quantitatively similar. The linearity of the method in the concentration range between QL and 0.20% is proved in the RU. Acceptance criteria for linearity are accomplished: correlation coefficient ≥ 0.999, Y-intercept confidence interval includes zero, residual plot presents no particular trend, %RSD of the response factor ≤ 10.0%.

Conclusion

From the comparison of the obtained results from both laboratories it can be concluded that all results are meeting the predetermined acceptance criteria limits. No deviations from the protocol were observed. Analytical method transfer report is generated by the transferring laboratory, reviewed and approved by both laboratories. The successfulness of the transfer is confirmed. The analytical transfer report qualifies Alkaloid QC laboratory to use the Cilostazol 100 mg tablets finished product methods of analysis, ensuring that the receiving laboratory has the procedure, knowledge and ability to perform it.

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Macedonian pharmaceutical bulletin, **62** (suppl) 179 - 180 (2016)
ISSN 1409 - 8695
UDC: 343.52:615.2
Short communication

Counterfeit medicines - threat to worldwide public health

Biljana Petrovska Jakimovska*, Biljana Nanova, Milkica Gligorova

Packaging department, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Pharmaceutical counterfeiting is an expanding threat to worldwide public health. It crosses geographic boundaries and impacts patients suffering from a variety of diseases. It is a crime that generates large profits for the counterfeiters even as it steals one of medicine's most important ingredients: trust. According to the World Health Organization (WHO), a counterfeit medicine is one that is deliberately and fraudulently mislabeled with respect to identity and/or source. Counterfeiting can apply to both branded and generic medicines and counterfeits may include products that are fraudulently packaged or mislabeled with respect to identity and/or source; contain no active ingredient, incorrect quantities or an undeclared active ingredient; contaminated with other materials; past their expiry date; contain no or incorrect patient information leaflets (WHO, 2010). The most counterfeit medicines are cancer medicines, cholesterol lowering medicines, antibiotics, hypertension medicines, hormones, steroids and copies of many commonly used pain killers and antihistamines. In developing countries, counterfeit medicines are drugs for the treatment of life-threatening conditions such as malaria, tuberculosis and HIV/AIDS.

Counterfeit medicines - reason and consequences

There are several reasons for counterfeiting. One of the main reasons is profit, because manufacturing of counterfeit medicines is very low cost and doesn't meet quality and safety standards. Inadequate legislation, regulations and enforcement results in supply systems vulnerable to counterfeit products and extremely low capacity to uncover and punish counterfeiters. The costs of legitimate medicines, both original and generic, may be too high for patients, and is one of the reasons why patients buy cheaper medicine with dubious quality.

The consequences of counterfeit medicines could be divided into three groups: consequences for the public and patients' health, consequences for pharmaceutical company and consequences for the country. Consequences for the public and patients health are life-threatening effects, does not provide effective treatment, needs prolonged hospitalization, cause direct damage or toxic effects. Counterfeit medicines cause direct financial losses for the company and loss of reputation and trust of patients. The consequences for the country include evading payment of taxes and customs duties and higher costs in the health system.

Activities of the pharmaceutical industry against counterfeiting of drugs

Pharmaceutical industry is one link of the chain that is fully committed in combating counterfeiting medicines and takes actions to protect their products. There are two main forms of product security and those are traceability and authentication. Traceability means ability to track drugs cheaply, securely, and efficiently across the globe, from manufacturer to patient, thus improving the safety of medicines. Tracking technologies allows pharmaceutical products to be tracked and monitored as they move from the manufacturer to the patient. The synonym for tracking technologies is " Track and Trace", and it incorporates serialization and pedigree. This technology implies an ability of the system to know where a product went (track) and where it came from (trace). Serialization is simply a process of assigning a unique number to a unit of production such that it can be identified later. Pedigree is process of recording most or all of the product history.

There are various authentication technologies that can be used at different levels of packaging and in different circumstances. Most security-printing deterrents can be grouped into three categories: overt, covert, and forensic.

* bjakimovska@alkaloid.com.mk

Overt features are easy to detect by sight, touch or smell. Examples of overt security-printing deterrents include color-shifting inks, alignments that are difficult to replicate, microprinting, optically variable microstructures (also known as holograms) and visible watermark. Covert features can be detected by machines but are not visible to the naked eye. Examples of covert security-printing deterrents include invisible inks (such as those visible only when exposed to ultraviolet light), magnetic printing, encrypted codes, hidden overprints, micro-displacement of glyphs and metal fibers. Forensic features require laboratory testing in order to be detected. Examples of forensic security printing deterrents include various embedded chemical compositions known as chemical taggants that are designed to uniquely identify an object (Davison, 2011)

Combining track-and-trace with secure printing dramatically improves protection against counterfeiting and increases efficiencies in product recalls and other supply chain challenges.

Global activities in combating counterfeit

There are three strategies that are applied globally in order to combat counterfeiting of medicines

• Providing tools, international norms, standards and guidelines to assist that medicines circulating in national and international commerce are safe, efficacious and of good quality

• Providing support to non EU and EU Member States to build national regulatory capacity

• Developing global activities to combat counterfeit medicines, in collaboration with all relevant stakeholders

In 2006, World Health Organization established International Medical Products Anti Counterfeiting Taskforce (IMPACT) and the main conceptual approach is a voluntary coalition of partners, with the purpose of coordinating international activities aimed at combating counterfeit medical products. IMPACT partners reflect the fact that combating counterfeiting of medical products cannot be successfully achieved by the health sector alone but requires a coordinated effort and effective collaboration among health sector, enforcement, border control, justice (all at different administrative levels), as well as health professionals, manufacturers, importers, distributors, media, and patients/consumers. IMPACT outputs include recommendations, policy advice and training materials that reflect consensus reached among IMPACT partners (IMPACT, 2010).

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Macedonian pharmaceutical bulletin, **62** (suppl) 181 - 182 (2016)
ISSN 1409 - 8695
UDC: 615.33.015.3:616.314.17
Short communication

Comparative evaluation of the efficacy of local administration of doxycycline and chlorhexidine in patients with periodontal disease using multivariate chemometric data analysis

Liljana Bogdanovska[1]*, Ana Poceva Panovska[1], Natalija Nakov[1], Marija Zafirova[1], Mirjana Popovska[2], Aneta Dimitrovska[1], Rumenka Petkovska[1]

[1]*Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University of Ss. Cyril and Methodius, Mother Theresa 47, 1000 Skopje, Macedonia*
[2]*Department of Oral Pathology and Periodontology, Faculty of Dentistry, University of Ss. Cyril and Methodius, Mother Theresa 17, 1000 Skopje, Macedonia*

Introduction

The process of evaluation of the efficacy of a given pharmacological treatment, in general, is performed in two subsequent steps: 1) monitoring numerous pre-defined clinical and laboratory parameters which provide information on treatment benefits and 2) analysis and interpretation of the obtained results. In order to get thorough understanding of the studied problem, the data obtained in step 2 are studied using chemometric algorithms for multivariate data analysis. Compared to the traditional univariate approach, the application of chemometric algorithms provides comprehensive overview of the studied data and results in greater statistical power even when there is small number of observations or missing data points (Helmy et al., 2012).

The treatment of periodontal disease consists of scaling and root planning followed by local application of antiseptics or antibiotics such as chlorhexidine (CHX) and doxycycline (DOX). The efficacy of the treatment is monitored using the following parameters: clinical indices (index of gingival inflammation (GI), periodontal pocket depth (PD) and clinical attachment loss (CAL)) or determination ofactivity/concentration of inflammatory biomarkers in gingival crevicular fluid (GCF) such as enzymes: alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) or cytokines: interleukin-1β (IL-1β) and tumor necrosis factor –α (TNF-α) (Aimmeti et al., 2012). However, the data analysis of the results obtained from monitoring treatment efficacy parameters is commonly performed using the traditional approach (Tu et al., 2009).

The aim of our study was to perform comparative evaluation of the efficacy of local periodontal treatment with DOX and local periodontal treatment with CHX using chemometric algorithm for multivariate data analysis, orthogonal projection to latent structures - discriminant analysis (OPLS-DA).

Materials and methods

The subjects enrolled in the study were divided in two groups: a group of 25 patients (DOX group) treated locally with 10% DOX gel (controlled-release gel formulation) and a group of 34 patients (CHX group) treated locally with 1% CHX gel (conventional-release gel formulation). DOX group was treated with 115 mg gel containing 10 mg of DOX and the GCF samples were taken 7 days after the local treatment. The CHX group was administered 330 mg gel containing 2 mg CHX and GCF samples were taken 30 min after gel application. GCF samples were collected from quadrants consisting of five periodontal pockets, before and after the local treatment. In brief, paper strips were placed in the selected periodontal pockets until mild resistance was felt and left in place for 30 s.

The study protocol was approved by the Ethics Committee at the Faculty of Dentistry, Skopje.

For determination of biomarker activity/concentra-

* lbogdanovska@ff.ukim.edu.mk

tion in GCF, five paper strips were placed in 500 μL PBS (phosphate buffer saline, pH=7.4) and the tube was centrifuged for 5 min at 4000 rpm (4 ˚C). The resulting solutions were divided in two aliquots of 250 μL. The aliquot for determination of enzyme activity was assayed immediately after collection whereas the aliquot for determination of cytokine concentration was added proteinase inhibitor cocktail and kept at – 80 ˚C until analysis. ALP, LDH and AST activity in GCF samples was determined using appropriate kits in accordance with the International Federation of Clinical Chemistry (IFCC) recommendations. The concentration of IL-1β and TNF-α in GCF samples was determined according to manufacturer's instructions, using commercial ELISA kits. The clinical indices PD and CAL were measured in mm, while the index of gingival inflammation was scored with values 1-3 (1-healthy tissue, 2-moderate inflammation, 3-severe inflammation). The OPLS-DA algorithm was applied using SIMCA 13.0.3 software (Umetrics, Umea, Sweden). The comparisons of significantly different variables between the DOX group and the CHX group, identified by the OPLS-DA model, were confirmed using non-parametric Mann-Whitney U test in the SPSS 17 software. $P < 0.05$ was considered statistically significant.

Results and discussion

OPLS-DA is a supervised chemometric algorithm widely applied to investigate different treatment effects. The properties of the OPLS-DA provide comprehensive description on the discrimination between classes of samples, especially in cases where subtle differences among classes are present. This algorithm separates the systematic variation in the matrix X (inflammatory biomarkers and clinical indices for the DOX and the CHX group, in our case) in two parts, one linearly related (variation of interest) to the matrix Y (the classification variables) and one orthogonally related (so-called orthogonal variation or structured noise) to the matrix Y. The partitioning of the X-data improves the interpretation of the model.

In the score plot of the developed OPLS-DA model, the samples from both treatment groups were divided in two different clusters, indicating that DOX and CHX show different effect on the examined variables. To confirm model validity, the method of cross-validation was used. The results from the cross-validation were R2X (cum) = 0.527, R2Y (cum) = 0.507 and Q2X (cum) = 0.530, indicating a good model.

Based on the VIP statistics from the cross-validated OPLS – DA model, statistically significant variables responsible for separation between both treatment groups were extracted. VIP ranks the overall contribution of each variable to the generation of the model. According to the criterion for the VIP statistics, a VIP value > 0.8 was used as a threshold value for determining importance of variables. The variables most strongly influencing the discrimination between the DOX treatment group and the CHX treatment group were periodontal pocket depth, AST activity and concentration of the cytokines IL-1β and TNF-α in GCF. The discriminatory variables identified by the OPLS-DA model were additionally confirmed at a univariate level by the non-parametric Mann Whitney U test ($P<0.05$) which revealed that the DOX treatment markedly decreased periodontal pocket depth, AST activity and concentration of the cytokines IL-1β and TNF-α in GCF compared to the CHX treatment. The different effects are due to the different mechanism of action of DOX and CHX. DOX possesses anti-inflammatory properties, thus is decreasing the inflammatory response presented by the pro-inflammatory cytokines IL-1β and TNF-α. This molecule also stabilizes cell membranes, which decreases the activity of intracytoplasmic enzymes, such as AST.

Conclusion

The comparative evaluation of the efficacy of the local periodontal treatment with DOX and the local periodontal treatment with CHX using the OPLS-DA algorithm revealed differences in the therapeutic effects of both treatments, at the same time providing insights into their mechanisms of action. The results from this study suggest that the OPLS-DA algorithm is a valuable tool for comparative evaluation of treatment efficacy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 183 - 184 (2016)
ISSN 1409 - 8695
UDC: 615.33.015.3.074:543.544.5.068.7]:616.314.7
Short communication

Development and validation of RP-HPLC-FLD method for determination of doxycycline in gingival crevicular fluid and saliva

Liljana Bogdanovska[1]*, Spiro Spasovski[2], Mirjana Popovska[2], Silvana Gjoseva[3], Katerina Goracinova[3], Natalija Nakov[1], Marija Zafirova[1], Aneta Dimitrovska[1], Rumenka Petkovska[1]

[1]*Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University of "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Macedonia*
[2]*Department of Oral Pathology and Periodontology, Faculty of Dentistry, University of "Ss. Cyril and Methodius", Mother Theresa 17, 1000 Skopje, Macedonia*
[3]*Institute of Pharmaceutical Technology, Faculty of Pharmacy, University of "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Macedonia*

Introduction

Doxycycline (DOX), a second generation tetracycline antibiotic, is widely used in the treatment of periodontitis. DOX accumulates in gingival fibroblasts and is further distributed in gingival crevicular fluid (GCF), a valuable matrix for monitoring concentration of drugs used in periodontal treatment (Lavda et al., 2004). Since GCF is excreted in low volumes, determination of drug's concentration must be performed using sensitive methods. Application of HPLC-UV methods for DOX determination in biological samples results in inadequate sensitivity. On the other hand, HPLC methods with fluorescent (FLD) detection provide the required sensitivity and are less affected by interference from other components from the sample matrix (Denic et al., 2013; Lu et al., 2004).

The aim of our study was to develop, optimize and validate RP-HPLC-FLD method for determination of DOX in GCF and saliva.

Materials and methods

Referent standards of DOX, tetracycline (internal standard, IS) and oxytetracycline were supplied by Sigma-Aldrich (Germany). Solvents (methanol and water) were of HPLC grade and reagents (CH_3COONa, EDTA disodium salt and $CaCl_2$) were of analytical grade. Whatmann 3MM chromatography paper strips, 2 x 5 mm (Whatman Lab sales Ltd., UK) were used for GCF collection.

Calibration standards for determination of DOX in GCF were prepared using serum instead of GCF, in the following concentrations: 20, 50, 100, 150, 200, 400, 500 and 1000 ng/mL. For determination of DOX in saliva samples, the concentrations of the calibration standards prepared in saliva were 20, 50, 100, 150, 200, 400, and 500 ng/mL. For both GCF and saliva samples, the concentration of the IS was 1 μg/mL. Methanol: water mixture (50:50, *V/V*) was used as an extracting solvent.

60 patients with chronic periodontitis were assigned in two groups (group I and II). Group I (n=30) received conventional short-term orally administered regimen (100 mg DOX, once a day for 21 days). Group II (n=30) received sub antimicrobial dose doxycycline (SDD) regimen (20 mg DOX, twice daily for 2 months). GCF samples were collected from 10 periodontal pockets (pocket depth: 3-5 mm) for 30 s, by insertion of the paper strips in the pocket and unstimulated saliva samples were collected in sterile containers (- 20 ˚C until analysis). GCF and saliva samples were collected 7, 15 and 21 days after beginning of therapy (group I) and 15, 30 and 60 days after beginning of therapy in patients from group II. The study protocol was approved by the Ethics Committee at the Faculty of Dentistry, Skopje.

Before analysis, 50 μL IS working standard solution and methanol: water mixture (50:50, *V/V*) as an extract-

* lbogdanovska@ff.ukim.edu.mk

ing solvent was added to each GCF sample up to volume of 500 µL. They were vortex for 30 s and then centrifuged at 1000 rpm for 5 min. For saliva samples, 100 µL of IS working standard solution was added to a 500 µL sample. The extraction solvent (methanol: water mixture (50:50, *V/V*) was added up to volume of 1000 µL. Saliva samples were vortex-mixed for 30 s and then centrifuged 5 min at 4000 rpm.

The HPLC analysis was conducted on Agilent 1200 RR series. The chromatographic separation was performed on a Purospher STAR RP 18e column (250 x 4.6 mm, 5 µm) at 30 ˚C. The mobile phase consisted of methanol and buffer solution (a mixture of 15 mM $CaCl_2$, 25 mM CH_3COONa and 25 mM Na_2EDTA, pH=8.1), 50:50, *V/V*. The flow rate was 0.8 mL/min. DOX and IS were recorded at 380 nm as excitation (λex) and 532 nm as emission wavelength (λem). 100 µL were injected on the column. The total runtime for the analysis was 10 min.

Results and discussion

During preliminary investigations, mobile phase composition, λex and λem, flow rate and the volume of injection were optimized. An IS was selected as well.

Several mobile phases containing methanol and buffer solution were investigated where the organic phase was varied from 20-50% (organic phase/buffer, *V/V*). The best result was obtained using mobile phase containing 50% methanol and 50% buffer solution (*V/V*). Mobile phase flow rate was investigated in the range from 0.6-1 mL/min and the final flow rate was set at 0.8 mL/min. The injection volume was tested and it was found that 100 µL was optimal for further analysis. DOX fluorescence intensity was estimated using the following λex: 375 nm, 380 nm, 385 nm and λem: 512 nm, 520 nm and 532 nm. The optimal value for the λex and λem were 380 nm and 532 nm, respectively. Two compounds were tested as an IS, tetracycline and oxytetracyline. Under the optimized conditions, oxytetracycline resulted in poorer peak symmetry and eluted early, so tetracycline was selected instead. After the entire optimization procedure the retention times for DOX and IS were 5.1 min and 6.5 min, respectively. Method validation was performed according to EMA Guideline on bioanalytical method validation (EMA, 2011).

Selectivity of the method was evaluated by comparison of blank GCF and saliva samples and spiked samples of GCF and saliva containing DOX and IS. No interfering peaks were found at the retention times of DOX and the IS in both samples, thus method selectivity was confirmed.

For GCF samples, linearity was observed within range 20-1000 ng/mL (R^2=0.9979). The within- run accuracy and precision ranged from 96.29-112.93% with a coefficient of variation (CV) within the range 2.60-4.66%. The between –day accuracy and precision was within the range from 98.29-104.23 with a CV from 2.32 – 7.05%. The mean extraction recovery (94.6-98.77%) confirmed an efficient extraction procedure.

For saliva samples, linearity ranged from 20-500 ng/mL (R^2=0.9965). The with-in run accuracy and precision ranged from 95.30-109.2% with a coefficient of variation (CV) within the range 0.81-4.26%. The between -day accuracy and precision was within the range from 99.00 -109.26% with a CV of 2.11 - 8.34%. The mean extraction recovery of DOX from saliva samples (96.13-98.5%) confirmed an efficient extraction procedure. Stability testing showed that GCF and saliva samples are stable after three freeze-thaw cycles, after 12 hours in the autosampler, 24 hours at room temperature and one month at – 20 ˚C.

The validated method was applied for determination of DOX concentrations in GCF and saliva samples from periodontitis patients. DOX concentrations in GCF samples for group I on day 7, 15 and 21 reached 2.24 ±0.32 µg/mL, 1.68 ± 0.19 µg/mL and 1.39 ± 0.17 µg/mL, respectively. The DOX concentration in saliva samples for the selected time points were 0.19 ± 0.03 µg/mL, 0.07 ± 0.01 µg/mL and 0.04 ± 0.01 µg/mL.

For group II, DOX concentrations in GCF samples on day 15, 30 and 60 were 1.72 ± 0.29 µg/mL, 1.67 ± 0.33 µg/mL and 1.38 ± 0.12 µg/mL, respectively. For saliva samples, DOX concentrations on day 15, 30 and 60 were 0.71 ± 0.10 µg/mL, 0.88 ± 0.22 µg/mL and 0.31± 0.07 µg/mL, respectively. As it can be seen from the results, the determined DOX concentrations in GCF are much higher compared to those in saliva which is consistent with the fact that gingival fibroblasts act as reservoirs of this drug (Lavda et al., 2004).

Conclusion

The data presented in this study indicate that the proposed method used for determination of DOX in GCF and saliva samples showed satisfactory selectivity, linearity, accuracy and precision. The method is therefore suitable and applicable for the analysis of DOX in GCF and saliva samples in patients undergoing conventional or SDD periodontal therapy.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 185 - 186 (2016)
ISSN 1409 - 8695
UDC: 615.254.1.074
Short communication

Phospholipids monitoring as a tool for elimination of matrix effect during LLE optimization

Natalija Nakov*, Jelena Acevska, Rumenka Petkovska, Zoran Kavrakovski, Aneta Dimitrovska

Institute of Applied chemistry and Pharmaceutical analysis, Faculty of Pharmacy, University "SS Cyril and Methodius", 1000 Skopje, Macedonia

Introduction

The concentration of indapamide for the needs of pharmacokinetic and bioequivalent studies should be determined in whole blood. Given the complexity of the blood as an extraction medium, matrix effect (ME) is the most critical aspect in the method development. For these reasons, particular attention should be paid during the optimization of the extraction procedure. There are several reported LLE procedures for extraction of indapamide using HPLC-UV or MS detection (Hang et al., 2006; Tang et al., 2005; Zendelovska et al., 2003). However they did not meet the expectations concerning the modern bioanalytical challenges towards sample preparation. In our previously published research, automated SPE-LC-MS/MS method for determination of indapamide in blood was developed (Nakov et al., 2013). Given the automation of the extraction procedure, the method resulted in low matrix effect and great performance consistency. Taking into considering that this type of equipment is not very disseminated across the laboratories, optimization of low matrix LLE was acceded.

The effectiveness of the LLE procedure in removing ME depends on the choice of a suitable organic solvent, because various organic solvents can remove different classes of phospholipids. Since the phospholipids are the main class of endogenous components responsible for the ME, monitoring of the specific phospholipids mass transitions was used as a tool for targeted optimization of the LLE procedure for indapamide extraction from human whole blood.

Materials and methods

The blood samples (0.5 mL) were spiked with 50 µL zolpidem tartarate (internal standards, IS) working solution of 300 ng/mL, 1 mL 5% $ZnSO_4$ and 1 mL 100 mM KH_2PO_4 (pH 6.8). The samples were vortex-mixed for 2 min and 1 mL organic solvent was added. The extraction was performed by vortex-mixing for 10 min. The samples were centrifuged at 4000 rpm for 15 min, the upper organic layer was transferred to another tube and evaporated in vacuum centrifuge for 60 min. The dried residues were dissolved in 1 mL methanol and 10 µL were injected into the LC-MS/MS system. The experiments were performed on Shimadzu LCMS 8030 triple quadrupole mass spectrometer in positive ESI mode, using Kinetex C18 (100 x 2.1mm, 1.7µm particle size) chromatographic column. Mobile phase was consisted of a mixture of 2 mM ammonium acetate (added 0.5 mL formic acid in 1L buffer) and acetonitrile in ratio 10:90. Analyses were conducted at a flow rate of 0.2 mL/min. Positive precursor ion scan m/z 184 was used for phospholipids monitoring. Indapamide and IS were quantified in selected reaction monitoring (SRM) using the transition of m/z 366.1→132.15 and m/z 309.0→236.10, respectively.

Results and discussion

The procedure of monitoring specific mass transitions of phospholipids (precursor ions scan of m/z 184) provides information about all precursor ions that fragment to trimethylammonium-ethyl phosphate ion (m/z 184), allowing detection of all phosphatidylcholine phospholipids, lysophospholipids and sphingomyelins. This procedure was used for identification of the class of phospholipids pres-

* natalijan@ff.ukim.edu.mk

ent in blood extracts and selection of an appropriate extraction solvent for LLE of indapamide. Two nonpolar (diethyl ether; mixture of n-hexane and dichloromethane) and two polar (5% MTBE in NH4OH and MTBE) extraction solvents were evaluated for elimination of ME and extraction recovery during the optimization of the LLE procedure. Post-extraction experiments were conducted to determine the degree of ME. The absolute ME was assessed from the responses obtained from blood extract containing indapamide added after the LLE and same concentration of indapamide added to neat solution. The post-extraction experiments showed that the nonpolar solvents gave high ME (30%). Intense peaks in the region from 1.5 - 4 min were found in the total ion chromatogram (TIC) of precursor ions scan of m/z 184. Several precursor ions (m/z 496, m/z 520 and m/z 512) were detected in both blood extracts using nonpolar extraction solvents. These precursor ions indicated the presence of lysophospholipids. Lysophospholipids belong to the class of early-eluting phospholipids and given the short retention time of indapamide (1.55 min), it was evident that this class of phospholipids was responsible for the ME.

The literature data showed that polar organic solvent are more effective in lysophospholipids removal (Chambers et al., 2007). Therefore, the subsequent experiments towards LLE optimization were conducted using more polar extraction solvents (alkaline MTBE and MTBE). The TIC of precursor ions scan of m/z 184 obtained from blood extract after LLE with alkaline MTBE, also showed presents of lysophospholipids. The absolute ME was reduced from 30% to 63.6%. However, if we consider the ME (91%) obtained using the automated SPE procedure (Nakov et al., 2013); the obtained result was still unsatisfactory. In addition, the extraction recovery was low (around 62%). The use of MTBE resulted in significant removal of lysophospholipids, which was confirmed with the phospholipids monitoring. The phospholipids signals obtained during the precursor ions scan of m/z 184 were with low intensity, which coincided with the post-extraction experiments. The ME obtained using MTBE as extraction solvent was 90.4%, suggesting that removal of the ME from complex biological material such as whole blood was achieved. The recovery of indapamide was found to be around 77% and the coefficient of variation of the LLE procedure was around 5%, thus confirming the acceptable repeatability.

Conclusion

In this research, it was found that MTBE is effective solvent for lysophospholipids removal from human blood, thus leading to elimination of ME during the LLE of indapamide. The monitoring of specific mass transitions of phospholipids, as well as the post-extraction experiments, confirmed that the optimized LLE yields clean extract same as the automated SPE, thus it could be used for extraction of indapamide from human blood during the pharmacokinetic and bioequivalent studies.

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Macedonian pharmaceutical bulletin, **62** (suppl) 187 - 188 (2016)
ISSN 1409 - 8695
UDC: 615.014.4
Short communication

Evaluation of stability data on pharmaceutical dosage form in order of extending the shelf life with application of statistical methods

Vasilka Dubrova - Koceva[1]*, Sonja Chortosheva[2], Hristina Babunovska[1], Sanja Despotovska[1], Dafinka Damcevska[1], Dragana Kafedziska[1], Marija Stojanovska[1]

[1]Alkaloid AD Skopje – Pharmaceutical, Chemical, Cosmetic & Botanical Industry, Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia
[2]Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University, Ruger Boskovic 16, 1000 Skopje, Republic of Macedonia

Introduction

Lately, the Shelf Life Extension Program (SLEP) for extending the shelf life of the pharmaceutical products has been gaining more actualization and application, particularly in the pharmaceutical industry (Lyon et al., 2000).

Data used for the statistical evaluation are collected from the examined parameters indicating the impact of the storage conditions on the quality, safety and efficacy of the product defined in the shelf life specification during the monitoring of the long-term stability of a final pharmaceutical product (Yoshioka and Stella, 2000). After completion of the stability studies the results are evaluated in accordance with the regulation (ICH, 2003a).

The goal of this paper was to explore the possibility of shelf life extension based on statistical evaluation of stability data of ongoing stability studies for final pharmaceutical product, Lisinopril tablets of 10mg, that contain active substances from different manufacturers, both with European quality certification (EDQM, 2015).

Materials and methods

The investigated batches of final pharmaceutical product, Lisinopril tablets 10 mg, with incorporated active substances from two different manufacturers, were examined over following stability indicating parameters: content of active substance, release of the active substance, related and degradation products and organoleptic characteristics (Diana, 2008).

Samples used in studies of stability are kept in stability chambers, with strictly defined and controlled monitoring of temperature and relative humidity (ΔC ± 2 °C, ΔRH ± 5%) with the software system Sympati (ICH, 2003a).

F-test method and analysis of variance (ANOVA) have been applied to the obtained analytical data in order to determine whether there are statistically significant differences between the batches in which the active substance is built from different manufacturers, or whether the quality of the active substance from various manufacturers affects the quality of the final product and the variation of the tested parameters (O'Brien, 1979). The interdependence between the results obtained with the long-term stability tests to 36 months is determined by using regression analysis. Regression analysis of the dependence of the tested parameters from the time of examination is performed by logarithmic regression, as a kind of curve that best corresponds to the nature of the results. Non-linear, i.e. logarithmic regression more suitably reflects the actual connection between the examination time and tested parameters, i.e. the content and release of the active substance. Based on the equation, the desired shelf life is provided and it is compared with the experimentally obtained value of the parameters tested for 48 months.

Results and discussion

Evaluation of the results of stability study within the prescribed period of use

The calculated F-value is 1.89 for the content of the active substance, i.e. 1.68 for the release of the active sub-

* vdubrova@alkaloid.com.mk

stance, which is a value lower than the tabular F-value = 19.0 on 2 degrees of freedom and statistical confidence of 99.0%, which means that there is no statistically significant difference between the variability of the content of the active substance and the release of the active substance in the tested batches of different manufacturers of the active substance.

According the Analysis of the variance, F-test value ($p = 0.968 > 0.05$ and $p = 0.847 > 0.05$), it can be concluded that among the average values of the content and release of the active substance in Lisinopril tablets 10 mg of both manufacturers there is no statistically significant difference.

Evaluation of the results of extended stability studies

The results obtained by determining the content of the active substance during long-term stability tests are incorporated in the logarithmic regression curve, from which extrapolating theoretical values for the content of the active substance are derived for the new proposed shelf life of 48 months. The same examined samples of batches of Lisinopril 10 mg tablets are left in the room for stability under the same conditions for another 12 months, in total 48 months.

By extrapolating the logarithmic regression curve theoretical values for the content of the active substance in Lisinopril tablets 10 mg are obtained after 48 months, with an interval of reliability at a statistical confidence of 95% and compared with the experimental values obtained. All experimental values were in the range of extrapolated values.

By extrapolating of the logarithmic regression curve, theoretical values for release of the active substance in Lisinopril tablets 10 mg after 48 months are obtained, with interval of confidence at a statistical confidence of 95% and compared with experimental values obtained. All experimental values were in the range of extrapolated values.

In the study for the stability of tested Lisinopril tablets 10 mg, the parameter "related content and degradation products" was monitored as well as the organoleptic characteristics of the same batches on which content and release the active component of both manufacturers was determined. Most of the obtained values for impurities were below limit of quantification, defined in the validation of analytical methods. These parameters were processed using risk analysis. Risk assessment of Lisinopril tablets 10 mg was performed by using Failure Mode Effects Analysis (FMEA) method to identify and assess the risk of extending the shelf life and introducing an active substance from a new manufacturer.

Conclusion

In line with the pre set goals of this paper, the influence of the choice of the active substance on the stability of Lisinopril 10 mg tablets is defined through monitoring the stability with defined testing up to 36 months, with possibility of extending the shelf life up to 48 months, in order to extend the period of use.

The results of the determination of the content and release of the active substance during the stability tests up to 48 months, indicate that none of the tested batches of Lisinopril tablets 10 mg exceeds the limit of the permitted deviation of the content of the active substance of 94% i.e. the limit of tolerance of the parameter of release does not affect the stability and quality of products and allows extension of the active substance of 85%. It can be concluded that the expiry date can be extender for an additional 12 months, without risk of occurrence of endangering the health of the patient, if using the active substance of the two manufacturers.

Monitoring and statistical evaluation of the results from the stability studies of the final pharmaceutical product has proved as useful approach for extending the shelf life of products with proven life cycle stability and possibility for incorporation of active substances from different manufacturers. This concept can be applied to any product of interest and established need for extension of the shelf life.

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Macedonian pharmaceutical bulletin, **62** (suppl) 189 - 190 (2016)
ISSN 1409 - 8695
UDC: 615.33.012
Short communication

Determination of clarithromycin residues on manufacturing equipment surfaces in cleaning validation process

Katerina Kochova*, Elena Petrovska, Gordana Trendovska Serafimovska

Quality Control Department, Pharmaceutical Company Replekfarm DOOEL, Kozle 188, Skopje, Republic of Macedonia

Introduction

"Cleaning validation is a process to ensure that equipment cleaning procedures are removing residues to predetermined levels of acceptability" (FDA, 1993).

In many cases the same equipment is used for manufacturing of different pharmaceutical products (ICH, 2000b). Well defined cleaning procedure must be used to avoid any contamination of a subsequent product with residues of a previous product, cleaning agents and microbial contaminants.

A new EU GMP requires that limits for the maximum acceptable carry over (MACO) of product i.e. API's residues should be based on toxicological evaluation, PDE (permitted daily exposure) concept (EMA, 2014).

There is a difference between the acceptance criterion usually used so far (1/1000 dose and 10 ppm), in accordance with FDA's guidelines and the determination the MACO by the PDE concept. For residues where toxicological data is available, the MACO calculation should be based on the no observed effect level (NOEL). NOEL is the amount of drug in mg that does not have any effect on the human health and is calculated by using LD50 of the drug. The principle of MACO calculation is that the calculated acceptable carry-over from the previous product, based upon the PDE, is transferred into the next manufacturing product (APIC, 2014; ECA, 2007).

For the control of a cleaning procedure, a method suitable for determination of the clarithromycin residues during the process of cleaning validation is needed.

In this research a sensitive analytical method based on high performance liquid chromatography was adapted and validated to determine residues of clarithromycin in swab samples.

Materials and methods

Varian HPLC System was used, equipped with Varian Prostar 240 pump, Varian 325 LC detector and Galaxie1.9.301.220 software for data handling. The experiments were carried out by HPLC using Discovery C18, (125 x 4,6 mm i.d., 5µm) column. The HPLC system was operated at isocratic mode using mobile phase composed of buffer solution (30mM phosphate buffer pH 3.8 - 4.0) and methanol in ratio 35:65 (%). The temperature was 45°C, UV detection was at 200 nm and injection volume 100 µL.

The swabbing procedure was optimized in order to obtain a suitable recovery of clarithromycin from stainless steel surfaces. Using the head of AlphaTM Swab Texwipe previously rinsed with methanol, the predefined 25 cm^2 surface area was wiped first in a horizontal and secondly in a vertical way, starting from the outside towards the centre.

Results and discussion

The HPLC method was appropriately adapted and validated according to ICH guidelines (ICH, 2000a). The method was validated through determining these validation characteristics: selectivity, linearity, precision, accuracy, quantification limit and detection limit. Predetermined maximal levels of acceptability for clarithromycin residues were 21 ppm in samples and 17.3 µg/cm^2 per surface area (LeBlanc, 2000). Achieved values for LOD of 0.59 µg/mL (ppm) and for LOQ of 1.9 µg/mL, indicate that the proposed HPLC method is enough sensitive for determining the established product residual limits. Linearity was studied in the concentration range 1 - 40 ppm, and the correlation coefficient (r^2) = 0.9998 obtained for the regression line demonstrates the excellent relationship between peak area and the concentration of clarithromycin. The selectivity was studied by comparing a blank solution and an clar-

* katerina.kocova@replek.com.mk

ithromycin test solution, which was prepared by wiping a stainless steel plate 5x5cm² spiked with 10 µg/cm² of clarithromycin according to optimized swabbing technique. The blank solution was prepared in the same way by wiping a clean plate. No sources of interference of the swab material were observed at the retention time of the analyte.

As sensitive sampling methods require development and must be applicable to each specific piece of equipment used, swab recovery was determined using spiking studies incorporating coupons of equipment surfaces. The swabbing procedure was optimized in order to obtain a suitable recovery of clarithromycin from stainless steel plates. The obtained "swab recovery" factor of 87.81% confirmed the appropriateness of the cleaning method, that recovery was taken into account in the results of cleaning validation.

Conclusion

HPLC method was developed and validated in order to determine residual amounts of clarithromycin after the cleaning procedure. The chromatographic technique was demonstrated to be sensitive, linear, accurate and precise in the concentration range studied. Direct sampling method was optimized to obtain effective and reliable recoveries. The detection limit for proposed liquid chromatography analytical method was sufficiently sensitive to detect the established acceptable level of the residues of clarithromycin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 191 - 192 (2016)
ISSN 1409 - 8695
UDC: 615.225.2.074:543.544.5.068.7
Short communication

Validation of RP-HPLC stability-indicating method for cilazapril and hydrochlorothiazide

Jasmina Šljivić*, Mira Zečević, Biljana Otašević, Ana Protić, Jelena Golubović

Department of Drug Analysis, University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia

Introduction

Hydrochlorothiazide is thiazide diuretic whose pharmacological effects are based on inhibition of the sodium and chloride reabsorption and enhancement of potassium secretion. Cilazapril is angiotensin-converting enzyme inhibitor. Hydrochlorothiazide and cilazapril are antihypertensive drugs which can be used as monotherapy or in fixed combinations (Skolnik et al., 2000; Sweetman, 2009).

Forced degradation studies are complementary part of drug development strategy being undertaken for identification of degradation products, to elucidate the degradation pathway and intrinsic stability of the drug. Investigation of degradation products formed under stress conditions is useful in development and validation of stability-indicating procedures and it is also necessary due to changes in toxicity, bioavailability or therapeutic effects of the dosage form (Alsante et al., 2007; ICH Q1A (R2), 2003; Kurmi et al., 2014).

To date, no information on stability of cilazapril and hydrochlorothiazide in therapeutic combination was found in the literature. Therefore, stability-indicating method was developed. In order to confirm its applicability in routine quality control of cilazapril and hydrochlorothiazide dosage forms, robustness and other validation parameters of the established method have been investigated.

Materials and methods

Satisfactorily chromatographic separation was achieved on Kinetex C18 (2.6 µm, 50 mm x 2.1 mm) column (Phenomenex Inc., Torrance, USA) with column temperature set to 25 °C with the mobile phase consisting of acetonitrile and buffer solution (20 mM ammonium formate, pH adjusted to 8.5 with formic acid) and pumped at 400 µL/min flow rate in following gradient elution mode: initial composition was acetonitrile : buffer solution (5:95, v/v) and final composition was acetonitrile : buffer solution (35:65, v/v). Time of the gradient elution mode was 15.38 minutes. The detection was performed using PDA detector at 215 nm, 250 nm and 270 nm. Cilazapril and hydrochlorothiazide solutions were subjected to acidic (1.0 M HCl), basic (1.0 M NaOH), thermal (70 °C) and oxidative (15-30% H_2O_2) degradation and appropriate stress solutions were used during method development and method selectivity investigation. Standard stock solutions of cilazapril and hydrochlorothiazide were prepared in concentrations of 1000 µg/mL and standard stock solutions of cilazaprilat and hydrochlorothiazide impurity B were prepared in concentrations 200 µg/mL, separately by dissolving standard substances in the mixture of acetonitrile-water (50:50, v/v). For method validation purposes, further dilutions of standard stock solutions were done with the mixture of acetonitrile and 20 mM ammonium formate buffer solution pH = 8.5 (20:80, v/v) in order to attain concentrations in the following ranges: 50.00-150.00 µg/mL for cilazapril, 125.00-375.00 µg/mL for hydrochlorothiazide, 0.50-1.50 µg/mL for cilazaprilat and 0.25-3.75 µg/mL for hydrochlorothiazide impurity B. Cilazil HCT® tablets (Pliva, Zagreb, Hrvatska) containing 5 mg of cilazapril monohydrate and 12.5 mg of hydrochlorothiazide were purchased and used for validation.

Results and discussion

Forced degradation studies on cilazapril and hydrochlorothiazide active substances were conducted prior to method development in accordance with the ICH regulatory guidelines (ICH Q1A (R2), 2003). Cilazapril appeared

* hadzicjasmina@hotmail.com

to be unstable towards acid and base hydrolysis which resulted in formation of cilazaprilat. Hydrochlorothiazide degraded after acid, base and thermal hydrolysis and formed degradation product was hydrochlorothiazide impurity B. Both degradation products were already reported in official monographs of cilazapril and hydrochlorothiazide (Ph. Eur., 2011) as their related organic impurities. After oxidative degradation of both active substances, unknown products arisen.

The stability-indicating UHPLC-UV-MS method was developed. The experimental conditions that enabled separation of cilazapril, hydrochlorothiazide and all of their degradation products were previously described. Method validation was carried out in accordance to ICH and FDA guidelines (Guidance for industry: Bioanalytical method validation, 2001; ICH Q2(R1), 2005).The selectivity of the method was confirmed by chromatographic screening for interfering substances. No interfering peaks were present in the chromatogram of dosage form sample at the retention times of cilazapril, hydrochlorothiazide, cilazaprilat, hydrochlorothiazide impurity B and unknown degradation products that were recorded in the chromatograms of stress samples.

The method robustness was considered during the method development and optimization procedure. Initial percentage of acetonitrile in mobile phase, final percentage of acetonitrile in mobile phase, time of gradient elution and column temperature were defined as critical variables. Investigated responses were retention factors of degradation products of cilazapril and hydrochlorothiazide denoted as $k_{283.6}$ and $k_{429.9}$, respectively, as the first and the last eluting substances. Initial percentage of acetonitrile in mobile phase and column temperature demonstrated more significant influence on observed responses while the influences of final percentage of acetonitrile in mobile phase and time of gradient elution was less significant. Small changes in value variables did not jeopardize responses of investigated substances.

The concentration range for testing of the linearity of the method was: 50.00-150.00 µg/mL for cilazapril, 125.00-375.00 µg/mL for hydrochlorothiazide, 0.50-1.50 µg/mL for cilazaprilat and 0.25-3.75 µg/mL for hydrochlorothiazide impurity B. The calculated calibration equations were y=29.7081x+18.9990, y=83.4604x+2180.4154, y=7.0676x-2.2344 and y=6.3525x+0.1990 for cilazapril, hydrochlorothiazide, cilazaprilat and hydrochlorothiazide impurity B, respectively. The correlation coefficients were 0.9999, 0.9998, 0.9977 and 0.9959 for cilazapril, hydrochlorothiazide, cilazaprilat, hydrochlorothiazide impurity B, respectively and they indicated that the relationship between peak area and the concentration was linear over investigated concentration range.

Experimentally determined value of limit of quantification for cilazaprilat and hydrochlorothiazide impurity B were 0.5 µg/mL and 0.25 µg/mL, respectively.

The precision of the method was considered as intra-assay and it was performed in six repetitive measurements where concentration levels were: 100 µg/mL for cilazapril, 250 µg/mL for hydrochlorothiazide, 1.0 µg/mL for cilazaprilat and 2.5 µg/mL for hydrochlorothiazide impurity B. Results were expressed as relative standard deviation values which were 0.35%, 0.25%, 4.61% and 3.23% for cilazapril, hydrochlorothiazide, cilazaprilat and hydrochlorothiazide impurity B, respectively.

Conclusion

Stability profile of cilazapril and hydrochlorothiazide was established and stability-indicating method was developed. The method showed good robustness and met all validation criteria.

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Macedonian pharmaceutical bulletin, **62** (suppl) 193 - 194 (2016)
ISSN 1409 - 8695
UDC: 615.33.074:543.544.5.068.7
Short communication

Development of fast, simple RP- HPLC method for determination of moxifloxacin in solid pharmaceutical dosage forms

Marjan Piponski*, Tanja Bakovska, Marina Naumoska, Emilija Janeva, Tatjana Rusevska Marija Globochki, Magdalena Piponska, Gordana Trendovska Serafimovska

Replek Farm Ltd., Quality Control Department, St. Kozle 188, 1000 Skopje, Republic of Macedonia

Introduction

Moxifloxacin is a fourth-generation synthetic fluoroquinolone antibacterial agent. It is monohydrochloride salt of 1-cyclopropyl-7-[(S,S)-2,8-diazabicyclo[4.3.0] non-8-yl]-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3 quinoline carboxylic acid (RxList, 2016). The analytical methods for its determination in pharmaceutical dosage forms are not so numerous and can be categorized generally into two groups: nonseparative UV-Vis spectroscopic methods, zero order (Dhumal et al., 2011; Sahu et al., 2011) or derivative (Dhumal et al., 2011) and separative, dominantly chromatographic methods (Abdelaziz et al., 2012; Dewani et al., 2011; Singh et al., 2014). These cited HPLC methods for quantification of moxifloxacin, found during literature search, show some unwanted features like skewed or asymmetric peaks and excessive retention achieved with 250 mm long columns.

Above presented facts prompted us to develop HPLC method for fast, simple, accurate, reproducible and rugged quantification of moxifloxacin in solid pharmaceutical dosage forms.

Materials and methods

The chemicals were Ph.Eur. grade. Potassium dihydrogen phosphate, ammonium dihydrogen phosphate, dipropylamine and 85% phosphoric acid were purchased from Sigma - Aldrich, USA, whereas methanol was purchased from Merck KGaA, Darmstadt, Germany. The demineralized water was Stilman produced and had conductivity less than 1 µS. Moxifloxacin working standard and Moxifloxacin film-coated tablets 400 mg were purchased from Replek Farm, Skopje, Macedonia.

The experiments were performed on three HPLC systems: Varian Prostar with DAD 330 detector controlled with Varian Star software version 6.21, UPLC Dionex with four-channel UV-Vis detector controlled with Chromeleon version 6.8 and UPLC Shimadzu Nexera with dual-channel UV-Vis detector controlled with Lab Solutions version 5.54.

Following HPLC columns were tested:

- RP Select B 125 mm x 4 mm, 5 µm (Merck Darmstadt, Germany);
- Discovery C8 150 mm x 4.6 mm, 5 µm (Supelco Bellefonte, USA);
- Discovery C18 100 mm x 4.6 mm, 5 µm (Supelco Bellefonte, USA);
- Discovery C18 125 mm x 4.6 mm, 5 µm (Supelco Bellefonte, USA).

The test and standard solutions were prepared by dissolving suitable amount of the active substance or powdered film-coated tablets in methanol or water to obtain concentrations in the range of 0.1 - 0.5 mg/mL.

Results and discussion

The characteristic UV-absorption spectrum of moxifloxacin with maximum at 293 nm permits use of almost all commonly used buffers in chromatography. Considering moxifloxacin pKa values (pKa1=6.25 and pKa2=9.29) (Lanqlois et al., 2005) and its well documented solubility in more polar solvents, phosphate buffers with pH value 3

* piponski99@gmail.com

in combination with methanol as mobile phase were used.

Two different phosphate buffers, 25-30 mM $NH_4H_2PO_4$ and KH_2PO_4 with pH=3 were tested. Slight insignificant advantage was given to ammonium dihydrogen phosphate compared to potassium dihydrogen phosphate, when peak symmetries were compared. The mobile phases were tested without and with added dipropylamine as a peak symmetry corrector by suppressing the ion-exchange interaction of residual silanol groups with protonated N-atoms in analyte molecule. The addition of 0.2% v/v dipropylamine in 25 mM ammonium dihydrogen phosphate at pH 3 improved the peak symmetry from 1.47 to 1.25.

Fluoroquinolones with their characteristic molecule structure are well known to chromatographists as problematic with yielding tailing peaks on the chromatograms, usually corrected with use of high percentage of amines in mobile phase composition, up to 1 - 2% v/v. This prompted our investigation, to choose chromatographic column which has high quality end-capped silica particles with lowest content of residual metal cations.

Toward aiming the goal, two C8-octylsilane columns and two C18-octadecylsilane columns from the same manufacturer but with different dimensions were tested.

The comparison of the chromatograms obtained with C8 and C18 columns from identical vendors showed that no significant preferences could be noticed. Both C8 and C18 can create perfect peak of moxifloxacin which can be fine adjusted with retention needed. It was clearly seen that lowering the percentage of methanol from 55% v/v to 40% v/v increases the peak retention and symmetry. Slight preference from the peak symmetry and less organic consumption point of view in our experiments showed C8 filled columns, that yielded moxifloxacin peak with t5% = 1.15. This fact generally does not prefer C8 over C18 columns, since peak symmetry could be sacrificed in aim to improve resolution, which is more important in chromatographic separations. Depending on the sample constituents complexity and target system suitability demands of the chromatographic method, C8 and C18 could be successfully interchanged and optimized for all demands.

C8 reversed phase column has stronger hydrophobic interactions than Phenyl-RP columns but weaker than C18. The HPLC method for related compounds of moxifloxacin, described in Ph.Eur.8 (2014) uses Phenyl-bonded RP column with TBAH cationic ion pair reagent in mobile phase, and it shows relatively close elution of related compounds with moxifloxicin. This is expected to be improved with use of more retentive C18 column.

The use of chromatographic columns having particles with wide pores also showed to be advantage in our investigations. Discovery C8 columns have pores with about 180 nm diameter, whereas RP Select B columns have 60 nm pores diameter, and generate wide skewed and distorted peak of moxifloxacin.

Discovery columns are better end-capped and more base-deactivated, which was shown to be advantage during our method development.

Finally, the proposed chromatographic conditions for the optimized method for quantification of moxifloxacin are: mobile phase composed of 25 mM $NH_4H_2PO_4$ pH=3 and methanol (65% : 35%, v/v), with flow rate 1.1 ml/min, HPLC column Discovery C8 150 mm x 4.6 mm with 5 µm particles, thermostated at 35 °C, detection wavelength at 293 nm and injection volume of 5 µl. Moxifloxacin peak elutes for less than 5 minutes with symmetry expressed as As_{USP} ~1.2.

Conclusion

The proposed method is fast, simple cost-effective, reproducible and rugged, intended for high-throughput routine assay analysis of moxifloxacin in solid pharmaceutical dosage forms. It can be further optimized and improved, using columns with different properties, better retention and selectivity, or changed ratio of mobile phase constituent, in order to achieve better retention of moxifloxacin peak and better resolution from its degradation products in order to make it suitable for use during stability testing of the pharmaceutical formulations.

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Macedonian pharmaceutical bulletin, **62** (suppl) 195 - 196 (2016)
ISSN 1409 - 8695
UDC: 615.33.074:543.544.5.068.7
Short communication

Positive chaotropic role in development of RP- HPLC method for quantification of norfloxacin in pharmaceutical dosage forms

Marjan Piponski, Tanja Bakovska*, Marina Naumoska, Marija Globochki, Irena Slaveska Spirevska, Stefan Stefov, Magdalena Piponska, Elena Petrovska, Gordana Trendovska Serafimovska

Replek Farm Ltd., Quality control Department, St. Kozle 188, 1000 Skopje, Republic of Macedonia

Introduction

Norfloxacin is a first generation synthetic fluoroquinolone, a broad spectrum antibacterial agent, approved for treatment of urinary tract infections, sexually transmitted diseases and prostatitits. Its IUPAC name is 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid (RxList, 2016). Majority of fluoroquinolone molecules, especially norfloxacin, are well known as problematic in terms of peak shape appearing on the chromatograms. They are often obtained as highly asymmetric, tailing peaks with calculated values of symmetry factor of about 2 or even greater. This fact complicates and compromises method accuracy based on the difficulties in determination of peak end on baseline. These problems are frequently solved with use of expensive high quality HPLC columns with double and triple end-capped bonded reversed phase silica matrixes and extensive base-deactivation of metal cations from silica particles. British Pharmacopoeial monograph (BP, 2013) for norfloxacin active pharmaceutical ingredient assay determination prescribes use of C16 column with gradient elution with water acidified to pH ~2 and acetonitrile at high column temperature of 60 °C and for norfloxacin tablets acidic isocratic elution with 0.1% v/v o-H_3PO_4 and acetonitrile with previous washing of column with different buffer, for 8 hours.

Many different methods are published (Bera et al., 2014; Chierentin and Salgado, 2013; Oliveira et al., 2009; Singh et al., 2013) for norfloxacin determination in pharmaceutical dosage form. All of them conflict with the mentioned problem of peak symmetry of norfloxacin and this problem is differently eliminated with use of amines, gradient elutions, high column temperatures, expensive high quality columns employment etc.

The aim of our study was to develop fast, simple accurate and robust HPLC method for determination of norfloxacin in pharmaceutical dosage forms, yielding chromatographic peak of norfloxacin with satisfying symmetry, without using gradient elution, high column temperatures and expensive high quality columns.

Materials and methods

Ammonium dihydrogen phosphate ($NH_4H_2PO_4$), 85% phosphoric acid and potassium hexafluorophosphate (KPF_6) were purchased from Sigma - Aldrich, USA, whereas methanol, acetonitrile and 70 % perchloric acid ($HClO_4$) were purchased from Merck KGaA, Darmstadt, Germany. All the chemicals used were Ph.Eur. grade. The demineralized water was Stilman produced and had conductivity less than 1 µS.

Common HPLC system was used, Varian Pro Star system with autosampler 410, Varian 325 dual-channel and Varian 335 photodiode-array detector, with ternary high pressure mixing pump, controlled by Galaxy software version 1.91.

Following two chromatographic columns were tested: RP Select B 75 mm x 4 mm, 5 µm and RP Select B 125 mm x 4 mm, 5 µm, both products of Merck Darmstadt, Germany.

Norfloxacin working standard and Norfloxacin film-coated tablets 400 mg were purchased from Replek Farm, Skopje, Macedonia.

Test and standard solutions were prepared by dissolv-

* tanja_bakovska@yahoo.com

ing suitable content of the active substance or powdered film-coated tablets in solvent composed of 5% v/v acetonitrile and 95% v/v 0.1% v/v H_3PO_4, to concentration of 0.4 mg/ml.

Results and discussion

During development of appropriate chromatographic method for quantification of norfloxacin with improved peak symmetry, we intended to investigate influence of participation of chaotropic salts in mobile phase constitution.

For this investigation we decided to use lower quality reversed HPLC column, RP Select B with two dimensions 75 mm x 4mm and 125 mm x 4 mm, with 5 µm particles, manufactured by Merck Darmstadt, Germany. These columns have a matrix which is single end-capped and partially base-deactivated silica particles with small, 60 nm wide pores. With these characteristics RP Select B is not appropriate choice for column in chromatographic method for norfloxacin determination, but use of this column should better manifest the influence of appropriate mobile phase on peak symmetry.

We performed two tests of same column matrix of RP Select B with different dimensions:

1. Using RP Select B 75 mm x 4 mm with 5 µm particles, mobile phase flow rate of 1.1 ml/min, chromatographic column temperature of 32 °C, injection volume of 5 µl, UV detection at 277 nm, and mobile phase consisted of 30% v/v methanol and 70% v/v mixture of 0.13% w/v $NH_4H_2PO_4$, 0.2% w/v KPF_6 and 0.1% v/v $HClO_4$ with pH value 2.39.

The chromatogram obtained under these conditions showed norfloxacin peak with retention time ~2.9 minutes, height ~570 mAU and symmetry expressed as As_{USP} ~1.25.

2. Using RP Select B 125 mm x 4 mm with 5 µm particles, mobile phase flow rate of 1.0 ml/min chromatographic column temperature of 30 °C, injection volume of 5 µl, UV detection at 277 nm, and mobile phase consisted of 35% v/v methanol and 65% v/v mixture of 0.13% w/v $NH_4H_2PO_4$, 0.2% w/v KPF_6 and 0.1% v/v $HClO_4$ with pH value 2.39.

The chromatogram obtained under these conditions showed norfloxacin peak with retention time ~3.75 minutes, height ~560 mAU and symmetry expressed as As_{USP} ~ 1.2.

With these results, the peak symmetry and retention correction features of potent chaotropes like KPF_6 was confirmed as beneficial. In our case, peak retention was not a primary problem. The main problem was the peak symmetry. According to theory and publication of Kazakievic and Lobrutto (2007), the beneficial features of the chaotrop KPF_6 are more significant with use of acetonitrile instead of methanol. But in our case, use of KPF_6 chaotrop showed much better peak symmetry correction benefit, than extended peak retention. Other important thing to mention is that use of 0.1% v/v $HClO_4$ contributes to peak symmetry too, since the ClO^{4-} anions are also chaotropic, but in lesser extent than PF^{6-} anions.

Further improvements of peak symmetry and retention can be achieved with increase of content of chaotropic agent KPF_6, but it is not necessary to extent run time for insignificant increase of peak symmetry. Better peak symmetry can be achieved with use of more expensive, new, high end-capped and well base-deactivated columns. Use of wider particle pore column is preferential when compared with 60 nm pores of RP Select B column.

Conclusion

New approach in creating fast, simple, accurate and robust RP-HPLC method was developed, which is very useful in high-throughput analysis per day, in pharmaceutical quality control routine analysis of norfloxacin pharmaceutical dosage forms. Method can be further adjusted and adapted for other intents of analysis, bioequivalence testing, pharmacodynamic, forensic analysis and similar.

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Macedonian pharmaceutical bulletin, **62** (suppl) 197 - 198 (2016)
ISSN 1409 - 8695
UDC: 615.33.074:543.544.5.068.7
Short communication

Forced degradation study of moxifloxacin in tablet formulation using RP-HPLC

Alma Salkić [1], Mira Zečević [2]*, Amra Butković [1], Jelena Golubović [2], Jasmina Šljivić [2]

[1] *ZADA Pharmaceuticals d.o.o. Bistarac Donji, Lukavac, Bosnia and Herzegovina*
[2] *Department of Drug Analysis, Faculty of Pharmacy, University of Belgrade,450 Vojvode Stepe, 11221 Belgrade, Serbia*

Introduction

In process of stability testing of a drug product it is very important to perform forced degradation studies. It is necessary to perform these studies in process of development the stability indicating method which can be applied latter for the analysis of samples generated from accelerated and long term stability studies in proposed shelf life to ensure that all possible degradation products that may occur in proposed shelf life in normal conditions of storage can be separated, detected and quantified (Djurdjevic et al., 2009; Singh et al., 2014; Sultana et al., 2011).

Moxifloxacin is a fourth-generation antibiotic drug of the fluoroquinolone familly, with higher activity against Gram-positive pathogens including *Streptococcus pneumonie*, than the other drugs from fluoroquinolone family (Dewania et al., 2011; Lalitha Devi and Chandrasekhar, 2009).

The objective of the present study was to report the stability of moxifloxacin in tablet formulations available on the local market, based on the information obtained from forced degradation studies, to develop and validate new stability-indicating HPLC method for quantitative analysis of moxifloxacin and its related substances in pharmaceutical dosage form tablet and to develop LC-MS method for the characterization of new degradation products in accordance with the information obtained from forced degradation studies.

Materials and methods

Three different tablet formulations (Formulation A: MOXI film tablets 400 mg-ZADA Pharmaceuticals, Formulation B: AVELOX film tablets 400 mg-Bayer, Formulation C: CENOMAR film tablets 400 mg-Hemofarm), purchased from the local market were tested. European Pharmacopoeia Reference Standards Moxifloxacin hydrochloride CRS and Moxifloxacin for peak identification CRS were used. Analytically pure moxifloxacin was obtained from Nosch Laboratories Limited, India. All chemicals and reagents used were of HPLC-grade purity.

A High Performance Liquid Chromatographic system (Thermo Surveyor HPLC) equipped with Finnigan Surveyor LC quaternary pump, Finnigan Surveyor Autosampler plus, Finnigan Surveyor PDA plus detector in isocratic mode was used for the analysis.

Experimental design was used to optimize experimental conditions. The method was optimized by analysis of the samples generated during the forced degradation studies. Experimental factors, independent variables, were selected as inputs and as dependent variables were study resolution between typical peaks and retention factor of moxifloxacin because latest eluted.

To assess the impact of some experimental factors on the responses of the system it was applied central composite design. The optimal chromatographic conditions were selected by calculating Desirability function.

After optimizing the experimental conditions the separation was conducted on a ZORBAX-SB Phenyl column (250x4.6 mm, 5 µm particle size) with mobile phase composed of methanol:buffer (0.5 g/L tetrabutylammonium hydrogen sulphate, 1.0 g/L monobasic potassium dihydrogen phosphate and 3.4 g/L phosphoric acid in water) in ratio 71:29, v/v; pumped at 1.5 ml/min flow rate. The column temperature was set at 50 °C and the detection at 293 nm using Diode Array Detector.

LC-MS method was developed for characterization of degradation products using HYPERSIL GOLD PFP 150x4.6 mm, 5 µm column with mobile phase composed

* mira.zecevic@pharmacy.bg.ac.rs

of ammonium formate (20mM): acetonitril pH=4; (70:30, v/v) pumped at 1 ml/min flow rate. The column temperature was set at 25 °C and the detection at 295 nm using Diode Array Detector.

The medicine was subjected to acid (4 mol/l HCl, 70 °C for 6 days) and base (4 mol/l NaOH, 50 °C for 6 days) hydrolysis, oxidative decomposition (3% H_2O_2), thermal and photolytic stress.

Forced degradation studies were conducted on the active substance, placebo mixture and binary mixtures of moxifloxacin with the excipients from the tablet formulations, in the same stress conditions, to determine the origin of the formed degradation products.

Results and discussion

New LC-MS compatible stability-indicating HPLC method for moxifloxacin was developed. Significant degradation was caused by acidic and basic conditions. The results were comparable in all tested tablet formulations. In the samples without stress impurities were not detected.

Under basic conditions (4 mol/l NaOH, 50 °C for 6 days) two degradation products have been detected (RRT 0.5 min. and 0.6 min.) in all three tested tablet formulations.

Under acidic conditions (4 mol/l HCl, 70 °C for 6 days) one degradation product (RRT 0.8 min.) was detected with significant area in all three tablet formulations. In formulation B it one additional degradation product on RRT 1.3 min. was detected, while in formulation C two additional degradation products (RRT 0.4 min. and 1.3 min.) were detected.

It was found that the degradation products formed in the presence of base are result of the interaction between moxifloxacin and excipients in the tablet formulation: lactose and cellulose, while the degradation products caused by acidic conditions are performed as a result of degradation of the active substance moxifloxacin.

Composition of formulation A and B included cellulose and lactose, while formulation C had only cellulose in its composition. All degradation products were identified as unknown.

Conclusion

An isocratic stability-indicating RP-HPLC method for quantification of moxifloxacin and its related substances in moxifloxacin tablets was optimized and validated. LC-MS method was found to be suitable for separation of degradation products formed under stress conditions. In all of the three tested tablet formulations unknown degradation products were found, which need to be identified.

The developed LC-MS method can be used for future research which objective will be the identification of formed degradation products.

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Macedonian pharmaceutical bulletin, **62** (suppl) 199 - 200 (2016)
ISSN 1409 - 8695
UDC: 615.214.24.074:543.544.5.068.7
Short communication

Simple RP-HPLC method for estimation of diazepam and benzyl alcohol in microclisme

Maja Vragolic[1], Branka Ivkovic [2]*, Olivera Cudina[2], Sote Vladimirov[2], Jasmina Brboric[2]

[1]*Pharmacy "Belgrade", Bojanska 16/IV, Belgrade, Serbia*
[2]*Department of Pharmaceutical Chemistry, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia*

Introduction

Diazepam (DZP) (7-chloro-1, 3-dihydro-1-methyl-5-phenyl-2H-1, 4-benzodiazepin-2-one) is a benzodiazepine (BZD) generally used as hypnotic, anxiolytic and muscle relaxant. DZP is also routinely prescribed as the standard first-line treatment for acute convulsions and prolonged status epilepticus (Martindale, 2009). Several methods for the analysis of BZDs have been reported. A number of chromatographic methods such as: thin-layer chromatography (TLC) (Hancu et al., 2011), gas chromatography and gas chromatographic–mass spectrometry (GC–MS) (Kudi et al., 1988) have been used in the analysis of DZP and other 1,4-benzodiazopines. Several high-performance liquid chromatographic (HPLC) methods have also been reported for the determination of diazepam and other BZDs (Magalhães et al., 2012; Moros et al., 2007). Benzyl alcohol (BA) is used as an antimicrobial preservative in pharmaceutical and cosmetic products and its concentration should not exceed limit values for each formulation type because it could produce fatal toxic effects, allergies and various other effects on the nervous system (Martindale, 2009). Benzyl alcohol is quantitatively estimated in raw material and pharmaceutical preparations by gas chromatography, derivative spectrophotometry, HPLC, amperometric enzyme electrode, polarography des and capillary electrokinetic chromatography (Khade et al., 2014; Sen, 2011). To the best of our knowledge no analytical method is available for the simultaneous determination of DZP and BA in pharmaceutical preparations. Therefore, it was considered necessary to develop a HPLC method for the quantification of these compounds in pharmaceutical dosage forms. We wish to describe a simple, rapid, economical and accurate method of analysis using reversed-phase HPLC to simultaneously determine DZP and BA in microclismes.

Materials and methods

The chromatographic analysis was performed using DionexUltiMate 3000 UHPLC focused. The HPLC grade acetonitrile was developed using ReproSil-Pur 120 C4, 150 x 4,6 mm, 3,0 µm chromatographic column with mobile phase containing a mixture of 0.1% (v/v) orthophosphoric acid in water and acetonitrile in the ratio of 70:30 v/v, respectively. Isocratic method was used with runtime of 10 minute. The flow rate of the mobile phase was 1.5 mL/min. The column temperature was maintained at 35°C and the eluted compounds were monitored at the wavelength of 254 nm. The injection volume was 20 µL. A standards stock solution of DZP and BA were prepared in methanol with a concentration of 2 mg/mL and 0.015 mL/mL. Working standard solutions were prepared from stock solutions by further dilution with water. The sample solution was prepared by dissolving 0.5 mL solution of microclisme in a 50 mL volumetric flask (US bath for 7 min) and diluting with water to volume. The sample solution was filtered through a 0.45 µm PTFE membrane filter.

Results and discussion

The proposed method was validated as per ICH guideline (ICH, 2003). The following validation characteristics were addressed: suitability, specificity, precision, linearity, accuracy, and robustness.

System suitability was checked for the conformance of suitability and reproducibility of chromatographic sys-

* blucic@pharmacy.bg.ac.rs

tem for analysis. The system suitability was evaluated on the basis of USP capacity factor, tailing factor and theoretical plates of DZ and BA and relative standard deviation (RSD) of six injections of standard solution. System suitability was determined before sample analysis. The acceptance criteria were % RSD should not be more than 2.0%, USP tailing factor should less than 2.0 and theoretical plate should be more than 3000 for DZ and BA peak from standard solutions. All critical parameters tested met the acceptance criteria [kDZ'=5.35 kBA'=1.14, AsDZP=1.4 and AsBA=1.1, NDZP=4800 and NBA=4253, RSDDZP=0.001 and RSDBA=0.0017). Specificity study to establish the interference of placebo was conducted. Study was performed on Placebo (Placebo contains excipients without DZP and BA). Chromatogram of placebo had shown no peaks at the retention time of DZP and BA, indicating that the excipients used in the formulation do not interferewith the estimation of DZP and BA. The precision of the method was verified by repeatability and intermediate precision at three concentration levels (DZP: 0.017 mg/mL to 0.023 mg/mL and BA: 0.118 µL/mL to 0.162 µL/mL). The intermediate precision of the method was also evaluated using different analyst and different instrument and performing the analysis on different days. The RSD (%) of result for DZP and BA was calculated. The % RSD for the result of DZP in was within 0.65% and in intermediate precision study was within 0.28%, The % RSD for the result of BA in was within 0.63% and in intermediate precision study was within 0.29%, which confirms the good precision of the method. Linearity test solutions were prepared by diluting the stock solutions to the required concentrations by covering the range from 0.014 to 0.026 mg/mL of DZP and 0.098 to 0.182 µL/mL of BA. Calibration curves were plotted between the responses of peak versus analyte concentrations. The correlation coefficient obtained was greater than 0.999 (for DZP is 0.9995 and for BA is 0.9992). The above result shows that an excellent correlation existed between peak area and concentration of DZP and BA. Accuracy of the method was evaluated in triplicate using three concentrations levels (80%, 100% and 120%) and confirmed by calculated recovery values. The percentage recoveries for DZP and BA were calculated and varied from 98.76 to 101.50 for DZP and from 98.15 to 101.96 for BA. To determine the robustness of the developed method, experimental conditions were deliberately altered and system suitability (SST) parameters for DZP and BA standard were recorded. The variables evaluated during the robustness testing were: composition of the mobile phase, column temperature and flow rate. The obtained results have shown that the method is robust for experimental variation of composition of the mobile phase and column temperature, but it is not robust for the change of the flow rate. Recovery value lies in the proper range of from 95% to 105%, ie. RSD does not deviate by more than ± 5% (RSD=0.64% for diazepam, RSD=0.49% of benzyl alcohol).

Conclusion

A simple and efficient reverse-phase HPLC method was developed and validated for quantitative analysis of DZP and BA inmicroclisme as pharmaceutical dosage forms. The method found to be precise, accurate, linear, robust and rugged during validation. The method could be used for routine analysis of production samples and to check the stability of the DZ and BA.

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Macedonian pharmaceutical bulletin, **62** (suppl) 201 - 202 (2016)
ISSN 1409 - 8695
UDC: 615.014.8.074:543.544.3
Short communication

GC-MS method for chemical characterization of pharmaceutical packaging materials

Vlado Petruševski[1]*, Suzana Trajković-Jolevska[2], Jasmina Tonić-Ribarska[2], Sonja Ugarković[1]

[1] *Research & Development Institute, ALKALOID AD - Skopje, Blvd. A. Makedonski 12, 1000 Skopje, Macedonia*
[2] *Faculty of Pharmacy, University of "Ss. Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Developing GC-MS methods for chemical characterization of pharmaceutical packaging materials presents significant challenge, since the sheer number of additives and their degradation products that can be found in packaging materials and subsequently separated and detected with this technique is enormous (Jenke, 2009). These methods need to be capable of simultaneous separation and detection of aliphatic fatty acids, alcohols, amides and their derivatives, compounds that are traditionally difficult to separate and detect, due to the large similarity in structure and their physico-chemical properties.

The aim of this paper was to develop a GC method for chemical characterization of semi-volatile additives and their degradation products in packaging materials for pharmaceutical use.

The GC method was developed using a commercially available system suitability mixture (Grob Test Mix), containing 11 compounds that belong to different chemical groups of compounds, which was suitable for the intended approach to develop a method that will be simultaneously capable of separation of different groups of packaging additives.

Materials and methods

Development was made using Grob DA 280 Column Test Mix (Restek Corp., Bellefonte, USA). The mixture is composed of 11 compounds (n-Decane (C10), Methyl decanoate (C10:0), n-Undecane (C11), Methyl undecanoate (C11:0), Methyl dodecanoate (C12:0), L(+)-2,3-Butanediol, 2,6-Dimethylaniline, 2,6-Dimethylphenol, 2-Ethylhexanoic acid, Nonanal and 1-Octanol), dissolved in methylene chloride (DCM).

All experiments were performed on Shimadzu GC-2010 Plus gas chromatograph and Agilent 7890B gas chromatograph equipped with Agilent 5977A single quadrupole mass spectrometer. MS identification of the detected compounds was performed by AMDIS spectral deconvolution and comparison of spectra with NIST library, using MassHunter software. For those compounds where library match was low, attempts to establish compound's identity were made via mechanistic fragment predictions of available spectral data, using MassFrontier v7.0 software.

Results and discussion

The development of the chromatographic conditions started using a non-polar GC WCOT chromatographic column (Agilent DB-5 ms; 30m x 0.25 mm x 0.25 μm), with helium as carrier gas at 2 ml/min (constant velocity, which has the advantage over constant pressure mode toward the end of the run, where increase in temperature would otherwise drastically reduce the flow, leading to prolonged runtime and reduced sensitivity because of decrease of column efficiency). The following temperature gradient conditions were chosen for initial separation of the Grob Test Mix: 40 °C (hold 2 minutes), 10 °C/min to 330 °C (hold 10 minutes), injector temperature was set at 250 °C and injection volume of 1 μL with split ratio of 1:10. Evaluation of the chromatograms quickly revealed that the method conditions aren't capable of separating the mixture, with multiple co-elutions observed.

In order to achieve specificity of the method, optimi-

* vpetrusevski@alkaloid.com.mk

zation of the conditions by variation of the carrier gas flow and temperature gradient was attempted. Besides the improvement in the separation of the components, is was found to be impossible to separate two critical pairs of compounds (n-undecane and n-nonanal; 2,6-dimethylphenol and 2-ethylhexanoic acid), even with extreme gradient changes.

A change in the stationary phase chemistry was the next logical step, therefore the Agilent DB-5 column was replaced with a non-polar Rxi-1 column (Rxi-1 MS 30m x 0.25mm x 0.25µm, Restek Corp., Bellefonte, USA), with the idea to promote different interactions between the stationary phase and the components from the critical pairs. The change in column chemistry proved to be successful, and minor changes to the temperature gradient conditions yielded complete baseline separation of all components from the Grob DA 280 Column Test Mix. The final chromatographic conditions were defined as: flow: 2 ml/min (constant flow); temperature gradient: 60 °C (hold 2 minutes), 10 °C/min to 80 °C (hold 2 minutes), 3 °C/min to 100 °C, 25 °C/min to 310 °C (hold 10 minutes); injection volume: 1 µL; split ratio: 1:5. The injector temperature was increased to 310 °C to obtain faster evaporation of the sample, better sensitivity and reduced peak width.

Besides the components from Grob DA 280 Column Test Mix, stearic acid and erucamide (cis-13 docosenamide) were chromatographically evaluated, and proved to be completely separated from the rest of the components from the Grob DA 280 Column Test Mix. These two compounds were evaluated not only because they are frequently used additives in the packaging materials, but also because they are candidate molecules for derivatization in gas chromatography.

Detection of the separated components was simultaneously performed with FID and MS detector using post-column splitter valve, in accordance with PQRI's recommendations (PQRI Leachables and Extractables Working Group 2006) to reduce the chances of missing an extracted compound, due to detector type specificities.

Low-density polyethylene (LDPE) and polypropylene (PP) plastics were evaluated by generating a number of extracts in water buffers, methanol and hexane and analyzed using the proposed method. Both detectors showed equivalent responses (as a number of detected compounds), using their default parameters, with the methanol and hexane extracts showing highest numbers of detected components of all extracts.

Conclusion

Gas chromatography is extremely important technique for analysis of extractables and leachables, due to the fact that it enables efficient separation and detection of an enormous number of organic compounds commonly encountered in packaging materials. Coupling gas chromatography with MS detection further enhances its capabilities toward identification of the detected compounds, giving a clearer picture of the additives profile of the tested material.

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Macedonian pharmaceutical bulletin, **62** (suppl) 203 - 204 (2016)
ISSN 1409 - 8695
UDC: 615.214.074:543.544.5.068.7
Short communication

Development of fast simple RP-HPLC method with UV detection for determination of Pregabalin in solid pharmaceutical dosage forms

Marjan Piponski, Tanja Bakovska, Marina Namoska, Tatjana Rusevska*, Irena Slaveska Spirevska, Elena Lazarevska Todevska, Stefan Stefov, Gordana Trendovska Serafimovska

Replek Farm Ltd., Quality control Department, St. Kozle 188, 1000 Skopje, Republic of Macedonia

Introduction

Pregabalin is described chemically as (S)-3-(aminomethyl)-5-methylhexanoic acid and is also known as β-isobutyl-γ-aminobutyric acid (beta-isobutyl-GABA). It is pharmacologically similar to gabapentin. Pregabalin is approved for the management of neuropathic pain associated with diabetic peripheral neuropathy and postherpetic neuralgia and for adjunctive treatment of partial onset seizures.

This drug has a specific chemical structure, without presence of any specific UV-chromophore, like, for example, cyclic structure with double bonds, absorptive group or bond in the molecule, which complicates its detection with UV absorbing detectors.

Patil et al. (2015) have developed spectrophotometric method for determination of Pregabalin, based on condensation reaction of Pregabalin with p-dimethylaminobenzaldehyde in an acidic medium, leading to formation of a complex that shows maximum absorbtion at 395.80 nm.

There are also many HPLC methods with UV detection. Some of them require precolumn chemical derivatization of Pregabalin, using 1-fluoro-2,4-dinitrobenzene as derivatiozation agent (Ahmadkhaniha et al., 2014). However, their application for routine pharmaceutical quality control of drug products is difficult and inappropriate. Among the other common HPLC/UV methods developed for this aim, the following deficiencies can be met: low capacity peak (Kasawar and Farooqui, 2010), inappropriate UV absorbing mobile phase components having lowUV-cut off, such as ammonium acetate buffer (Balaji et al., 2014), highly asymmetric peaks (Ashu et al., 2011; Kannapan et al., 2010) and even not-acceptable declared sensitivity (Saeed et al., 2014).

The aim of this work was to overcome above mentioned obstacles, and to develop HPLC method for Pregabalin determination in solid pharmaceutical dosage forms, for routine analysis, in quality control laboratories, in pharmaceutical industry.

Materials and methods

All the chemicals used were Ph.Eur grade. Acetonitrile and sodium hydroxide were products of Merck KGaA, Darmstadt, Germany, whereas potassium dihydrogen phosphate and 37% hydrochloric acid was purchased from Sigma Aldrich, USA. The demineralized water was Stilman produced and had conductivity less than 1 µS. Pregabalin capsules and Pregabalin working standard were purchased from Replek Farm, Skopje, Macedonia.

Analyzes were conducted on HPLC system Varian Prostar with ternary high pressure mixing pump, autosampler 410 with column oven and DAD 330 detector controlled by Varian Star software version 6.21 and UPLC system Shimadzu Nexera with low pressure mixing quaternary pump, autosampler, column oven, controller and dual-channel UV-Vis detector, controlled by Lab Solutions software version 5.54.

Following HPLC columns were used:

- Hypersil C18 ODS 100 mm x 4 mm, 3 µm (Thermo scientific, USA);
- RP Select B 75 mm x 4 mm, 5 µm (Merck Darmstadt, Germany);

* tatjana.rusevska@replek.com.mk

- Purospher C18e STAR 125 mm x 4 mm, 5 µm (Merck Darmstadt, Germany);
- Discovery C18 125 mm x 4,6 mm, 5 µm (Supelco Bellefonte, USA).

The final proposed chromatographic conditions are: Discovery C18 chromatographic column, mobile phase consisted of 7% v/v acetonitrile and 93% v/v of 25 mM KH_2PO_4 (pH 7.0) with flow rate 1.0 mL/min, column temperature 30 °C and 20 µL injection volume.

Test solution was prepared by dissolving 1 capsule in 500 mL volumetric flack and diluted with 0.1 M hydrochloric acid to volume.

Results and discussion

The choice of HPLC column had strong impact on Pregabalin peak height, shape and capacity. Therefore, during method development four different HPLC columns were tested: 3 µm particle column Hypersil C18 ODS, RP Select B with 5 µm, Purospher C18e STAR with 5 µm and Discovery C18 with 5 µm particles. Best results were achieved using Discovery C18 column matrix, which was somewhat expected from particles having 180 nm wide pores, well base deactivated (metal cations purified) and end-capped of free silanol groups of silica chromatographic matrix. Purospher columns have highest purity (lowest quantity of metal cations) chromatographic matrix but the wide pore particles of Discovery yield higher and narrower peak of Pregabaline, which makes this column more appropriate for achieving good sensitivity of the method.

Increasing the injection volume was another way to improve sensitivity, accompanied by creation of deeper baseline disturbances even when highly UV transparent mobile phase composition was used. Similarity between sample solvent and mobile phase improved the chromatogram appearance, since the presence of water in sample always generates deep negative peaks when working at very low UV wavelength signal. It is essential to dissolve the sample in mobile phase in aim to avoid negative peaks on the whole picture of chromatogram. Additionally, the method sensitivity could be decreased if the sample is additionally diluted with water or mobile phase.

The use of UV-DAD detector enabled comparison of the increase of method sensitivity by lowering monitoring wavelength from 215 nm up to 200 nm. It was found out that signal monitoring wavelength at 210 nm was adequate for most types of pharmaceutical analysis of Pregabalin in solid pharmaceuticals, i.e. for assay and dissolution determination.

Pregabalin molecule has two pKa values, 4.2 and 10.6, corresponding to the carboxylic acid and the amine groups, respectively. Mobile phase buffer with pH = 7.0 was tested and yielded satisfying chromatohraphic peaks of the analyte with retention time ~ 2 - 5 minutes depending on the HPLC column characteristics and length and the exact ratio of the inorganic and organic part in the mobile phase composition.

The optimal chromatographic conditions were obtained using Discovery C18 chromatographic column, mobile phase consisted of 7% v/v acetonitrile and 93% v/v of 25 mM KH_2PO_4 (pH 7.0), and 20 µL injection volume.

Conclusion

The method was found to be simple, fast and cost-effective, thus suitable for high-throughput routine determination of Pregabalin in solid dosage forms in pharmaceutical quality control laboratories. The method could be further improved if needed for higher sensitivity and higher throughput of analysis per day.

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Macedonian pharmaceutical bulletin, **62** (suppl) 205 - 206 (2016)
ISSN 1409 - 8695
UDC: 615. 356:577.164.1].074
Short communication

Comparison of new developed UV/VIS-spectrophotometric and HPLC method with UV/VIS detection for determination of Vitamin B12 in various pharmaceutical dosage forms

Tanja Bakovska*, Marina Naumoska, Marjan Piponski, Emilija Janeva, Elena Petrovska, Elena Lazarevska Todevska, Hristina Andonoska, Tatjana Rusevska, Gordana Trendovska Serafimovska

Replek Farm Ltd., Quality control Department, St. Kozle 188, 1000 Skopje, Republic of Macedonia

Introduction

Vitamin B12, or Cyanocobalamin is a part of the group of B-complex vitamins. Its chemical name is 5,6-dimethyl-benzimidazoyl cyanocobamide. It belongs to a group of cobalt-containing compounds, known as corrinoids (Eitenmiller et al., 2007; Hurst, 2002; NCBI).

Vitamin B12 (cobalamin) functions as a coenzyme. It is involved in the red blood cells formation and nervous system functioning, in DNA synthesis and regulation, and also in fatty acid metabolism and amino acid metabolism. Vitamin B12 is synthesized only by bacteria and it is present only in food bacterially fermented or obtained from animals that has this cobalamin from their gastrointestinal microflora (Institute of Medicine (US) Standing Committee, 1998).

Microbiological method is one of the oldest methods for measuring the concentration of Vitamin B12. Although this technique has developed through time, still it has several drawbacks such as: relatively low specificity, low precision, it is time consuming and requires very well trained technicians.

Several authors have developed different HPLC methods for determination of Cyanocobalamin, alone, or in combination with other vitamins and/or active substances (Chen et al., 2010; Perveen et al., 2009; Radhika et al., 2012).

Cyanocobalamin is the commercial form of Vitamin B12 and specifications are found in the Codex for usage as food, and in the USP and BP for pharmaceutical use. The official Pharmacopoeia monographs for Vitamin B12 prescribe UV-spectrophotometric assay determination, as an analytical procedure for quantification of our analyte of interest, at 361 nm.

The aim of our work is to propose two different methods for routine analysis of the active compound whether it is consisted in dietary supplement or pharmaceutical dosage form, as an alternative to each other in quality control laboratories in pharmaceutical/chemical industry.

Materials and methods

All used chemicals were of Ph.Eur. quality and the demineralized water was Stilman produced and had conductivity less than 1 μS. Pottasium phosphate monobasic (KH_2PO_4) and 85% phosphoric acid were purchased from Sigma - Aldrich, USA, whereas acetonitrile was purchased from Merck KGaA, Darmstadt, Germany. Cyanocobalamin working standard, the pharmaceutical dosage forms and dietary supplements were purchased from Replek Farm, Skopje, Macedonia.

Spectrophotometric method includes "VARIAN Carry Win 50" UV-VIS-spectrophotometer, 1-cm quartz cell at wavelength range 190 - 750 nm, with resolution 0.5 nm and scan rate of 300 nm/min.

The HPLC determination was performed on Varian Pro Star HPLC system, with autosampler 410, Varian 325 dual-channel and Varian 335 photodiode-array detector, with ternary high pressure mixing pump, controlled by Galaxy software version 1.91.

The test and standard solutions for both proposed methods were prepared by dissolving suitable amount of

* tanja_bakovska@yahoo.com

the active substance or powdered film-coated tablets in demineralized water, as solvent, to concentration of 0.02 mg/ml for spectrophotometric determination and 0.1 mg/ml for HPLC determination.

Results and discussion

Cyanocobalamin is the most stable form of vitamin B12. It has optimum stability at pH range 4.0 - 4.5, and therefore the extraction is usually carried out in this range. Severe alkaline and acid conditions, ultraviolet (UV) or strong visible light, and oxidizing agents inactivate the vitamin (Eitenmiller et al., 2007; Hurst, 2002).

Cyanocobalamin is water-soluble vitamin, therefore water is a good extraction solvent. In UV and visible spectrophotometry, aqueous solutions of Cyanocobalamin exhibit maximums in UV and visible region at 278 nm, 361 nm, and 550 nm. The spectrophotometric method we propose prescribes signal measurement at wavelength of 550 nm of aqueous solutions with concentration ~0.02 mg/ml. Spectrophotometric method for determination of Cyanocobalamin has low cost and acceptable specificity in comparison with radio ligand assay. However, it is not suitable for complex samples, where the sensitivity is relatively low, so it is not used routinely for determination of Cyanocobalamin in combined pharmaceutical products.

According to available literature, Vitamin B12 is a weak base that stays approximately neutral at pH from about 5 to 10. The pKa values reported are 3.3 and 9.3 (Trang, 2013). Since it is preferred to avoid use of mobile phase with pH in proximity of the pKa values of analytes, if possible up to at least ± 1 pH unit, we chose to use mobile phase containing buffer with pH ~2.5 and organic constituent (acetonitrile) in variable amount, in order to achieve optimal retention of the analyte. The optimized chromatographic method uses mobile phase consisted of 13% v/v acetonitrile and 87% v/v (20 mM KH_2PO_4, buffer adjusted to pH = 2.5 with 85% phosphoric acid). The tests are performed on a LiChropher RP Select B chromatographic column (125 x 4 mm with 5 µm particles), with UV detection at 550 nm. Volume of injection used is 50 µl, with mobile phase flow rate of 1.5 ml/min.

Cyanocobalamin peak elutes with retention time ~5 minutes, with symmetry expressed as AsUSP ~0.9.

Almost the same results were obtained when the two developed methods were used in analysis of tablets containing Cyanocobalamin. The absolute difference between values for Cyanocobalamin assay obtained using the proposed UV-spectrophotometric and HPLC method was insignificant (far lower than 2%), which indicates that both can be used in quality control laboratories for Cyanocobalamin determination.

Conclusion

The two developed methods are suitable for routine analysis of the active compound Cyanocobalamin in dietary supplements or pharmaceutical dosage forms, and can be used as an alternative to each other in quality control laboratories in pharmaceutical/chemical industry.

Both methods are simple, fast, cost-effective, sensitive, accurate, reproducible and rugged. We suggest use of the spectrophotometric method as a routine for industrial application and analysis of Vitamin B12 in more simple matrixes. The chromatographic method is more selective and can be used either in routine analyses or in more complex analyses and can be further optimized for purpose of analysis of various multivitamin dosage forms.

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Macedonian pharmaceutical bulletin, **62** (suppl) 207 - 208 (2016)
ISSN 1409 - 8695
UDC: 615.015.4.074
Short communication

Analytical approach in development of a new drug product formulation

Aleksandra Petrovska*, Marija Veliia Veli, Veronika P. Jakimovska, Sonja Ugarkovic

Institute for Research and Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

The objective of this study was to develop a new drug product formulation with amino acid as active substance. The direct detection by ultraviolet absorbance of amino acids is possible only at low wavelengths, where sensitivity is poor and interference in background absorption is potentially high (Petritis et al., 1999). There are two reported spectrophotometric methods with UV detection at 210 nm of this active substance in pharmaceutical preparation (Onal and Sagirli, 2009). Several derivatization methods have been proposed, by using derivatizing reagents such as potassium iodide for spectrophotometric detection (Gujral et al., 2009), fluorescamine, 2,4-nitrofluorobenzen and 2,3,5,6-tetrachloro-1,4-benzoquinon (Shaalan, 2010) for spectrofluorometric detection. The HPLC methods with precolumn (Vermeij and Edelbroek, 2004) and post column derivatization of this active substance with o-phthaldialdehyde (Douou et al., 2010) and derivatization with fluorescamine (Martinc et al., 2010) were also reported. All these methods with derivatization and fluorescence detection cannot be applied for determination of related and degradation products, because the main degradation product produced by cyclization between amino and carboxylic acid group has lactam ring in the structure, which is a strong chromophore group. The ultraviolet absorption method is suitable for its detection (Dee-Noor et al., 2009).

To establish degradation pathways of the active substance, its stability in a combination with excipients in final formulation mixture and detection of interactions between the drug and excipients, all in order to develop the most stable drug product, forced degradation study was performed by applying different stress conditions (Baertschi et al., 2005).

Materials and methods

Testing formulation I was consisted of amino acid as active substance and excipients in a ratio of 1:3 (% w/w), respectively, with filler 1, which was excipient without carbonyl group in the chemical structure.

Testing formulation II was consisted of the same filler 1, but active substance and excipients were in a ratio of 3:1 (% w/w), respectively.

Testing formulation III was consisted of amino acid as active substance and excipients in a ratio of 1:3 (% w/w), respectively, but with filler 2, which is reducing sugar with carbonyl group in the chemical structure.

The following forced degradation study was applied:

a) Thermal degradation: the samples were kept in drying oven at 80 °C for 20 days;

b) Thermal/humidity degradation: the opened and capsulated samples sprayed in thin layer on Petri of 1:3 (% w/w),ept in pharmaceutical stability chamber at 40 °C and 75% RH for 90 days;

c) Photo degradation: the samples were kept in photo stability chamber under maximum radiation of the xenon lamp (765Wh/m2) for 35 hours;

d) Acid hydrolysis: the samples were treated with 0.5M hydrochloric acid at 50 °C for 2 hours;

e) Base hydrolysis: the samples were treated with 0.5M sodium hydroxide at 50 °C for 2 hours;

f) Oxidative degradation: the samples were treated with 1% (v/v) hydrogen peroxide at room temperature for 1 hour.

All samples, control and treated, were analyzed by the in-house developed HPLC method for determination of related and degradation products in the drug product.

* apetrovska@alkaloid.com.mk

Results and discussion

Considering all of the reported data for the Maillard reaction between carbonyl group from reducing sugar and amino group (Qiu et al., 2005) the main goal was to find the adequate filler in the development of the drug product formulation containing amino acid as active substance. The first differences caused by the type of excipient used as filler and the second differences caused by the content of excipients in the formulation, have been fully identified and understood.

The active substance was unstable under elevated temperature, acid and base hydrolysis. No degradation of excipients was found under all applied stress conditions. It has been observed different modes of degradation according to the type of formulation mixture. Under thermal degradation the main degradation product, lactam, was formed in the same amounts, regardless of the type of filler and the content of the excipients in the formulation. Further, in formulation III one unspecified impurity with RRT 2.37 was slightly increased, which is the product of the Maillard reaction between amino acid and reducing sugar used as filler 2. Under thermal and humidity degradation, many differences between the formulations were observed because the samples were treated in two different ways: as opened capsule masses sprayed in thin layer on Petri's dishes and as capsulated samples. Two degradation pathways, cyclization to lactam and the interaction between amino and carbonyl group were enhanced due to several factors for instance, the higher content of the excipients in terms of the active component, the hygroscopic characteristics of an another filler, filler 3, used in amount of 33% w/w and the capsule shell, especially in the formulation I and III.

As was expected, the acid hydrolysis causes the reaction of cyclization of amino acid to lactam (Dee-Noor et al., 2009), where the most susceptible formulations in our study were formulation I and III.

After the base hydrolysis, lactam was increased in greater, but in the same amounts in all samples. Besides lactam, formulation I and III with the higher content of the excipients were shown as much unstable, degraded to a few unspecified impurities, where total impurities of 10.56% and 12.01%, respectively, were obtained. The effect of photo degradation was insignificant. Under oxidation, the content of the active substance in all samples was not decreased. All samples treated with mild oxidative conditions, 1% H_2O_2, room temperature for 1 hour, degraded to several products with higher ultraviolet absorption than active substance, due to the new formed chromophore groups in the structure.

Conclusion

In this study, it has been shown that the amino acid as active substance in the presence of reducing sugar used as filler in the drug product formulation undergoes a Maillard reaction over time, especially enhanced by increased temperature and humidity. In order to produce the most stable formulation during the manufacturing process and during the proposed shelf-life of the drug product, the proposed filler without carbonyl group in the chemical structure was proved as a suitable excipient, avoiding the drug-excipient interaction, and further, lower content of excipients in terms of the active component in the drug product formulation, has been suggested.

Acknowledgment

We are grateful to Prof. Dr. Aneta Dimitrovska from Faculty of Pharmacy, Cyril and Methodius University, for valuable discussion and support of this research.

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Macedonian pharmaceutical bulletin, **62** (suppl) 209 - 210 (2016)
ISSN 1409 - 8695
UDC: 615.12:614.35
Short communication

Strengthening the position of OMCLs

Jelena Acevska[1]*, Katerina Brezovska[1], Liljana Ugrinova[1], Suzana Trajkovic Jolevska[1], Aneta Dimitrovska[1], Richard Wanko[2], Kevin O'Donnell[3]

[1] *Center for Drug Quality Control, Faculty of Pharmacy, University Ss.Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2] *Biological Standardisation, OMCL Network & HealthCare Department (DBO), EDQM - Council of Europe, Strasbourg France*
[3] *Health Products Regulatory Authority, Dublin, Ireland*

Background

"An Official Medicines Control Laboratory (OMCL) is a public institution, which performs laboratory testing of medicinal products on behalf of National Competent Authority (NCA) and in fulfilment of other national obligations, independently from the manufacturer or Marketing Authorisation Holders (MAH). Testing is carried out in the interest of official market surveillance of medicinal products in relation to the safety of human patient and/or animals, prior to and/or after marketing of the respective medicines and is demonstrated to be free from conflicts of interest" (Directive 2001/83/EC, Directive 2001/82/EC, PA/PH/OMCL (07) 89 14R, 2015).

The role of the OMCLs is focussed on the independent quality control of medicines and it includes a number of different pre and post-marketing surveillance programmes. These extend to the sampling and testing of branded and generic medicines, unlicensed (unauthorized) medicines, illegal medicines (counterfeits / medicines with falsified labelling), vaccines, blood and plasma-derived medicinal products, and active substances. OMCL surveillance work also includes examinations of product packaging and labelling. It supports the evaluation of the quality part of MA files, as well as pharmacovigilance assessments, quality defect investigations, and GMP inspections. In the framework of the European Pharmacopoeia, the work of OMCLs plays a vital part, in relation for example to monograph development activities, etc. Beside medicines, a number of OMCLs also perform laboratory testing on cosmetics, medical devices, diagnostics, food, feed (premixes) for veterinary use, and other "borderline products" (PA/PH/OMCL (07) 89 14R, 2015).

Public and stakeholder awareness of the importance of OMCL work

The public and animal health benefits of having a well-established and resourced system of quality control surveillance of medicines placed on the market are numerous - such work helps to independently assure the quality of the medicines on the market, and it results in regulatory actions when sub-standard or illegal medicines are found in the market. However, the benefits of this work are not always apparent to the various stakeholders, especially in relation to the routine surveillance day-to-day testing work that is carried out. This lack of awareness can lead to questions among stakeholders about the usefulness of the work of OMCLs, and about the level of resources and funding that OMCLs require.

Furthermore, it may not be evident to the public how a reduction in the funding of an OMCL (or the downsizing of an OMCL) may affect public and animal health protection (PA/PH/OMCL (14) 123 R, 2015).

Therefore, a common need for raising the national and wider awareness of the benefits of the OMCL and the GEON was acknowledged.

Public relation efforts

Recently, GEON began renewed efforts to strengthen its stakeholder-interaction strategies and its communication activities so as to more effectively disseminate in-

* jelena_petrusevska@ff.ukim.edu.mk

formation on the benefits of having a strong OMCL Network in place for the independent surveillance of the quality of medicines.

Despite some earlier successes of GEON public relation (PR) efforts towards the general public as well as in the relation with supranational bodies (PA/PH/OMCL (14) 123 R, 2015), it was envisaged that a new communications strategy is required for the GEON. There are various elements to this work, including the need to identify what key messages about OMCLs and the GEON need to be made and to what stakeholders. While the general public if of course a very important stakeholder, it is unclear at the present time exactly what PR activities should be directed towards the general public. It may be more beneficial to focus communication strategies on those stakeholders directly involved in regulatory, legal and funding activities.

Best communication practices

If a country is dedicated to strengthening Quality Management (QM) & Quality Control (QC) systems, then each measure for developing the capacity of the national OMCLs within national strategic programmes should be considered. As the OMCLs are usually part of bigger organizations, special efforts should be made to address the stakeholders that provide the OMCL directly with resources.

This can be done by means of presenting data underpinning the performance of OMCLs e.g. by highlighting particularities and/or added values. Each success story should be released to the public, so higher awareness amongst the concerned stakeholders is achieved.

Each opportunity for joint national, bilateral and international projects that facilitate recognition of the importance of OMCLs and involvement in critical issues of the health care system should be utilized.

Social network platforms should be populated with information on regular OMCL activities and lessons learned on health hazardous issues, especially those affecting patients directly (i.e. in case when medicine, medical devices or 'food supplements' are purchased from untrusted sources, such as internet or teleshops).

The best communication practices from each individual OMCL could help developing a common 'tool kit' on international level, which could in revert help OMCLs to better reposition themselves within their national competent authorities for the benefits of all. In this context the European Directorate for the Quality of Medicines & HealthCare (EDQM), under the auspice of Council of Europe, has a central role in the process of strengthening the role of OMCLs and its Network, as it compiles the best communication practices from the GEON, and identifies key points to elaborate European wide strategy. Target partners in this respect would be: Co-ordination Group for Mutual Recognition and Decentralised procedures - Human and veterinary (CMDh and CMDv) within Head of the Medicine Agencies (HMAs), Committee for Medicinal Products for Human and Veterinary Use (CHMP and CMVP) within European Medicine agency (EMA),etc.

Perspective

The authors wish to highlight the importance of the work done by OMCLs within the national and international health systems, but also to point out that the role of OMCL is very often underestimated which as a consequence might result in negative impact on the global patient safety in particular regarding the combat against substandard and falsified medicines. Additionally, the authors acknowledge that other types of laboratories with comparable scope of activities are experiencing similar pressure with respect to cost/resource cuttings. The aim of this paper is to motivate each OMCL and other quality control laboratories to consider activities which could help raising public awareness, and in particular the awareness of stakeholders which decide about and provide resources to these laboratories. The importance of investments to develop OMCL capacities in order to meet new analytical challenges against substandard and falsified medicines must become part of national strategies, as it would pronounce a benefit for the health system in a cost/effective way.

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Macedonian pharmaceutical bulletin, **62** (suppl) 211 - 212 (2016)
ISSN 1409 - 8695
UDC: 615.015.4:519.87
Short communication

Mathematical modeling of drug dissolution from prolonged-release drug product

Blagica Manchevska*, Packa Antovska, Irena Brashnarska, Sonja Ugarkovic

Research and Development Institute, ALKALOID AD,
Blv Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

The process of dissolution is of fundamental importance for the bioavailability and therapeutic efficacy of drug products. The process of drug release in an aqueous body fluid includes different physical phenomena as wetting of the particle's surface, breakdown of solid state bonds, solvation, diffusion as well as convection in the surrounding bulk fluid. Drug release rate from modified release drug products can be controlled by diffusion, degradation, swelling followed by diffusion and dissolution. Different mathematical models may be used as an instrumental tool for understanding the drug release form different drug delivery systems (Siepmann and Siepmann, 2013). Some of the models were used to explore and explain the release mechanisms of prolonged-release drug product.

Materials and methods

Dissolution tests of prolonged-release tablets with BCS Class I have been performed, using basket apparatus at 100 rpm, in 500 mL phosphate buffer with pH 6.8 at 37±0.5 °C for 24 hours. Dissolution profiles were obtained by sampling 10 mL at 8 time points (1, 2, 4, 6, 8, 10, 12, 24 hours). Quantification of the dissolved drug is performed with HPLC method with UV detection at 242 nm, mobile phase is solution of sodium edetate and acetic acid, methanol and acetonitrile (60:20:20 v/v/v), pump flow 2.0 mL/min, column temperature 40 °C and injection volume 100 µL. Mathematical modeling of the dissolution data was performed with DDSolver (Zhang et al., 2010). Three formulations with different rate-controlling excipients were evaluated. A number of mathematical models have been assessed for evaluation of drug release data: zero order, first-order, Higuchi, Korsmeyer-Peppas, Hixon-Crowell, Hopfenberg, Peppas-Sahlin, Weibull and Gompertz.

Results and discussion

The average values (in percentage) of the dissolution profile of formulation 1 are: 7.78, 14.69, 26.87, 38.02, 48.22, 56.82, 64.87 and 97.71, formulation 2: 4.55, 8.71, 17.5, 27.88, 38.23, 50.64, 60.69 and 93.9 and formulation 3: 4.36, 8.84, 18.46, 26.94, 35.55, 45.12, 54.38 and 88.85.

Concerning the model selection criteria, DDSolver provides a number of statistical criteria for evaluating the goodness of fit of a model, among which the most popular are adjusted coefficient of determination ($R^2_{adjusted}$), Akaike Information Criterion (AIC), and Model Selection Criterion (MSC) (Zhang et al., 2010).

The Adjusted Coefficient of Determination is an adjustment for the Coefficient of Determination that takes into account the number of variables in a data set.

AIC is a measure of the relative quality of statistical models for a given set of data. Given a collection of models for the data, AIC estimates the quality of each model, relative to each of the other models. When comparing models with different numbers of parameters, the model with a lower AIC value can be considered to be the better model.

MSC is a modified reciprocal form of the AIC and is independent of the scaling of the data points. When comparing different models, the most appropriate model will be that with the largest MSC.

Zero order kinetics describes the systems where the drug release rate is independent of its concentration and can be used to describe the dissolution of several types of modified release dosage forms and dissolution from dosage forms that do not disaggregate and release the drug

* bmanchevska@alkaloid.com.mk

slowly (assuming that area does not change and no equilibrium conditions are obtained) (Costa and Sousa Lobo, 2001). $R^2_{adjusted}$ is 0.91-0.98, AIC 46.85-61.05 and MSC 1.95-3.40.

Dosage forms that follow first order dissolution profile, such as those containing water-soluble drugs in porous matrices, release the drug in a way that is proportional to the amount of drug remaining in its interior, in such way, that the amount of drug released by unit of time diminishes (Costa and Sousa Lobo, 2001). $R^2_{adjusted}$ is 0.96-0.98, AIC 45.29-53.53 and MSC 2.82-3.70.

Higuchi describes drug release as a diffusion process based in the Fick's law, square root time dependent and can describe drug dissolution from several types of modified release dosage forms, such as some transdermal systems and matrix tablets with water soluble drugs (Costa and Sousa Lobo, 2001). $R^2_{adjusted}$ is 0.87-0.94, AIC 56.84-63.97 and MSC 1.66-2.41.

Korsmeyer-Peppas is exponential equation often used to describe the drug release from polymeric systems, when the release mechanism is not well known or when more than one type of release phenomena could be involved (Costa and Sousa Lobo, 2001). The n-value from the Korsmeyer-Peppas model indicates (0.70-0.85) anomalous diffusion or nonfickian diffusion which refers to combination of both diffusion and erosion controlled rate release (Peppas 1985). $R^2_{adjusted}$ is 0.98-0.99, AIC 35.76-46.04 and MSC 3.65-4.63.

Hixon-Crowell model describes the release from systems with a change in surface area and diameter of particles or tablets. When this model is used, it is assumed that the release rate is limited by the drug particles dissolution rate and not by the diffusion that might occur through the polymeric matrix (Costa and Sousa Lobo, 2001). $R^2_{adjusted}$ is 0.98-1.00, AIC 28.81-46.03 and MSC 3.66-5.53.

The release of drugs from surface-eroding devices with several geometries was analyzed by Hopfenberg who developed a general mathematical equation describing drug release from slabs, spheres and infinite cylinders displaying heterogeneous erosion (Costa and Sousa Lobo, 2001). $R^2_{adjusted}$ is 0.99-1.00, AIC 19.69-37.60 and MSC 4.59-6.54.

Peppas-Sahlin model (Zhang et al., 2010) demonstrates the following goodness of fit: $R^2_{adjusted}$ 0.99-1.00, AIC 26.09-42.81 and MSC 4.01-5.83.

Weibull model demonstrates the following goodness of fit: $R^2_{adjusted}$ around 1.00, AIC -8.91 to 21.67 and MSC 6.36-9.72. This is an empiric model, not deducted from any kinetic fundament, therefore, could only describe, but does not adequately characterize, the dissolution kinetic properties of the drug. This model is useful for characterization of release profiles of matrix type drug delivery (Costa and Sousa Lobo, 2001).

The Gompertz model (Costa and Sousa Lobo, 2001) is suitable for characterization of release profiles of drugs having good solubility and intermediate release rates. $R^2_{adjusted}$ is 0.99-1.00, AIC 16.83-37.30 and MSC 4.63-6.86.

Conclusion

Mathematical models are generally designed to develop new drug delivery systems based on the release characteristics, to predict the effect of design parameters on the resulting drug release rate, to optimize the release kinetics, to elucidate the underlying mass transport mechanisms, to accurately predict the drug release profile and improve the overall therapeutic efficacy and safety of these drugs.

The mathematical models with better goodness of fit are Weibull, Gompertz, Hopfenberg, Peppas-Sahlin, Hixon-Crowell and Korsmeyer-Peppas and they characterize the dissolution kinetic and help to better understand the underlying drug release mechanisms. Also the amount of drug release percent by each mechanism at each time can be also calculated.

The drug release profiles from all formulations can be best expressed by Weibull's equation, as the plots showed highest goodness of fit. This is an empiric model that can describe the dissolution kinetic properties.

The results from the mathematical modeling indicate that more than one type of release phenomena is involved. Hopfenberg model indicates that erosion is one of the release mechanisms. Korsmeyer-Peppas model indicates that there is combination of both diffusion and erosion controlled rate release. Hixon-Crowell model includes dissolution as one of the release mechanisms. When the prolonged-release tablets come in contact with the dissolution medium, they take up water and swell, forming a gel layer around the matrix and simultaneously erosion occurs.

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Macedonian pharmaceutical bulletin, **62** (suppl) 213 - 214 (2016)
ISSN 1409 - 8695
UDC: 615.277.074:543.544.5.068.7
Short communication

Validation of RP-HPLC method for determination of exemestane and its impurities in pharmaceutical dosage forms

Branka Ivković*, Aleksandra Jonić, Jelena Žunić, Sote Vladimirov, Milkica Crevar Sakač, Zorica Vujić

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, Belgrade, Serbia

Introduction

The nonsteroidal aromatase inhibitors are competitive inhibitors that bind to the enzyme active site by coordinating the iron atom present in the heme group of the P450 protein. Exemestane is 6-methylenandrosta-1,4-diene-3,17-dione a newer steroidal aromatase inhibitor. It is an enzyme activated irreversible inhibitor of aromatase. It is orally available and highly selective for aromatase. Its structure reflects only minor structural modifications to the natural substrate, androstenedione. Plasma estrogen levels are reduced by 85% to 95% within 2 to 3 days, and effects last 4 to 5 days. Exemestane does not inhibit any of the major cytochromes P450 and has essentially no interaction with steroid receptors, with only a very weak affinity for the AR. The 17-hydroxyexemestane reduction product, however, has much higher affinity for the AR than the parent (still several fold less than DHT, 0.28% for parent vs. 30% for metabolite). The clinical significance of the affinity is likely minimal because of the low levels of the metabolite produced (Pagani et al., 2014).

Literature survey reports that Exemestane can be determined by different analytical techniques such as HPTLC (Mane et al., 2010), UV-spectrophotometry (Angalaparameswari et al., 2012), GC-MS (de Albuquerque Cavalcanti et al., 2011), LC-MS (Allievi et al., 1995), HPLC (Konda et al., 2011; Ksycinska et al., 2011) and UPLC (Reddy et al., 2011) in pharmaceutical dosage forms. In these described methods C18 (modified silica gel with a hydrocarbon chain of 18 carbon atoms) stationary phases were used. In our work C12 stationary phase was used. Using C12 stationary phase we have developed a method for the quantification of exemestan and its impurities in the tablets. The developed method was validated as per ICH guidelines with respect to specificity, linearity, LOD, LOQ, accuracy, precision and robustness.

Materials and methods

The experiments were performed on the chromatographic system Agilent HP1200 with DAD detector. The analytical columns used in this study were in examining of chromatographic conditions were used: reference standards exemestane, impurities B and C, the chromatographic column Synergi 4µm MAX-RP 80A 150 x 4.60 mm (C12). The optimum conditions for separation and determination of exemestane and its impurities were achieved with the mobile phase: water /acetonitrile in the ratio 50/50 (v/v), at a flow rate of 1.2 mL / min and the column temperature was maintained at 30 °C. Stocks of standard solutions were prepared in the methanol and storage in the fridge. To evaluate the linearity of the developed method, five solutions of exemestan and its impurities were prepared in the concentration range from the 50 µg/mL to 150 µg/mL, 0.49 µg/mL to 49 µg/mL and 0.54 µg/mL to 54 µg/mL for exemestan, imp. B and imp. C. The accuracy of the method was confirmed by preparing three series of solutions containing appropriate placebo, exemestan and impurities in three levels: low level 80%, medium level 100% and high level 120%. To prove the precision of the method, three different solu-

* blucic@pharmacy.bg.ac.rs

tions of exemestan and impurities were prepared. The values for LOD and LOQ were obtained by calculating the signal / noise ratio. Sample solution was prepared in the mobile phase in the final concentration 75 μg/mL.

Results and discussion

The proposed method was validated as per ICH guideline. The system suitability was evaluated on the basis of resolution between exemestan and impurities (Rs >1.5). In the specificity study chromatograms of placebo had shown no peaks at the retention time of exemestan and impurities. The precision of method was verified by repeatability and intermediate precision at three concentrations levels (1-3 μg/mL for impurities B and C, 80-120 μg/mL for exemestan). The RSD (%) of results for exemestan and impurities were calculated (RSD for exemestan is 0.06% to 1.41%, for impurity B 0.41% to 1.41% and impurity C 0.56% to 1.52% RSD < 2%). The intermediate precision of the method was also evaluated using different analyst and different instrument and performing the analysis on different days (RSD < 3% for both impurities and exemestan). The LOD and LOQ for impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations (LOD is 0,049 μg/mL and LOQ 0,12 μg/mL for both impurities). The method was linear over the range of concentrations: for impurities B 0.49 μg /ml to 4.90 μg /ml (r =0.999), impurity C of 0.54 μg /ml to 5.40 μg / ml (r = 0.999) and for exemestane 50.90 μg /mL to 152.70 μg / mL (r =0.999) In the based on the obtained results, the identification and quantification of impurities in the investigated exemestane tablets examined the impurities C is present in quantities of less than 0.10%, while the amount of B < LOQ. On the basis of validated methods to determine the content of exemestane in the exemestane tablets of 95.68%.

Conclusion

It can be concluded that the defined RP-HPLC method is rapid and efficient for purity testing of exemestane in the raw material and pharmaceuticals dosage forms.

Acknowledgements

We gratefully acknowledge the financial support from the Ministry of Science and Education of the Republic of Serbia, grants OI 172041.

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Macedonian pharmaceutical bulletin, **62** (suppl) 215 - 216 (2016)
ISSN 1409 - 8695
UDC: 615.015.4
Short communication

Dissolution method development for generic drug products

Marija Petrovska*, Ivana Mitrevska, Tina Achkoska, Irena Brashnarska, Packa Antovska, Dejan Kuneski, Sonja Ugarkovic

Research & Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

The rate and extent in which the amount of drug substance dissolves over a period of time is called dissolution. Dissolution testing has emerged in the pharmaceutical field as a very important tool to characterize drug product performance. Dissolution test is required to study the drug release from the dosage form and its in vivo performance. Dissolution test is used to assess the lot to lot quality of drug product. Before selecting the proper dissolution medium should be determined physical and chemical characteristics of the active substance (Ph. Eur., 2015). Dissolution test procedures can be a challenging process, on multiple fronts. The aim of the study was to develop a dissolution test for the quality control for new generic immediate-release drug product with one active substance. Sink conditions, drug solubility in different dissolution media, different filter type, different volume of the dissolution medium and different apparatus were tested.

Materials and methods

The dissolution procedure has several distinct components. These components include a dissolution medium, an apparatus, the study design (including acceptance criteria) and the mode of assay. All of these components must be properly chosen and developed to provide a method that is reproducible for within laboratory day-to-day operation and robust enough to enable transfer to another laboratory.

Selection of dissolution medium

When selecting the dissolution medium, physical and chemical data for the drug substance and drug product need to be considered, e.g. the solubility and solution state stability of the drug as a function of pH value. The solubility of the active substance is pH dependent and the increase of pH would enhance its solubility and consequently the dissolution rate, particularly at pH > pKa of the compound.

Sink conditions have been determined by in house method. Generally, when developing dissolution method, one of the goals is to obtain sink conditions. According to the European Pharmacopoeia (Ph. Eur., 2015), "sink conditions" normally occur in a volume of dissolution medium that is at least 3 to 10 times the saturation volume, therefore sink conditions are maintained throughout the duration of the test film-coated tablets in the specified medium, phosphate buffer at pH 7.2. The dissolution media temperature is fixed i. e. 37.0 (±0.5) °C (Ph. Eur., 2015).

Media deaeration is usually required, and can be accomplished by heating the medium or (more commonly) filtering the medium or placing it under vacuum for short period of time. Bubbles can cause particles to cling to the apparatus and vessel walls. On the other hand, bubbles on the dosage units may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate (USP, 2015).

Selection of dissolution apparatus/ agitation

The choice of apparatus is based on the dosage form performance in the in vitro test system. For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. For immediate-release and capsule (both soft gel and hard gel) formulation , USP-I i.e. basket apparatus at 100 rpm or USP-II i.e. paddle apparatus at 50 or 75 rpm are recommended. Coning in dosage form can be reduced by increasing the paddle speed, thus reducing the artifact and improving the data (USP, 2015).

Apparatus II (paddle) rotating at 75 rpm was selected as most appropriate. Different paddle speeds (50 rpm and 75 rpm) and different Apparatus (Apparatus 1 and Appara-

* mpetrovska@alkaloid.com.mk

tus 2) were tested. On 50 rpm conning was noticed which leads to incomplete release of active ingredient from tablets and higher variability in dissolution rates.

Results and discussion

Dissolution is evaluated by measuring rate release profile or the amount dissolved over time. For immediate-release dosage forms, dissolution time from 30 min to 60 min is recommended (British Pharmacopoeia, 2015). In our experimental case multiple time points were performed for establishing the dissolution time. Observations are especially useful during method development and optimization. USP chapter <1092>contains additional information on observations (USP, 2015).

Sampling probe can affect the hydrodynamics of the system and so that can change the dissolution rate. Regarding the position of sampling, the probe should be sampled at approximately half the distance from the basket or paddle to the dissolution medium and not closer than 1 cm to the side of the flask. Adsorption of the active substance onto filter needs to be evaluated. Filter material must be saturated with the active substance by repeated passage to avoid losses that might go undetected during the test sampling. Accumulation of the particulate matter on the surface may cause significant error in the dissolution testing.

Sample solutions were prepared in proposed dissolution medium. The sample solutions were prepared using film-coated tablets into 900 mL dissolution medium at 37.0 ± 0.5 °C and with at 75 rpm for 45 minutes. Different aliquots (0.3 and 6 mL) were filtered using different filter types (0.45-µm), diluted suitably and analyzed spectrophotometrically. A filter type is found to be acceptable for use if the results of the filtered portions are within 98-102% of the original concentrations of the unfiltered standard solution and the centrifuged sample solution.

Acceptance criteria must also be considered during test development. Acceptance criteria are derived in the form of "Q-factors" a minimum amount dissolved at a given time as a percentage of the labeled content (ICH, 1999).

Assaying the results

There are two common ways of analyzing dissolution test samples, spectrophotometric (UV) determinations and HPLC. The selection of the optimal wavelength for analysis was based on the wavelength of maximum absorption in the UV spectrum of the active substance. Cells with path lengths ranging from 0.02 to 1 cm were used.

Optimization of Dissolution Test

The dissolution experiments were conducted in a six-station bath dissolution apparatus by subjecting six immediate-release tablets to 900 mL of proposed dissolution medium, paddle dissolution apparatus, and stirring speeds of 75 rpm. The temperature was maintained at 37 ±0.5 °C. Aliquots of 10 mL were withdrawn manually at 5, 10, 15, 20, 30, and 45 min, and replaced by the same volume of the medium heated at 37 ±0.5 °C. The sample was filtered using regenerated cellulose (RC), 0.45-µm filter and analyzed spectrophotometrically at 221 nm. Acceptance criteria for not less than 85% (Q+5%) of the labeled content for 45 min was set.

Discriminatory properties of the dissolution method

Finally, the dissolution test procedure should be discriminating enough to be capable of distinguishing significant changes in a composition or manufacturing process that might be expected to affect in vivo performance. In general, a properly designed dissolution test should result in reproducible data.

The proposed dissolution methodology has shown to be suitable for quality control of tablets manufactured using the commercial process and is capable of discriminating changes in the critical manufacturing process steps and formulation composition. To elucidate the discriminatory power of the dissolution method, a formulation containing different quantity of the active ingredient than the final tested product, was analyzed using the proposed dissolution method and the dissolution profiles of both formulations were compared.

Conclusion

The obtained results indicate that pH 7.2 as a dissolution medium of choice, together with the selected dissolution method parameters, are reflective of the product characteristics and are able to distinguish differences in formulation of the product.

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Macedonian pharmaceutical bulletin, **62** (suppl) 217 - 218 (2016)
ISSN 1409 - 8695
UDC: 615.233.074:543.544.3
Short communication

Validation of GC method for determination of ethanol, methanol, toluene and benzene as residual solvents in pholcodine monohydrate drug substance

Olivera Blaževska*, Vlado Petruševski, Ana Petkovska, Monika Stojanovska, Gjorgji Petruševski, Irena Brašnarska, Biljana Šapkareva, Sonja Ugarković

Institute for Research and Development, Alkaloid AD – Skopje, bul. Aleksandar Makedonski br. 12, 1000 Skopje, Macedonia

Introduction

During extraction and synthesis of pholcodine monohydrate, organic solvents are used. For that purpose a HS-GC-FID method for determination of ethanol, methanol, toluene and benzene in pholcodine monohydrate has been developed and validated. Even though benzene is not used during any of the manufacturing stages, it is a regulatory requirement to monitor for its eventual presence in the product, since benzene is a known degradation product of toluene. Furthermore, benzene is the most toxic among these solvents (Class 1) and it is recommended to be avoided in the production of drug substances, in accordance to ICH Q3C (R5) (ICH, 2011). Methanol and toluene show severe toxicity (Class 2), hence their usage should be limited, and ethanol is the least toxic of them (Class 3) (Ph.Eur., 2014). As residual solvents are highly volatile compounds, HS-GC-FID is the most convenient technique for their determination and quantitation (Ph.Eur., 2014).

This study describes the development and validation of method for routine analysis of four residual solvents (ethanol, methanol, toluene and benzene) in pholcodine monohydrate drug substance.

Materials and methods

Ethanol, methanol, N,N-dimethylsulfoxide (DMSO) and N,N-dimethylformamide (DMF) - GC grade were obtained from Merck Millipore (Darmstadt, Germany), toluene and benzene-from Sigma Aldrich (Sent Louis, USA) and pholcodine monohydrate API was obtained from Alkaloid AD Skopje, Macedonia.

The method was developed, optimized and validated on a Shimadzu 2010 Plus gas chromatograph, equipped with FID detector and PAL AOC-5000 autosampler for headspace and liquid samples (Shimadzu Corporation, Kyoto, Japan), with helium as a carrier gas in the split mode. The separation of the components was performed using Agilent DB-624 (30 m x 0.53 mm x 3 μm) WCOT capillary column.

Results and discussion

A standard stock solution in N,N-Dimethylformamide containing the investigated residual solvents in API pholcodine monohydrate was prepared in such a way that the final concentrations of the solvents were approximately 2500 ppm for ethanol, 1500 ppm for methanol, 440 ppm for toluene and 1 ppm for benzene (concentration level 100%). The sample solution was prepared by weighing approximately 500 mg of pholcodine monohydrate in a 20 ml headspace vial, followed by addition of 1 mL N,N-Dimethylformamide as diluent.

Chromatographic method development started with initial gradient conditions: 0 to 10 minutes isocratic segment at 45 °C, then linear temperature gradient of 10 °C/min to 220 °C and finally and isocratic segment at 220 °C for 2.5 minutes, for total chromatographic runtime of 30 minutes. These conditions provided complete separation of the four tested solvents and the diluent, with adequate column performance and good peak shape for all analytes, howev-

* oblazeska@alkaloid.com.mk

er with unnecessarily long run time. Judging by this conclusion, chromatographic conditions was performed by increasing the initial column temperature to 50 °C, shortening the initial isocratic segment to 3 minutes and selecting more aggressive temperature gradient of 20 °C/min.

The method was validated within the ICH guidelines. Parameters included in the validation procedure were: specificity, linearity and range, accuracy, repeatability and intermediate precision, limit of detection, limit of quantitation and robustness.

In order to determine the specificity of the optimized method, a blank solution (DMF), sample solution and individual standard solutions of the investigated residual solvents, as well as their mixture, were injected onto the GC column. DB-624 column (30 m length x 0.53 mm i.d. x 3 µm film thickness) provided sufficient baseline separation of all residual solvents, including the diluent, fulfilling the criterion for the resolution between ethanol and methanol peaks being not less than 4. The retention time for residual solvents individually and in spiked standard solution was determined. There was no co-elution between the observable peaks.

Linearity evaluation was prepared by quantitative dilutions of the stock solutions of ethanol, methanol and toluene in order to obtain solutions in the range from quantification limit to 150% of working concentration (Lokhannde et al., 2012). The average value for the area under the peak obtained from the three injections for each solvent was plotted against the corresponding concentration in ppm, hence calibration curves were obtained. The correlation coefficients were above 0.990.

The accuracy of the analytical method was confirmed by evaluation of the recovery. The recovery test was carried out at three concentration levels: 50%, 100% and 150% of the limits for each residual solvent. The percentage recovery ranges was from 82.12% to 104.41%.

Repeatability of the system as a validation parameter was evaluated on the basis of the results for the relative standard deviation from six successive injections. The RSD for at least of six injections was not more than 15% according to Ph.Eur. (Jahnavi and Saravanan, 2012).

To evaluate the intermediate precision, six sample solutions were prepared individually using single batch of pholcodine monohydrate drug substance as per test method, and were applied in two consecutive days according to the same procedure. The difference between the results obtained in two different days was less than 20%, so acceptance criteria was fulfilled and the method was confirmed being precise.

The limit of detection and limit of quantification were found to be specific for each solvent.

Robustness was tested by introducing small variations in the method parameters: carrier gas flow (± 0.1 ml/min), injector temperature (± 5 °C) and detector temperature (± 5 °C). For each set of variation, six replicate injections of the standard solution with concentration level of 100% were performed. The obtained results for all variations indicated that studied variations of GC-HS conditions will not cause any significant changes in system suitability and the method robustness was confirmed.

Conclusion

During the validation procedure, carried out according to ICH guidelines, highly selective HS-GC-FID method was developed and validated for the identification and quantification of residual solvents present in pholcodine monohydrate API through an understanding of the synthetic process, nature of solvents and nature of stationary phases of columns. The residual solvents, ethanol, methanol, toluene and benzene were determined and quantified. The developed method was found to be: specific, accurate, precise and robust as per ICH guidelines, hence can be used for routine quality control of residual solvents in pholcodine monohydrate API.

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Macedonian pharmaceutical bulletin, **62** (suppl) 219 - 220 (2016)
ISSN 1409 - 8695
UDC: 615.276.074:543.544.5.068.7
Short communication

Evaluation of drug-excipient interaction in formulation of ibuprofen topical gel by High Performance Liquid Chromatography

Elena Kazandzievska*, Slavica Mitrevska, Irena Brasnarska, Liljana Krsteska, Dejan Kostovski, Marina Kajdzanoska, Sonja Ugarkovic

Research & Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) used to reduce fever and treat mild to moderate pain or inflammation caused by minor injury, menstrual cramps, toothache, back pain, arthritis or headache. There is a great interest to develop non-oral dosage forms of ibuprofen to reduce its common side effects which are related to gastric disorders. Using topical gel means that the total amount of ibuprofen in the body remains low; this in turn means reducing the mentioned side effects.

In general excipients are known to facilitate the administration and release of active component as well as to protect and stabilize formulation from the environment. However, excipients can also give rise to inadvertent or unintended effects such as increased degradations of the drug. Knowledge of the chemistry of the active substance and excipients can often minimize formulation surprises (Bharate et al., 2010). Assessment of possible interactions (incompatibility) between the active substance (i.e. ibuprofen) and different excipients along with the evaluation of thermal stability are crucial parts of a normal study prior to the formulation of a medicine (Lira et al., 2007).

Excipients used for formulating gel for topical use often consist of gelling agents (cellulose derivates; carbomers; poloxamers), solvents in which the active ingredient is dispersed or solubilized (water, alchohol), pH stabilizers which neutralize polymers as a means of gel formation (organic amines, NaOH), penetration enhancers and cooling agents (menthol).

The aim of this study was to evaluate binary/ternary mixtures prepared between ibuprofen and some of the most used excipients for gel topical formulations and predict possible interactions by evaluating the related and degradation products of ibuprofen using HPLC stability indicating method, when exposed to different stress conditions for a predetermined period of time.

Materials and methods

Materials

Active pharmaceutical ingredient (API) ibuprofen used for preparation of the mixtures was of pharmacopoeial quality (with Certificate of Suitability to the monographs of the European Pharmacopoeia, CEP). European Pharmacopoeia Chemical Reference standard ibuprofen impurity BCRS was used. Excipients used for preparation of the binary/ternary mixtures are all pharmacopeial grade. All other chemicals used in this study were obtained commercially as HPLC or analytical grade reagents.

Preparation of binary and ternary mixtures

Binary and ternary mixtures were prepared by mixing equal masses of ibuprofen and the excipient (1:1 and 1:1:1), except Propylene glycol (1:2). Diisopropylamine was pre-hated at temperature of 40-45 °C. Due to aggregate form of the excipients, some mixtures were liquids, solid state and slurry mixtures.

One of the approaches to investigate drug-excipient interaction is using drug and excipients under thermal methods of analyses (Patel et al., 2015). All binary/ternary mixtures and pure API, were stored under stress conditions, examining the effect of temperature and humidity at different conditions (25 °C/60% RH and 40 °C/75% RH) for 14 days.

* ekazandzievska@alkaloid.com.mk

Methods

Evaluation of related and degradation products of ibuprofen in binary/ternary mixtures, was performed using validated HPLC method. The equipment consisted of Agilent 1260 binary pump, 1260 diode array detector, and detection wavelength on 214 nm. Samples were analyzed on Zorbax Extend C18 column (150 mm x 4.6 mm column ID, 5µm particle size). The mobile phase consisted a mixture of 1ml o-H_3PO_4 85% in 2L water and acetonitrile (64:36, v/v) and run through HPLC system at a flow rate of 2 ml/min at room temperature.

Results and discussion

The HPLC method used in this study was selective since no other peaks due to excipients appear on the retention time of ibuprofen. The limit of detection and quantification were found to be 0.12 µg /ml and 0.40 µg/ml, respectively.

Related substances present as impurities in ibuprofen can originate from the synthesis and from degradation. According to Ph. Eur. (04/2008:0721) specified impurities in ibuprofen API are ibuprofen impurity A, impurity J and impurity N. All other impurities are limited as other/unspecified impurities.

The results obtained from the HPLC analyses revealed that pure drug ibuprofen and the mixtures with Carbopol 940 and levomenthol, were stable during 14 days under both storage conditions since no significant change in the degradation profile were noted. There were no other peaks related to degradation products of drug or excipients.

Binary mixture of ibuprofen and propylene glycol revealed significant increase of unknown impurities (RRT 1.57; RRT 1.67; RRT 1.74) at 40 °C/75% RH. This may be expected having in mind the chemical properties of propylene glycol as oxidative reagent which is easily degraded under presence of high temperature, acidic or basic contaminants and the extreme mass ratio (1:2) between ibuprofen and propylene glycol.

Regarding the corresponding binary mixtures, the ternary mixture, composed of ibuprofen, diisopropylamine and Carbopol 940 shows different degradation profile. While 25 °C/60% RH for 14 days did not reveal significant change in the degradation profile, 40 °C/75% RH revealed rise of unknown impurities (RRT 0.11 and RRT 0.12) which are not noted as degradation products in the binary mixtures. As for RRT 0.38 which occurs in significant amount in the binary mixture with diisopropylamine (40 °C/75% RH), in the ternary mixture it is reduced for 60% on 40 °C/75% RH, and not detected on 25 °C/60% RH, for the same period of time.

It is well known that the chemical compatibility of an API in a binary mixture may differ from that of a multicomponent prototype formulation. Although the degradation is evident at elevated temperature or at unrealistically high concentration ratio of excipient to API, they may not be seen at ambient (real) temperature or at excipient to API ratio for the duration of the product shelf-life (Bharate et al., 2010). However such information can be very helpful in analyzing any instability issues with commercial formulations or during the development of new formulations.

Conclusion

Traditionally, excipients have been regarded as inert. In this study, excipients which were commonly used for gel topical formulation of ibuprofen were evaluated for interactions possibility by estimating the degradation profile of ibuprofen in the mixtures under stress conditions.

No significant change of known impurities was observed during 14 days stress conditions in all samples. Binary mixtures with organic amines (triethanolamine and diisopropylamine) and propylene glycol, reveled significant increase of unknown impurities on accelerated conditions of 40 °C/75% RH. However the behavior of ternary mixture, composed of ibuprofen, Carbopol 940 and DIPA is completely different then the binary mixtures (mild rising of unknown impurities which are not noted in the binary samples and extreme reducing of the significantly raised unknown impurities).

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Macedonian pharmaceutical bulletin, **62** (suppl) 221 - 222 (2016)
ISSN 1409 - 8695
UDC: 615.074:543.544.5
Short communication

A quality by design approach for liquid chromatography method development for determination of assay of drug product

Tina Achkoska*, Ivana Mitrevska, Marija Petrovska, Irena Brasnarska, Sonja Ugarkovic

Research & Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Analytical method development can be a time-consuming process that can last a long time. Methods are commonly developed using the traditional approach called "one-factor-at-a-time" (OFAT) which is carried out by selecting one instrument parameter to study while holding all other parameters constant. The best performing level of the study parameter is identified by visual inspection of the trial chromatograms. The parameter is then constant at this level, and a new instrument parameter is selected for the next trial. The OFAT process is repeated parameter by parameter until an adequately performing instrument method is obtained (Krull et al., 2008). This type of development may create an adequate method but provides a limited understanding of method capabilities and method robustness.

Another, systematic screening approach that evaluates a number of stationary phases, pH ranges and organic modifiers provides a more thorough approach to method development. A Quality by Design (QbD) approach to method development uses statistical design of experiments (DoE) to develop a robust method 'design space'. The design space defines the experimental region in which changes to method parameters will not significantly affect the results (ICH, 2009; Lukulay et al., 2009). This approach builds-in robustness to the method as the method is being developed. A better understanding of the overall method capabilities and limitations in development ensures a greater chance of successful method validation, transfer and routine use (Heyden, 2006). Method development using software affords considerable time savings for the analyst and the use of quality by design can produce a significantly more robust and quality submission to regulatory authorities.

In this study, a quality by design approach using method development software for determination of assay of drug products is presented.

Materials and methods

The study was carried out on an Agilent 1290 infinity system equipped with a 6-position column manager and solvent selection valve to allow for automated exploration of a wide range of conditions. Fusion AE Method Development software was used in conjunction with Chemstation to facilitate a more comprehensive QbD approach method development. For the first phase of initial screening 6 different columns were used: Sun fire C8, 150 x 4.6 mm, 5 µm; Halo Phenyl-Hexyl 150 x 4.6 mm, 5 µm; Zorbax Eclipse XDB C18 150 x 4.6 mm, 3.5 µm; Zorbax SB AQ 150 x 4.6 mm, 3.5 µm; X Select CSH Fluoro-Phenyl 150 x 4.6 mm, 3.5 µm and X Select HSS C18 100 x 4.6 mm, 3.5 µm. Organic modifiers acetonitrile and methanol were varied from 5 to 30 percent. The buffer solution which included a 5 mM sodium dihydrogen phosphate monohydrate and a 10 mM 1-pentasulphonic acid sodium salt was varied in pH range from 3.0-8.0. The column temperature (25 °C), flow rate (1 mL/min), injection volume (20 µL) and wavelength (233 nm) were maintained constant. The samples were prepared by spiking placebo, active substance and all known impurities of the active substance in a concentration of 1.0 percent of the concentration of the active substance. The samples were diluted with the buffer solution with pH value depending on the pH value of the mobile phase of the experiments in order to avoid eventual peak distortion or other chromatography problems caused by differences in sample solvent and mobile phase.

* tachkoska@alkaloid.com.mk

Results and discussion

After defining the variables of interest and the region of variation, an experimental design was generated within Fusion AE. A partial factorial statistical design was selected by the software to obtain the maximum amount of information with the least number of experimental runs (Heyden and Dejaegher, 2007). The experimental design was then transmitted to Chemstation software where all methods were automatically generated and ready to run. After the end of all the experiments the chromatograms were integrated and the results were imported back into Fusion AE which was used to model the data and to generate an initial method for subsequent optimization. The critical quality attributes (CQA), like retention time (3-8 min), Max Peak-Resolution (>1.5) and USP peak tailing (0.9-2.0) were established and the software carried out some possible solutions.

The contour plots of the modeled data show the unshaded (white) region as the acceptable region where all the critical quality attributes (CQA) are within the above designated criteria. By choosing the contour plot for each column, the type of organic modifier and changing the factors on each axis, the software gives predictions how the critical attributes are changing as a function of the factors on x and y axis. With the usage of the point prediction tool the most optimal method conditions which were located in the unshaded region of the contour plot were selected. These conditions were then exported to chromatography data system and additional experiments were performed. The best chromatography and the "widest" unshaded region was obtained with column Sun Fire C8 150 x 4.6mm with particle size of 5 µm with acetonitrile as organic modifier. The optimal percent of the organic modifier was 5 percent and the pH value of the buffer was 3.0.

The DoE contour plot helped to determine the settings for the next phase of the development of the method when the concentration of buffer solution was tested. For this purpose a 20 mM sodium dihydrogen phosphate monohydrate and a 5 mM 1-pentasulphonic acid sodium salt buffer with pH value of 3.0 was used and analyzed with the above specified chromatographic conditions. It can be seen that with a higher concentration of sodium dihydrogen phosphate monohydrate and lower concentration of 1-pentasulphonic acid sodium salt a better resolution between the peak of the active substance and the nearby eluting impurity is observed.

Conclusion

Chromatographic analytical method development normally begins with selection of the analytical column, the pH of the mobile phase and the organic solvent type. When using the OFAT approach in the initial phase of development, there is a possibility that this approach will provide limited coverage of the design space and there will be no understanding about the interaction effects between the instrument parameters. On the other hand a quality by design approach employs a statistically designed experiment to address the design space comprehensively and enables all important variable effects to be understood.

The initial column-solvent screening experiment carried out by using the quality by design approach and software capabilities can identify the correct analytical column, pH, and organic solvent type to use in the next phase of method development. The chromatographic conditions for the determination of the assay of drug substance set in this phase of the method development were: column Sun Fire C8 150 x 4.6 mm with particle size of 5 µm; mobile phase (buffer 20 mM sodium dihydrogen phosphate monohydrate and a 5 mM 1-pentasulphonic acid sodium salt buffer with pH value of 3.0 with acetonitrile in ratio of 95 : 5 percent); flow rate (1.0 mL/min); column temperature (T=25 °C), injection volume (20 µL) and wavelength (λ=233 nm).

The automated method development approach using Fusion AE software provides a better performing and more robust method in less time compared to manual method development.

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Macedonian pharmaceutical bulletin, **62** (suppl) 223 - 224 (2016)
ISSN 1409 - 8695
UDC: 615.357:577.175.3].076
Short communication

Comparison of method A and method B described in Ph.Eur. for determination of bacterial endotoxins in pharmaceutical preparation containing somatropine

Branislava Janeva, Sandra Zinoski, Katerina Starkoska*

Institute for Public Health of the Republic of Macedonia, Medicines Quality Control Department, "50 Divizija" No 6, 1000 Skopje, Republic of Macedonia

Introduction

The test for bacterial endotoxins is used to detect or quantify endotoxins originating from gram-negative bacteria using amebocyte lysate from horseshoe crab (Limulus polyphemus or Tachypleustridentatus). There are three techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex (Ph.Eur., 2014).

Material and methods

The testing was performed in accordance with Ph.Eur 8.0/2.6.14 by using Method A. Gel-Clot method: limit test and Method B. Gel-Clot method: quantitative test where permissible endotoxin limit concentration (ELC) in pharmaceutical preparation containing somatropine is < 5IU/mg. The maximum valid dilution (MVD) is 1:400. One International Unit (IU) of endotoxin is equal to one Endotoxin Unit (EU).

The following reagents, equipment and inventory were used in the test: Limulus amebocyte lysate (LAL), Reagent, λ = 0,125 EU/ml from Charles River Endosafe, USA; Endotoxin control standard, E.coli 055:B5 from Charles River Endosafe, USA; LAL water from Charles River Endosafe, USA; Thermoblock for incubation (37 ± 1 °C) from Lab-Line Instruments, USA; Vortex mixer from Barnstead, USA; Micropipettor from 5-50 µl; 50-200 µl and 200-1000 µl from Socorex, Switzerland; sterile and apyrogen extensions for multipipettors from Socorex, Switzerland; sterile and apyrogen glass test tubes from Charles River Endosafe, USA; sterile and apyrogen glass test tubes for dilution from Charles River Endosafe, USA and universal indicator paper pH 0-14 from Sigma-Aldrich.

Method A

Diluting used for preparation testing, which is not higher than MVD is 1:300.

The following test solutions were prepared:

Solution A: Diluted test solution being diluted less than MVD (1:300).

Solution B: Calibration curve of the endotoxin control standard (CSE) and diluted test solution (1:300) - concentrations equivalent to 2λ, λ, λ/2 and λ/4 (test for interfering factors).

Solution C: Calibration curve of the control endotoxin standard (CSE) and LAL water-concentrations equivalent to 2λ, λ, λ/2 and λ/4 (Confirmation test for the declared lysate sensitivity).

Solution D: LAL water as a negative control.

Procedure: Preparation tested on MVD (1: 400) – solutions 1, 2, 3 and 4.

The test is valid if the following condition is met:

The both replicates in the solutions 2 and 3 of the procedure are positive, while being negative in the solution 4.

The tested preparation meets the test if there is a negative result for both replicates in solution 1. If a positive result is obtained on both replicates of solution 1diluted on MVD, it does not meet the test. If a positive result is obtained on both replicates of solution 1 diluted to dilution less than the MVD, the test will be repeated with dilution which does not exceed the MVD or at the level of MVD.

* k.starkoska@iph.mk

Method B

Dilutions for preparation testing, and which are not higher than MVD 1:1; 1:2; 1:4; 1:8; 1:10; 1:100; 1:200 and 1:400.

The following test solutions were prepared:

Solution A: Test solution diluted lower than MVD (test for interfering factors).

Solution B: Undiluted test solution containing CSE at the concentration of 2λ (Positive Product Control).

Solution C: Calibration curve of the endotoxin control standard (CSE) and LAL water - concentrations equivalent to 2λ, λ, λ/2 and λ/4 (Confirmation test for the declared lysate sensitivity).

Solution D: LAL water as a negative control.

The test is valid if the following three conditions are met:

1. The both replicates of solution D (negative control) are negative.

2. The both replicates of solution B (positive product control) are positive.

3. Geometric mean of the final points in solution C is in the range of 2λ to λ/2.

Results and discussion

Method A

The obtained test results in Solution A are satisfactory i.e. the preparation does not contain detectable endotoxins. In Solution B with the dilution of 1:300 the interference factors exceeded, while the obtained result 0.2102 EU / ml is within the permissible limits of 2 λ to λ/2 (0.25 EU / ml to 0.0625 EU / ml). The declared lysate sensitivity in solution C is confirmed and the obtained result 0.2102 EU/ml is within the limits of 2 λ to λ/2 (0.25 EU/ml to 0.0625EU/ml). The obtained negative results in Solution D satisfy the test. In the mentioned procedure, both replicates in solution 2 (Positive Product Control) and solution 3 (Positive Water Control) are positive, while the both replicates in solution 1 (Negative Product Control) and solution 4 (Negative Water Control) are negative.

Method A

The obtained result 0.003525 EU/ml i.e. 0.0003525 EU/mg (allowed < 5IU/mg) in Solution A meets the quality specifications and the preparation does not interfere with the test. The obtained results of the both replicates in solution B (Positive Product Control) are positive and the results satisfy the test. Declared lysate sensitivity in solution C was confirmed and the obtained result 0.0884 EU/ml is within the limits of 2 λ to λ/2 (0.25 EU/ml to 0.0625 EU/ml). The obtained results of the both replicates in solution D (Negative Water Control) are negative and the results satisfy the test.

Conclusion

The obtained value of the lysate sensitivity determined by Method A.Gel-Clot method: the limit and test method B.Gel-Cloth method:quantitative test is within the limits of 2λ to λ/2.

The advantage of Method B compared to Method A is the option it has to determine concentration of endotoxin on unknown sample (a sample with unknown composition declaredand endotoxin limit concentration not listed).

Using the Method A, with dilution of 1:300, the interference factors exceeded and the preparation meets the test i.e. neither false positive nor false negative results were obtained.

Using the Method B, with dilutions of 1:200, the factors of interference started exceeding, while completely exceeded at dilution 1:400, which leads to a conclusion that Method B confirms the results of Method A since the dilution of 1:300 in Method A with exceeded interference factors in the range between 1:200 and 1:400.

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Macedonian pharmaceutical bulletin, **62** (suppl) 225 - 226 (2016)
ISSN 1409 - 8695
UDC: 615.477:347.5(497.7)
Short communication

Liability for damage caused by using medical devices

Vlatko Kokolanski*, Katerina Anchevska-Netkovska, Zoran Sterjev, Suzana Trajkovikj Jolevska

Faculty of Pharmacy, University of Cyril and Methodius, Skopje, str. Majka Tereza No 47 Skopje, Republic of Macedonia

As technological innovations advanced so did the medical devices that became part of surgery (so called invasive surgery) from the mid 19th century onward. There are many medical devices that are used in diverse surgical procedures on a daily basis (medical equipment and instruments). Medical devices covers a wide spectrum of different technological devices starting from simple depressors to complex, highly-technical and computerized devices. They play a crucial role in healthcare, whether in small, rural healthcare facilities or in expert medical diagnostic and treatment centers (Radisic, 2008). An additional proof of their necessity and significance is the fact that many governments across the world provide more than one billion Euros for medical purposes (WHO, 2003).

The purpose of this paper is to address the necessity of legal regulation of the types of liabilities of the entities involved for damage caused by using the medical devices in the R. Macedonia.

The technological advance of the modern medical devices is one of the most important elements of an efficient healthcare system. However, specific medical devices can be hazardous to the health of the medical staff or patients. There are numerous risks such as: improper alignment of the medical device, improper and inadequate usage of it, inadequate maintenance, and inadequate supervision before and after usage, breaking down of the medical device as well as the existence of dysfunctional medical devices. The medical devices that are most harmful for the patients' inner and outer body are the ones with specific usage, features, positioning and way of usage. (Tupanchevski et al., 2012)

Having in mind the increased usage of different medical devices that are harmful to the patients' overall health, the liability of the inflicted harm is also a big legal problem. Furthermore, this represents a complex social and legal problem, not just because it hasn't been regulated by specific legal norms, but because it has been expanding rapidly as well.

The liability for the damage caused by the use of medical devices in most legal systems as it is the case with Republic of Macedonia is not separately regulated. On the contrary, the liability is regulated by several laws of different legal disciplines. Namely the types of liability for the caused damage from using of medical devices can be the following: a) disciplinary liability (for which the most severe sanction includes the health worker's loss of licence), b) civil liability (that results in liability for compensation due to individual error which leads to a violation of patient's rights) c) misdemeanor liability (which usually entail a fine for inflicting minor injuries) and d) criminal liability (in cases where besides individual interests, it is necessary to ensure the protection of the wider social interest). However, despite this classification, the abovementioned forms are mutually intertwined and complementary to some extent, which means, whenever possible, it is necessary to avoid multiple punishments (Davitkovski et al., 2009).

The health workers are not solely responsible for the damage caused by using different types of medical devices. The manufacturer or the importer, the health institution and the people in charge of the health institution, should also take legal liability.

Civil liability is determined and accomplished by general legal rules liable for any caused damage. Above all, these are rules for personal liability, which is based on the guilt of the health worker and the liability rules of the legal entity (healthcare facility) for any damage caused by the employee to a third party. This liability arises as a consequence of violating contractual or legal duties of health professionals related to medical devices, and qualifies as a

* kokolanski@live.com

medical error. Nevertheless, the rules of objective liability (liability regardless of fault) are no exception in terms of rules of personal liability, but have a special scope of application that is defined by general terms such as: dangerous object and dangerous activity. The rules of liability for dangerous objects or dangerous activities have their field of application, and in that context can be used in all circumstances under which the damage is caused by the use of medical devices that have been labeled as dangerous objects. Therefore, apart from health workers, the manufacturer of the medical device and the holder of the dangerous object are also deemed as liable for the damage (Radisic, 2008). The compensation for the harm caused by medical devices is established in the Law on Obligations (Assembly of the Republic of Macedonia, 2001).

Criminal liability of health professionals, legal entities and responsible persons in the legal entity is established in the Criminal Code, in the chapter – criminal violations of people's overall health are provided the following crimes: Unscrupulous treatment of the ill (Article 207), as well as manufacturing and using of harmful devices for treatment (Article 212), (Assembly of the Republic of Macedonia, 1996).

Misdemeanor liability of the entities is established in Chapter VI Penalty Sanctions Law on Medicines and Medical Devices (Assembly of the Republic of Macedonia, 2007). Disciplinary liability of health workers stems from the codes of professional ethical duties and rights of health care workers, who make the respective chambers, and whose obligation for the Chambers derives from Article 250, Paragraph 3 of the Law on Health Protection (Assembly of the Republic of Macedonia, 2012).

The liability for the damage that may be caused by the use of medical devices is governed by several laws. The manufacturer or the importer of medical devices, the responsible person in the legal person, public health officials and the health facility are deemed liable criminally, civilly, and disciplinary for the inflicted damage.

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Macedonian pharmaceutical bulletin, **62** (suppl) 227 - 228 (2016)
ISSN 1409 - 8695
UDC: 615.074:543.544.5.068.7
Short communication

A quality by design based analytical method development for determination of impurities in new pharmaceutical drug product

Ana Georgieva*, Irena Brašnarska, Sonja Ugarkovič

Institute for Research and Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Regulatory authorities FDA and ICH are encouraging and promoting implementation of Quality by Design (QbD) principles in the pharmaceutical industry environment (ICH, 2008; ICH, 2009; Reid et al., 2013; Sangshetti et al., 2014). It is a current trend among pharmaceutical industry that a method development process is done by implementing an Analytical Quality by Design (AQbD), as a part of risk management, pharmaceutical development, and pharmaceutical quality system. The application of QbD concept to analytical method is justifiable, because of many variables that significantly affect the method results (Peraman et al., 2015).

There is no single QbD approach to chromatographic method development. A systematic approach that is broadly-applicable includes the following steps: establishing the critical quality attributes (CQAs), creating an experimental design that includes screening and optimization, defining an operational design space and defining a control strategy (McBrien, 2010; Salman and Vinayak, 2014).

Quality is guaranteed with robustness and reproducibility of the method that with this approach is built in method development stage. Submission is based on product knowledge and assured by analytical target profile (ATP). Method flexibility, meaning allowed movement within method operable design region (MODR) and continuous improvement that can be done without prior regulatory approval, are one of the benefits that come along with this approach.

Materials and methods

QbD approach for development of a high pressure reversed phase liquid chromatography (HPLC) method for determination of impurities in a drug product that contains two active ingredients and five known impurities was performed. Several common critical parameters in HPLC - gradient time, pH of the aqueous eluent, type of organic modifier, temperature and stationary phase were evaluated within the quality by design framework by the means of computer modelling software Fusion AE. Agilent 1290 Infinity automated method development system equipped with a column and solvent selection valves was used. As for initial chemistry screening of our experiment setup, four different column chemistries were tested: Zorbax SB-CN, Zorbax Eclipse XDB-Phenyl, Zorbax Eclipse XDB-CN, and Zorbax Eclipse Plus Phenyl-Hexyl, to maximize selectivity. Five different pH levels of 50 mM ammonium formate buffer in a pH range of 3.5-5.5 were tested and two organic modifiers, acetonitrile and methanol. Column temperature was varied in the interval 20-50 °C. Gradient time was set variable, from 28 to 30 minutes. As constant variables were set flow rate (0.7 ml/min), injection volume (20µl), initial and final hold time (2 and 10 minutes, respectively), initial and final hold percent organic (25 and 100 percent, respectivelly).

An experimental design was converted in redy to run sequence with 62 chromatografic runs, along with columns equlibratin stages. We run the experiment and analyze the data. The experimental results were import back to Fusion AE and processed to generate an initial method for subsequent optimization. We set a specific goal for each response along with lower and upper acceptability limits for the solution search as follows: number of peaks - target 7, number of peaks that have USP resolution ≥ 1.50, target 6 and number of peaks that have USP Tailing ≤ 2.0, target 7.

* ageorgieva@alkaloid.com.mk

Results and discussion

As stage one of our experiment based on QbD principles, analytical target profile was defined. The goal of this analytical method development process was getting a robust method for determination and quantification of related compounds of our drug product. The criteria for the successful separation (defined critical quality attribute) is tree fold: detection of all peak of interest, maximum critical resolution and USP tailing factor.

The software carried out a number of solution searches equal to the number of runs in the experiment design, with the specific goal settings of the experiment run as the starting point. The software then ranked the result of each search in terms of relative goodness (closeness to meeting or exceeding all goals). It then displayed the best overall answers in decreasing rank (LC Method Development Tutorial, Fusion QbD Quality by Design Software, S-Matrix Corporation, 2014, Version 9.7). All parameters were studied in combination, thus defining a multi-dimensional design space. Fusion AE enable visualization of the effect of each factor on the separation. This visualization of the design space was found very helpful in thorough understanding on the impact of each variable on method performances (Monks et al., 2011). By changing the factors on each axis, the design space was explored in detail. Column Zorbax SB-CN and acetonitrile were found to be optimal, as the acceptable performance region for these variables was found to be the broadest. The initial method was further optimized in experiment where secondary effectors such as column temperature, initial gradient slope and strong solvent percent and flow rate were varied. After processing the results, the final optimized method was generated.

Conclusion

HPLC analytical procedure for related and degradation products in new pharmaceutical product was developed, using the AQbD approach. Column Zorbax SB-CN, 50 mM ammonium formate buffer pH 4.5 as weak and acetonitrile as strong solvent in a gradient time of 28 minutes and column temperature of 20 °C were established as input variables that fulfil the goals set at the very beginning, thus defining a well-known and understandable multi-dimensional method operable design region.

This kind of approach to analytical method development gave us better understanding of method variables, since they were studied in combination. Given the large amount of chromatographic information contained in generated models, these offer a sound foundation for method troubleshooting and to justify continuous future improvement.

An automated method development using Fusion AE software take less time compared to manual method development, but also requires a huge understanding in selection of input variables and output response. QbD means the right analysis at the right time, based on science and risk assessment. This way of understanding and practicing the AQbD is current area of focus and needs to be implemented.

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Macedonian pharmaceutical bulletin, **62** (suppl) 229 - 230 (2016)
ISSN 1409 - 8695
UDC: 615.33.074:543.544.5.068.7
Short communication

Optimization of an UPLC method for determination of moxifloxacin hydrochloride and its related substances

Marija Zafirova*, Gabriela Petrovska, Liljana Ugrinova, Liljana Bogdanovska, Vasil Karcev, Katerina Brezovska, Aneta Dimitrovska, Suzana Trajkovik Jolevska

Center for Drug Quality Control, Faculty of Pharmacy, University "Ss. Cyril and Methodious", Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Moxifloxacin is a fourth generation fluoroquinolone antibacterial agent, active against Gram-negative and some Gram-positive bacteria, developed primarily for the treatment of community acquired pneumonia and upper respiratory tract infections (Balfour and Wiseman, 1999). Various procedures have been developed for the synthesis of the drug during which different process-related impurities are formed. Isolation and structural identification has been achieved for many of the synthesis-related impurities (Bayer, 2000; Kumar et al., 2004, Petersen, 2006). A number of methods have been developed for the determination of moxifloxacin hydrochloride and its related substances, based on reverse-phase high performance liquid chromatography (HPLC) (Dewani et al., 2011; Djurdjevic et al., 2009; Singh et al., 2014). Ultra high performance liquid chromatography (UPLC) is a relatively new technique which offers several advantages over conventional high performance liquid chromatography (HPLC), especially substantially decreased time of analysis and reduced solvent consumption while retaining chromatographic efficiency, resolution and sensitivity (Swartz, 2005). The aim of our study was to develop and optimize an UPLC method for determination of moxifloxacin hydrochloride and its related impurities.

Materials and methods

Moxifloxacin hydrochloride and its five impurities: (1) 8-hydroxy quinolonic acid, (2) 8-methoxy quinolonic acid, (3) ethyl quinolonic ester, (4) 8-hydroxy moxifloxacin and (5) N-methyl piperazine were used for this study. Stock solutions were prepared in methanol at the concentration level of 1 mg/ml for moxifloxacin and 0.1 mg/ml for each of the selected impurities. The resolution solution of moxifloxacin and its related impurities was prepared by diluting the stock solutions in mobile phase A, to the final concentration of 0.4 μg/ml for each compound.

The chromatographic analysis was conducted on Shimadzu Nexera UPLC system equipped with PDA detector. The separation was performed on an Acquity UPLC BEH C18, 50 x 2.1 mm, 1.7 μm chromatographic column, using gradient elution of mobile phase B (%): 0-2 min: 19%; 2-6 min: from 19% to 67%; 6-8 min: from 67% to 27%; 8-8.1: from 27% to 19%; 8.1-9 min: 19%. Mobile phase A was a mixture of aqueous phase pH=2.2 (containing 0.16 mol/L phosphoric acid and 1 ml/L trifluoroacetic acid, adjusted to pH 2.2 with triethylamine) and methanol (85:15 v/v). Mobile phase B consisted of a mixture of methanol and water (80:20 v/v). The column temperature was maintained at 50 ˚C and the flow rate was 0.3 ml/min. Injection volume was 5 μL and the total run time for analysis was 9 min. Moxifloxacin and its impurities were recorded at 296 nm.

Results and discussion

Optimization of the method started by varying the composition and the pH of the mobile phase, using isocratic and gradient elution. Two aqueous mobile phases were examined, one containing potassium dihydrogen phosphate, and the other containing phosphoric acid, at three pH values (2.2; 5.5; 6.0), maintaing the column temperature at 25 ˚C. The studied compounds were partially separated by isocratic elution, therefore to achieve full separation, gradient elution was used. The best separation and peak symmetry were observed with the aqueous phase containing phosphoric

* marijazafirova@ff.ukim.edu.mk

acid at pH=2.2, therefore it was selected for further analysis. Under these conditions, 8-methoxyquinolonic acid and ethylquinolonic ester impurities were not resolved at all. In order to achieve their separation, column temperature was gradually increased. Satisfactory resolution was achieved at 50 ˚C. In order to obtain adequate sensitivity the injection volume was also varied. Optimal results were obtained using injection volume of 5 μL. According to the literature data, the wavelength was optimized in the range from 290-296 nm (Djurdjevic et al., 2009). The wavelength of maximum absorption was selected according to the recorded UV spectrum of the moxifloxacin hydrochloride, which was 296 nm. Different diluents for preparation of the resolution solution were tested as well. The best peak shape of the studied compounds was observed using mobile phase A as diluent. The final step in the optimization process was the selection of the best conditions for gradient elution. Therefore, the ratio of mobile phase B, the flow rate and the gradient steps were optimized. Best peak shape and resolution with optimal run time was acquired using gradient elution of mobile phase B (%): 0-2 min: 19%; 2-6 min: from 19% to 67%; 6-8 min: from 67% to 27%; 8-8.1: from 27% to 19%; 8.1-9 min: 19%. Using the finally optimized chromatographic conditions a critical resolution between 8-methoxy quinolonic acid and ethyl quinolonic ester of 1,8 was obtained. All the peaks of the studied compounds have a good symmetrical shape, with maximum tailing factor of 1.3 for the peak of 8-hydroxy moxifloxacin. The number of theoretical plates (tp) shows suitable column efficiency, with a minimum NTP of 1770 tp/column for the peak of N-methyl piperazine. The obtained values for signal to noise (minimal value of 11.9 for the peak of 8-hydroxy moxifloxacin) confirmed the sensitivity of the method. All of the aforementioned parameters indicate on a satisfactory performance of the chromatographic system.

Conclusion

An UPLC method for quantitative determination of moxifloxacin hydrochloride and its related impurities was developed and optimized. The method is simple and sensitive and the studied impurities were separated with excellent resolution in only 9 minutes. The results from our study indicate that, after proper validation, the proposed method could be used for routine analysis of moxifloxacin hydrochloride and its related substances as an active ingredient and in pharmaceutical dosage forms as well.

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Macedonian pharmaceutical bulletin, **62** (suppl) 231 - 232 (2016)
ISSN 1409 - 8695
UDC: 615.281.014.8
Short communication

Photo stability study design of drug product containing fluoroquinolon as active compound

Veronika Popovska Jakimovska*, Marija Velichkovska, Aleksandra Petrovska, Irena Brashnarska, Biljana Shapkareva, Suzan Memed-Sejfulah, Sonja Ugarkovich

Institute for Research and Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Fluoroquinolone as active compound belongs to a class of synthetic antimicrobial agents. Fluoroquinolones absorb radiation energy in the ultraviolet range. A loss of antibacterial activity was observed after UV exposure, as a result of changed colour, decreased assay and increased degradation products, suggesting photodegradation of the active substance. Regarding to this, selection of an appropriate packaging to protect the drug product from light and a storage advice is necessary to be established.

The photodegradation itself has not yet been documented, as well as no information has been so far provided on the nature of the photoproducts. For some fluoroquinolones, however, metabolites have been identified with reduced antibacterial activity (Eva-Maria et al., 1993).

In order to demonstrate that appropriate light exposure does not lead to unacceptable changes in dosage form, photostability testing was performed on the drug solution, as part of formal stability study ICH guideline Q1A (R2) (Blessy et al., 2014).

Materials and methods

Photo stability study was conducted according to ICH guideline Q1B ”Photo stability Testing of New Drug Substances and Products”- Option 1. Testing was performed on one batch of Alkaloid’s drug product, solution packed in glass colorless ampoules as primary packaging and cardboard boxes as secondary packaging, using following methods:

Determination of colour of solution: Ph.Eur 2.2.2 method and visual method

Determination of assay and related and degradation compounds of the drug product: validated in-house HPLC methods.

The formulation was examined at confirmatory storage conditions: exposure time in the visible range is 7.1 hours, which corresponds to an illumination of 1.2 million lux hours and in the UV range 2.9 hours, which corresponds to an integrated near ultraviolet energy of 200 Wh/m^2.

In first phase of photostabilty study, samples and placebo, were exposed outside the immediate pack, placed in a quartz Erlenmeyer’s with stoppers, alongside with protected sample and placebo (solutions placed in amber glass volumetric flasks) used as a dark control to evaluate the contribution of thermally induced change.

In the second phase, the samples and placebo were exposed in primary packaging to the same conditions.

In the last, third phase, the samples and placebo were exposedin the secondary packaging as intended for marketing, also to the same conditions.

After treatment, samples were examined for any changes in physical properties: i). colour of solution with visual method; ii). determination of assay with HPLC method and iii). determination of degradation products with HPLC method.

Results and discussion

In this study, photo stability of the drug product and degradation pathways of the active substance was fully determinate and understood.

After the exposure for 2.9 hours to an integrated near ultraviolet energy of 200 Wh/m^2, the assay of the active component was slightly decreased in both samples, the sam-

* vpopovska@alkaloid.com.mk

ples outside of primary packing and samples in the primary packaging. The decrease in the assay was followed with changed levels of impurity amount significantly for the specified impurity C, susceptible to light degradation (Hani et al., 2015; Mohammad and Zaynab, 2014; Ummeet al., 2013) which has increased to 0.43 and 0.47%, respectively.

All samples have shown change in colour, compared with Ph.Eur. Reference standard solution GY4, used for determination of the colour.

After the exposure for 7.1 hours to an illumination of 1.2 million lux hours in the visible range, the obtained results from the opened samples and samples in the primary packaging were very similar. The assay of the active component was decreased to 97.82% and 97.50% respective to the increased amount of the specified impurity in the level above the qualification threshold, 0.76% and 0.74%, respectively. Also, both samples have shown change in the colour of the drug product.

No changes were noticed in the amount of the unknown impurities in all treated samples at end of exposure.

Alongside with the drug product, placebo solution was also stressed in order to exclude those impurities that are not degradation products, which was found stable during the photo stability study.

In the dark control samples and samples in the secondary packaging, no changes have been observed at the end of exposure period.

Data review of the tests on the exposed drug product outside the immediate packaging as well as in the primary packaging suggest that the drug product is sensitive to light and should be kept in the secondary packaging. The secondary packaging, cardboard box, provides sufficient protection, where the physical properties, assay and impurity levels are comparable to the untreated samples.

Conclusion

From the results for colour, assay and related and degradation products, obtained from conducted photo stability study on the drug product, it has been concluded that the drug product, containing fluoroquinolone, is susceptible to photo degradation and loss of antibacterial activity. No photo degradation was observed at the third phase of performed study, confirming that product should be kept in its original package in order to be protected from light.

With regard to data presented, the SmPC and labelling sections for the drug product should be amended with a storage advice: ‘Store in the original package to protect from light’.

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Macedonian pharmaceutical bulletin, **62** (suppl) 233 - 234 (2016)
ISSN 1409 - 8695
UDC: 615.214.2.074:543.544.5.068.7
Short communication

Investigation of chromatographic behavior of aripiprazole and its five impurities

Nevena Maljurić, Ana Protić, Biljana Otašević, Jelena Golubović*, Jovana Krmar, Mira Zečević

Department of Drug Analysis, University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia

Introduction

Aripiprazole is an atypical antipsychotic drug, recently approved for the treatment of acute manic and mixed episodes associated with bipolar disorder and as an additional treatment of depression (Thakkar et al., 2011). It acts primarily as partial agonist of D2 receptors. It is also a partial agonist of the 5-HT1A receptors, and like other atypical antipsychotics displays an antagonist profile binding to the 5-HT2A receptors. Aripiprazole has moderate affinity for histamine and alpha adrenergic receptors, and no appreciable affinity for cholinergic muscarinic receptors 1-2 (Ravindra et al., 2014). Chemically, it is 7-[4-[4-(2, 3-dichlorophenyl) piperazin-1-yl] butoxy]-3, 4-dihydro-1H-quinolin-2-one.

Organic impurities can arise during the manufacturing process or storage of a drug substance, and include starting materials, intermediates and degradation products (ICH, 2006). In this study, retention behavior of aripiprazole and its five impurities was investigated. The following impurities were analyzed: hydroxy quinoline impurity (Impurity A): 7-hydroxy-3,4-dihydro quinolin-2 (1H)-one; piperazine impurity (Impurity B): 1-(2,3-dichlorophenyl)piperazine hydrochloride; diquinoline impurity (Impurity C): 7,7'-(butylenedioxy)di-3,4-dihydroquinolin-2(1H)-one; chlorobutoxyquinoline impurity (Impurity D): 7-(4-chlorobutoxy)-3,4-dihydroquinolin-2(1H)-one and N-oxide impurity (Impurity E): N-oxide-7-[4-{4-(2,3-dichlorophenil)-1-piperazinyl}butoxy]-3,4-dihydroquinolin-2(1H)-one.

β-Cyclodextrins (β-CDs) are macrocyclic oligosaccharides composed of seven α-D-glucopyranose units joined in a cone-shaped structure (Chen et al., 2004). They exhibit a hydrophobic cavity delimited by two rims, a wide and a narrow one, composed of secondary and primary hydroxyl groups (Fifere et al., 2012). This structure enables β-CDs to generate inclusion complexes with a wide variety of hydrophobic organic compounds in aqueous solutions. In analytical chemistry β-CDs could be used as mobile phase additives, in order to reduce the amount of organic solvents and introduce the concepts of green chemistry.

The aim of the present study was to investigate the retention behavior of aripiprazole and its five impurities using High-performance liquid chromatography (HPLC) method and applying the concepts of green analytical chemistry. The influence of β-CD on retention behavior of aripiprazole and its five impurities was examined, in order to shorten retention times with smaller percentage of acetonitrile, as an organic modifier.

Materials and methods

All experiments were carried out on Thermo Scientific, Dionex Ultra 3000 equipped with PDA detector. Chromatographic separation was performed at non-polar C18 analytical column Chromolith RP-18e column (100 mm × 4.6 mm, macropore size 2 μm, mesopore size 13 nm) at temperature varying from 25 °C to 40 °C. Injection volume was 5 μL. Mobile phase was prepared by dissolving an appropriate amount of β-CD in water to achieve 10 mM and 15 mM β-CD solutions. The pH was adjusted using formic acid. The mobile phase was degassed in ultrasonic bath and filtered through membrane filter prior to use. The flow rate was 1 mL/min. The detection was performed at 235, 254 and 285 nm using PDA detector.

Stock solution of aripiprazole was prepared by dis-

* golub@pharmacy.bg.ac.rs

solving 500 μg of standard substance in 1 mL of HPLC water. Working solution was prepared from appropriate volume of stock solution and mixture of β-CD and acetonitrile (80 : 20, v/v). The same procedures were used for preparing stock and working solutions of the impurities, but the concentration of stock solutions was 100 μg/mL. All the reagents used were of analytical grade except water and acetonitrile which were of HPLC grade.

Results and discussion

Investigation of retention behavior of aripiprazole and its five impurities was done by varying the pH value of the mobile phase in the range from 2 to 4, the β-CD concentration from 10 mM to 15 mM, column temperature from 25 °C to 40 °C and acetonitrile as an organic modifier from 25% to 30%. Under the chosen acidic conditions, aripirazole was in cationic form, as well as its impurity B and impurity E. Impurity A was in molecular form, but its retention time was short, which could be explained by the polarity of a molecule (logP = 1.28). Impurities C and D were in molecular forms, which probably caused longer retention times. Percentage of acetonitrile was the factor with the most significant impact on retention times of analyzed compounds. Although aripiprazole is a lipophilic compound (logP = 4.79), its satisfactory elution time was achieved with 25% of acetonitrile. The same amount of acetonitrile was used for impurities, which were also mostly lipophilic. LogP values of impurities B, C, D and E are 2.79, 2.95, 2.52 and 4.18, respectively.

It was recognized from the recent literature reports that reducing the consumption of organic solvents introduces an environment-friendly approach to drug analysis (Cielecka-Piontek et al., 2013). The mobile phase consisting of β-CD could play an important role in reducing the amount of acetonitrile. Taking into consideration the structure of β-CD, there was a possibility for building inclusion complexes with aripiprazole or its impurities, which could shorten the time of analysis. The higher column temperature also shortens the retention times of all the analyzed compounds. The change in β-CD concentration from 10 mM to 15 mM did not have a significant influence on the retention times. Based on all described facts it was decided to work with 15 mM β-CD. Also, it was decided that the percentage of acetonitrile could be 30% and the column temperature should be set at 40 °C. Retention time of aripiprazole under these circumstances was 5.343 min, impurity A 1.717 min, impurity B 2.067 min, impurity C 10.917 min, impurity D 10.773 min and impurity E 5.940 min. From the obtained UV spectra the most appropriate wavelength for monitoring the compounds was decided to be 254 nm. Finally, although aripiprazole and its five impurities differ in structure and polarity, using HPLC method with β-CD solution as mobile phase additive, they could be separated in single analytical run.

Conclusion

The present study shows that β-CD has an important influence on retention behavior of aripiprazole and its five impurities. Reducing the amount of organic solvents by using β-CD solutions as mobile phases is a great advantage and step forward to limit the exposure to toxic agents, without decreasing the sensitivity and resolution of determination. Under these circumstances the proposed HPLC method could be successfully used for separation of aripiprazole and its five impurities. Besides, another advantage of proposed method is the compliance with the novel trend in analytical method development according to which it is recommended to use methods suitable for adopting the environment-friendly approach to pharmaceutical analysis.

Acknowledgment

These results are part of the Project no. 172033, financed by the Ministry of education, science and technological development of the Republic of Serbia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 235 - 236 (2016)
ISSN 1409 - 8695
UDC: 615.015:663.81
Short communication

Alcohol induced dose dumping for prolonged-release drug product

Elena Davitkovska*, Blagica Manchevska, Dusica Angelovska, Irena Brasnarska, Packa Antovska, Biljana Sapkareva, Sonja Ugarkovic

Research and Development Institute, ALKALOID AD,
blvd Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Alcohol Induced Dose Dumping (ADD) is a term that describes the unintended, rapid release in a short period of time of the entire amount or a significant fraction of the drug contained in a prolonged-release dosage form. Depending on the therapeutic indication, the therapeutic index and other characteristics of the drug, dose dumping can pose a significant risk to patients, either because of safety issues or diminished efficacy or both. Generally dose dumping is observed as a result of an impairment of the release-rate-controlling mechanism. Some prolonged-release oral dosage forms contain drugs and excipients that exhibit higher solubility in ethanol solutions compared to water solutions. Such products can be expected to exhibit a more rapid drug dissolution and release rate in the presence of ethanol. Therefore, when a prolong-release product is consumed with alcohol, the prolong-release mechanism could be adversely affected, which could lead to dose dumping.

Where appropriate, based on the risk of dose dumping when a prolonged-release drug product is consumed with alcohol, the prolonged-release drug products labeling includes warnings against the consumption of alcohol while taking the drug product. Even with significant warnings in the labeling, the consequences of concomitant alcohol use need to be considered for certain drug products because alcohol use may still be likely and such alcohol use may lead to dose dumping, which could result in serious adverse events especially for certain narrow therapeutic index drugs.

Mitigating the risks of alcohol-induced dose dumping is an issue that puts great demands on formulators, regulatory agencies and the industry at large. Over the last few years, the pharmaceutical industry has become increasingly concerned with the topic of alcohol-induced dose dumping, which can lead to dangerous or even potentially lethal side effects. The goal of the regulatory approach should be to minimize the risk of alcohol-induced dose dumping from prolonged-release dosage forms, irrespective of any warnings on product labeling and instructions by health care providers (Meyer et al.,2005). To address the problem, regulatory guidelines have been implemented, resulting in the need for new formulation approaches.

Guidelines in Europe and the United States

In the European Union, all prolonged-released formulations must be evaluated for alcohol-induced dose dumping risk. Effects of alcohol for generic oral formulations, in vitro studies of the release in alcohol solutions should be performed. Where accelerated active substance release is seen in vitro either at high or low alcohol concentrations over a short period of time or at lower alcohol concentrations over a longer period of time, the product should be reformulated. If the alcohol effect cannot be avoided and it is present also in the reference product, the applicant should justify / demonstrate that it lacks of clinical relevance or discuss the possible clinical relevance in comparison to the reference product. EMA guidelines show a path for decision-making for formulators (Friebe et al., 2005).

Differences are present in US and EU regulations. According to EMA guideline the prolonged-released product must undergo in vitro testing in the presence of 20% alcohol concentration (EMA, 2014). On the other hand, the United States' Federal Drug Administration (FDA, 2015), requires in vitro testing in concentrations up to 40% alco-

* edavitkovska@alkaloid.com.mk

hol for a list of defined generic formulations (Friebe et al., 2005).

Nevertheless, the applicant should discuss the significance of any out of specification results, particularly at the early time points, together with consideration of the risks of dose dumping and accelerated release. Appropriate warnings in the summary of product characteristics should be proposed and justified (EMA, 2015).

With respect to increasing globalization of pharmaceuticals, harmonization of the regulatory guidelines for in vitro ADD resistance testing is highly desirable. It should be based on a common understanding of the physiological impacts of alcohol consumption and realistic exposure times.

Materials and methods

Comparative dissolution tests of prolonged-release tablets containing BCS Class I active substance in three different media have been performed: buffer pH 6.8 (solution of sodium hydroxide and potassium dihydrogen phosphate in water R) also proposed for routine testing, buffer pH 4.5 (solution of sodium acetate R and acetic acid in water R) and medium pH 1.2 (solution of hydrochloric acid and sodium chloride in water R), each of them with and without 20% ethanol. The level of alcohol in the medium mimics level that can be reached in the fluids of the gastrointestinal tract following alcohol consumption.

Dissolution tests have been performed, using basket apparatus (Apparatus I) at 100 rpm, in 500 ml aforementioned media at 37±0.5 °C for a total period of 24 hours. Dissolution profiles were obtained with sampling at 8 time points on 12 individual units.

The dissolution rate of the prolonged-release drug product has been determined using in-house validated HPLC method.

Results and discussion

In vitro dissolution studies with alcohol have been performed to evaluate whether ethanol ingestion may modify the release characteristics of the drug product.

To demonstrate in vitro similarity of the dissolution profiles performed in media with and without ethanol, $f2$ statistic method was employed.

The results of the performed comparative dissolution tests of prolonged-release tablets in the dissolution medium proposed for routine testing, but with alcohol, are within the specification limits.

Furthermore the $f2$ statistic method indicates similarity between the dissolution profile in the presence of alcohol and the dissolution profile of the same batch in the same media in absence of alcohol, for all three dissolution media.

Conclusion

The conducted in vitro dissolution studies demonstrate that there is no potential risk of alcohol-induce dose dumping for the tested prolonged-release drug product.

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Macedonian pharmaceutical bulletin, **62** (suppl) 237 - 238 (2016)
ISSN 1409 - 8695
UDC: 615.014:004.38
Short communication

AlkaSAP computer system validation

Sonja Sterjevska*, Nada Popstefanova, Darko Atanasoski, Miroslava Ilievska

Quality Assurance, Alkaloid AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Computers are widely used during development and manufacturing of drugs and medical devices. Proper functioning and performance of software and computer systems play a major role in obtaining consistency, reliability and accuracy of data. Therefore, computer system validation (CSV) should be part of any good development and manufacturing practice. It is also requested by EU and FDA regulations and guidelines through the overall requirement that "equipment must be suitable for its intended use".

Specific requirements for computers can be found in section 211.68 of the US cGMP regulations (USC, 2008).

- Automatic, mechanical, or electronic equipment or other types of equipment, including computers, or related systems that will perform a function satisfactorily, may be used in the manufacture, processing, packing, and holding of a drug product. If such equipment is so used, it shall be routinely calibrated, inspected, or checked according to a written program designed to assure proper performance. Written records of those calibration checks and inspections shall be maintained.
- Appropriate controls shall be exercised over computer or related systems to assure that changes in master production and control records or other records are instituted only by authorized personnel.
- Input to and output from the computer or related system of formulas or other records or data shall be checked for accuracy.
- The degree and frequency of input/output verification shall be based on the complexity and reliability of the computer or related system.
- A backup file of data entered into the computer or related system shall be maintained except where certain data, such as calculations performed in connection with laboratory analysis, are eliminated by computerization or other automated processes. In such instances a written record of the program shall be maintained along with appropriate validation data.
- Hard copy or alternative systems, such as duplicates, tapes, or microfilm, shall be designed to assure that backup data are exact and complete and that it is secure from alteration, inadvertent erasures, or loss shall be maintained.

Specific requirements for computers and electronic records and signatures are also defined in FDA's regulations 21 CFR Part 11 on electronic Records and Signatures (USC, 1997).

The Good Automated Manufacturing Practices Forum (GAMP) has developed guidelines for computer validation. All these guidelines and publications follow a couple of principles:

- Validation of computer systems is not a onetime event. It starts with the definition of the product or project and setting user requirement specifications and cover the vendor selection process, installation, initial operation, going use, and change control and system retirement.
- All publications refer to some kind of life cycle model with a formal change control procedure being an important part of the whole process.
- There are no detailed instructions on what should be tested. All guidelines refer to risk assessment for the extent of validation.

Validation of computer systems is not a once off event. Annex 11 of the European GMP directive is very clear about this: Validation should be considered as part of the complete life cycle of a computer system. This cycle includes the stages of planning, specification, programming, testing, commissioning, documentation, operation, monitoring and modifying" (EC, 2010).

Scope

The scope of this work is to present the validation of ERP SAP computer system used in Alkaloid AD Skopje known as AlkaSAP system.

* ssterjevska@Alkaloid.com.mk

Background

AlkaSAP system was implemented in Alkaloid AD Skopje in 2007. Within AlkaSAP there are several 'modules', each of which contains transactions in order to perform necessary tasks to complete business processes. Several of these tasks and processes are considered critical or have regulatory implications to Alkaloid.

The SAP implemented modules in Alkaloid AD Skopje in 2007 were:

1. Sales & Distribution (SD)
2. Production Planning (PP)
3. Quality Management (QM)
4. Materials Management (MM)
5. Financial Accounting (FI)
6. Controlling (CO)

During 2014 additional SAP module concerning human capital management (HCM) was implemented.

Validation of the AlkaSAP

Validation of the AlkaSAP system was made in accordance with GAMP 4. All validation activities were defined in Validation Master Plan (VMP). The validation programme was divided into phases according to the validation life cycle. Each phase was divided into tasks and for every task there were actions, responsibilities and associated procedures.

Specification / Design Phase

During this phase the following documents were created:

Validation Master Plan (VMP); User Requirement Specification (URS); Functional Specification (FS); Risk Assessment (RsA); Hardware Design Specification (HDS); Software Design Specification (SDS); Code Review (CR);

Testing phase

Both hardware and software elements were tested as part of the AlkaSAP implementation.

Hardware Acceptance Testing

Hardware Acceptance Testing (including Infrastructure) was conducted successfully on all servers.

Document reference: CSV VMP AlkaSAP HATS

Software Acceptance Testing

All bespoke and functional application software identified in the Functional Specification or Software Design Specification was tested against the Software Acceptance test Specification.

Document Reference: CSV VMP AlkaSAP SATS

Software Performance Test Specification

All software identified in the Functional Specification or Software Design Specification was given a risk priority and was tested against the Software Performance Test Specification:

Document Reference: CSV VMP AlkaSAP SPTS

In accordance with risk priority given in the Risk Assessment tests were executed as Detailed Functional Tests; User Acceptance Tests; Business Scenarios

Validation Report

The Validation Report has been issued as a phased report for the purpose of 'go-live' for the AlkaSAP and summarizes the validation programme activities.

Document Reference: CSV VMR AlkaSAP

Ongoing Operation and Evaluation

In order to control and maintain the validation status of the AlkaSAP system, the following procedures are established and written:

Policies and Procedures for Security and Access to servers

Policies and Procedures for Back Up, Restore and Archive

Procedures for Training and Operating of the system

Periodic review

Periodic Reviews of the System is covered by Alkaloid Guideline ensuring SOPs, validation documentation and records are audited on a regular basis to evaluate the effectiveness of controlling procedures.

Summary

Alkaloid AD Skopje started 'go-live' with the validated AlkaSAP computer system. The validation test results were of sufficient detail and quality to establish that this computer system does what it purports to do.

AlkaSAP still remains status of "validated system" which can be proved with regular periodic review of the system.

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Macedonian pharmaceutical bulletin, **62** (suppl) 239 - 240 (2016)
ISSN 1409 - 8695
UDC: 633.888:615.212.7.074
Short communication

Determination of cannabidiol and Δ^9tetrahydrocannabinol in *Cannabis sativa* L. preparations present in the European market by HPLC/DAD

Maja Shishovska[1]*, Dragica Doneva[1], Zorica Arsova-Sarafinovska[1,2], Katerina Starkoska[1*]

[1]*Institute for Public Health of the Republic of Macedonia, Medicines Quality Control Department, "50 Divizija" No 6, 1000 Skopje, Republic of Macedonia*
[2]*University "Goce Delcev", Stip, Republic of Macedonia*

Introduction

Cannabis sativa L. is a medicinal plant, known and used for a long time. There are 400 – 500 compounds that have been identified in its extracts, and among them approximately 70 are C_{21} terpenophenols, members of a group known as cannabinoids, specific for this plant (ElSohly and Slade, 2005; Fischedick, et al., 2009; Turner, et al., 1980). The constituent of *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (Δ^9THC), is the primary psychoactive cannabinoid. Given that the main pharmacological and psychoactive effects have been attributed to this compound, the most of the studies have been focused on its effects (Costa, 2007). The effects of *Cannabis sativa* L. are not solely due to Δ^9THC because cannabidiol (CBD) was found to cause pharmacological effects (Russo and Guy, 2006). It was shown that CBD and other cannabinoids achieve synergy with Δ^9THC causing potentiation of benefits, antagonism of adverse effects, summation, pharmacokinetic advantages, and metabolism (Russo and Guy, 2006).

Many countries became more liberal towards medicinal use of *Cannabis sativa* L. (Baker et al., 2003). Recently there has been an increasing interest in development of cannabinoids and *Cannabis sativa* L. preparations as legitimate medicines for a variety of medical applications. Some of them include, but are not limited to, multiple sclerosis, chronic pain, glaucoma, asthma and cardiovascular conditions, and as an antiemetic (Williamson and Evans, 2000). Cannabidoids are very potent compounds and their control in *Cannabis sativa* L. preparations is very important, especially because their potential users are patients which already have serious health problems.

The aim of this study was to apply our in-house HPLC/DAD method on the *Cannabis sativa* L. preparations present in the European market and to control their quality.

Materials and methods

The samples of *Cannabis sativa* L. preparations were purchased from the European market, produced by Endoca (Pharmaceutical Company, Denmark). The standard substances were obtained from Lipomed AG (Switzerland). The samples of standard substances were: Δ^9-THC (3-pentyl-6,6,9-trimethyl-6a,7,8,10a-tetrahydro-6*H*-dibenzo(b,d) pyran-1-ol), delivered as 1 mL ampule solution (5 mg/ mL prepared in ethanol, with purity 98.52%, *m/m*) and CBD ((-)-*trans*-2-p-mentha-1,8-dien-3-yl-5-pentylresorcinol) as the solid substance (99.73%, *m/m*). The solvents used during the analysis were with HPLC grade.

For the chromatographic analysis Agilent Technologies HPLC system 1200 series (Germany) was used. The analysis was performed on HPLC-column: Purospher® Star RP18e (150 mm x 4.6 mm ID, 5 µm) from Merck KGaA (Germany) using mobile phase composed of acetonitrile and water in gradient mode with acetonitrile from 50%, *V/V*, to 80%, *V/V*, at flow rate 1.5 mL/min, temperature 30 °C, detection at 220 nm, injection 10 µL, in 31 min run time.

The working solutions of standard substance CBD were prepared in methanol in two concentration ranges:

* k.starkoska@iph.mk

95.48 µg/mL – 286.43 µg/mL and 103.13 µg/mL – 309.40 µg/mL. The Δ^9THC working solutions were prepared in methanol in concentration range: 9.81 µg/mL – 19.63 µg/mL.

The sample solutions were prepared by liquid/liquid extraction technique using a mixture of methanol and chloroform (9:1). When the layers were clearly separated, the sample from the methanolic layer was applied in HPLC/DAD system.

Results and discussion

Five different *Cannabis sativa* L. preparations purchased from the European market were analyzed. All were prepared from hemp oil with various amounts of CBD as active compound and naturally contains Δ^9-THC, as an impurity. According to the certificates the amount of CBD was: 3%; 5%; 10 %; 15% and 30%, *m/m*, and expected impurity as Δ^9THC was limited at maximum of 0.20%, *m/m*.

For preparation of the samples we used the recommended United Nations Office on Drugs and Crime method (UNODC Manual, 2009). For identification and quantification of cannabinoids we used our in-house chromatographic gradient method which we have proposed for hemp seed oil analysis (Shishovska et al., 2014) because of the similarity of samples in origin and form.

In the analyzed samples of hemp oil preparations cannabidiol and Δ^9THC were identified and quantified. Identifications were done by comparing of the retention times and UV spectra of standard compounds with retention times of peaks at the chromatograms of samples and their UV spectra. The retention times of CBD and Δ^9THC peaks were 15.9 min and 21.0 min, respectively.

For the quantification, data obtained from the chromatograms were calculated using the method of the calibration curve (constructed as concentration, c (µg/ml), versus peak area, A (mAU)). The analyzed working solutions of the active compound CBD and the trace compound Δ^9THC showed high linearity in the working ranges. The estimated coefficient of the linearity for CBD working solutions at concentration range: 95.48 µg/mL – 286.43 µg/mL was 1.000 ($A = 23688\ c + 16.188$); while for the CBD working solutions at concentration range: 103.13 µg/mL – 309.40 µg/mL it was 0.9998 ($A = 39613\ c + 31.129$), and for the working solutions of standard substance Δ^9THC at concentration range: 9.81 µg/mL – 19.63 µg/mL it was 0.9997 ($A = 25269\ c - 7.0738$).

It was found that content of cannabidiol in analyzed samples is from 109.7% to 125.4%, *m/m* of the declared values. The assays of Δ^9THC traces at the samples vary according the concentration of the active compound. As quantities of CBD at samples were lower (3 – 5%), the assay of Δ^9THC is under the allowed maximum limit (0.02% – 0.14%), but at samples with higher concentrations of CBD (10 – 30%) the Δ^9THC traces are above the allowed maximum limit (0.45% – 1.14%).

Conclusion

The satisfactory chromatographic data proved that our in-house gradient HPLC/DAD method can be successfully used for determination of the active substance CBD and traces of Δ^9THC in *Cannabis sativa* L. preparations. The results obtained for the analyzed samples of *Cannabis sativa* L. preparations showed higher amounts of CBD than declared value in the certificate (differences range from 9.7% *m/m* to 25.4%, *m/m*). In two samples, the amount of Δ^9THC found was under allowed maximum limit of 0.20%, while in three samples the amount was above that value. Not all of the samples tested have composition as declared in the certificate, although they all origin from a European manufacturer and are present in the European market.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 241 - 242 (2016)
ISSN 1409 - 8695
UDC: 615.281.074
Short communication

Generation and combined study on the chemical structure of nitrofurantoin radical anion

Angelina Popova, Simeon Stoyanov, DenitsaYancheva*

Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., build. 9, 1113 Sofia, Bulgaria

Introduction

Nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin) is antimicrobial compound, used extensively as a prophylactic for urinary tract infections in humans and animals (Maaland and Guardabassi, 2011). It is reported that the modes of action underlying DNA damage or cytotoxicity induced by nitrofurantoin in rodent liver and lungs may involve ROS generation by reduction to nitro radical anion (Suntres and Shek, 1992). A recent study showed that the drug exhibits carcinogenicity in the kidneys of male rats and the structure of the nitro furan plays a key role in the induced genotoxicity (Kijima et al., 2015).

Most nitroaromatic drugs, containing nitroquinone, nitroimidazole or nitrofuran moiety, are considered to exert their toxic effect by nitro reduction (Boelsterli et al., 2006). One-electron reduction of the nitro group catalyzed by nitro-reductase gives rise to nitro anion radical, the chemical instability of which promotes production of various ROS such as superoxide anion and hydroxyl radical via its electron-donating ability (Wang et al., 2008).

Despite these drawbacks, new nitrofuran derivatives are still being developed as antimicrobial agents (Zorzi et al., 2014). Therefore, improved knowledge on the structure and reactivity of nitrofurantoin radical anion could help reduce the toxicity of the new nitrofuran antimicrobial agents.

This reports motivated us to generate electrochemically the nitro radical anion of nitrofurantoin and study its chemical structure by spectroscopic IR methods and DFT computations. The radical anion was electrochemically generated and the spectral and structural changes arising from the conversion were described based on IR spectra and DFT calculations. The structural variations, electron charge distribution over molecular fragments and IR frequency shifting were discussed.

Materials and methods

The electrochemical generation of the radical anion of nitrofurantoin was carried out in a special CaF_2 cell, provided with platinum electrodes build in the polyethylene spacer. 4.5 V were applied to the cathode in the solution cell containing 0.1 mol/l nitrofurantoin and equimolar amount of tetraethylammonium bromide in DMSO-d6. Electrochemical generation of the radical anion was continued for a period of 75 min and then the polarity of the electrolysis cell was reversed in order to regenerate the parent compound. The process of nitrofurantoin reduction and regeneration was monitored by recording IR spectra in 10 min intervals. The IR spectra were measured on a Brucker Tensor 27 FT spectrometer at a resolution of 2 cm^{-1} and 64 scans.

All theoretical calculations were performed using the Gaussian 09 package of programs. Geometry and vibrational frequencies of species studied were performed by analytical gradient technique without any symmetry constraint. All the results were obtained using the density functional theory (DFT), employing the B3LYP (Becke's three-parameter non-local exchange correlation) method in conjunction with 6-311+G(2df,p) basis set. In order to account for the ifluence of the medium, we used the Integral Equation Formalism Polarizable Continuum Model (IEF-PCM) on the same level of theory with inclusion of DMSO.

Results and discussion

The initial IR spectrum of nitrofurantion in DMSO-d6 solution, containing tetraethylammonium bromide as

* deni@orgchm.bas.bg

electrolyte salt, showed that the asymmetric and symmetric stretching vibrations of the nitro group are observed at 1521 and 1350 cm^{-1}. The stretching vibration of the N-C bond linking the nitro group to the furan ring is found at rather high wavenumber - 1211 cm^{-1}, respectively. A few minutes after applying the current, the solution in cathode space became brown, and the bands of the anion radical of nitrofurantoin appeared in the IR spectrum. More prolonged electrolysis (75 min) caused strong increase of the bands of the anion radical, while the bands of the neutral compound vanished. Reversal in the polarity of the electrolysis cell resulted in gradual decrease of the IR bands of the radical anion and reappearance of the neutral molecule absorptions. After 75 min of reversed electrolysis the initial spectrum of the parent compound was completely restored without the presence of any additional IR bands. This fact unambiguously demonstrates that the observed spectral changes are due to the reduction of nitrofurantoin to radical anion and not to other chemical transformations.

The conversion of nitrofurantoin into radical anion is related to strong frequency decreases in the assymetric N-O stretching: $\Delta vas(NO_2)$ = 220 cm^{-1}, strong frequency decreases in the symetric N-O stretching: $\Delta vs(NO_2)$ = 209 cm^{-1} and strong frequency increase in C-NO_2 stretching: Δv(C-NO2) = 273 cm^{-1}. Based on the calculated spin density, the odd electron is localized mainly on the nitro group (c.a. 70%) and in smaller extends – on the furan ring (c.a. 30%). The radical anion formation leads to simultaneous shortening of the C-N bond and lengthening of the N-O bonds.

Conclusion

The observed frequency shifts arising from the conversion of nitrofurantoin into radical anion are larger than those found with the conversion of dinitrobenzenes and cyanobenzonitriles. It is evidence that larger structural variations in the nitrofuran moiety occur upon conversion into radical anion than in case of dinitrobenzenes and nitrobenzonitriles. The localization of the spin density over the nitro group is a sign for high reactivity of the formed nitrofurantoin radical anion and strong ability to initiate production of various ROS via electron donation. The importance of aminohydantoin moiety for the stability and reactivity of the nitrofurantoin radical anion has to be elucidated by studying other nitrofuran derivatives with modified side chains.

Acknowledgements

The financial support of this work by the National Science Fund of Bulgaria (Contracts RNF01/0110), Science Fund is gratefully acknowledged.

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Macedonian pharmaceutical bulletin, **62** (suppl) 243 - 244 (2016)
ISSN 1409 - 8695
UDC: 616.34-002-085.37-074:543.544.5.068.7
Short communication

Bioanalytical HPLC method for therapeutic drug monitoring of azathioprine metabolites during inflammatory bowel disease

Bojana Danilova, Dragana Mladenovska, Matea Miceska*, Jasmina Tonic Ribarska

Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Inflammatory bowel disease is an idiopathic disease caused by a dysregulated immune response to host intestinal microflora, with a high incidence in younger adults. Generally, its treatment is based on immunosuppressive drugs. Azathioprine is thiopurine metabolite with immunosuppressive and cytotoxic effects, which is used in the treatment of inflammatory bowel disease and childhood acute lymphoblastic leukemia. Unfortunately, about one third of patients under azathioprine therapy, have need for modifying or terminating the treatment because of adverse effects or low therapeutic outcome. As a prodrug of 6- mercaptopurine (6-MP), azathioprine acts through the formation of thioguanine and methylthioinosine nucleotides, which are capable of incorporating in DNA strains like "false" bases (Dervieux and Boulieu, 1998). Hence the immunosuppressive effect, target cells of azathioprine main metabolites - 6-thioguanine nucleotide (6-TGN) and 6-methylmercaptopurine (6-MMP) are the "fast-growing" cells, mainly lymphocytes, though the bone marrow and the hepatic cells are not excluded. Consequently, the risk-benefit ratio of azathioprine therapy is dose-dependent and here lies the crucial need for therapeutic drug monitoring (TDM) (Vikingsson, 2012). On the other hand, intra- and inter-individual variations seem to reflect individual differences in 6-MP absorption and metabolism, resulting in different bioavailability and distribution of the active metabolites in patients receiving similar doses. The purpose of this study is to establish a specific, sensitive HPLC method for simultaneous determination of 6-TGN and 6-MMP found in erythrocytes after azathioprine administration.

Materials and methods

Chemicals and reagents. 6-MMP, 6-TGN and dithiothreitol (DTT- derivatization agent) were purchased form Sigma-Aldrich Inc., St. Louis, USA. Methanol, phosphate buffer (pH 3,5), perchloric acid, hydrochloric acid, all HPLC grade, were obtained from Merck, Darmstadt, Germany. For all analysis, HPLC grade water was used. Zorbax Eclipse XDB C18 column, 150 x 4.5mm, 5 μm was supplied by Agilent Technologies.

Erythrocyte samples. Blood samples were collected from healthy volunteers (used for method validation) and from patients under azathioprine therapy (used like testing samples). Erythrocytes were extracted by centrifugation at 4 °C. Further on, the eppendorf tubes were normalized on $4x10^9$ erythrocytes, and frozen at -80 °C until the analysis.

Preparation of standard solutions. Stock solution of the analytes (5 μg/ml 6-TGN and 40 μg/ml 6-MMP) were prepared by dissolving each compound in 0,1 M HCl. Working solutions were prepared in 6 different concentrations (concentration range between 0,04 μg/ml to 2,50 μg/ml for 6-TGN and 0,25 μg/ml to 20 μg/ml for 6-MMP) from stock solutions by dilution with 0,1 M HCl. Calibration standards were made by spiking the blank erythrocytes with appropriate volume of working solutions of 6-TGN and 6-MMP at six different concentration. Samples for quality control (QC) were prepared in 4 different concentrations that cover the range of the calibration curve (LLOQ, low QC, medium QC, high QC appropriate for 6-TGN and 6-MMP).

Sample preparation. Sample solutions were made by adding DTT in each eppendorf tube which contains 500 μl of erythrocytes from patients under azathioprine therapy. In each eppendorf tube 50 μl 70% Perchloric acid (acid hydrolysis and deproteinization agent) was added and centrifuged at 3000 rpm, for 15 minutes at 4 °C. After centrifu-

* matea.lero@gmail.com

gation thiopurine nucleotides are obtained. Acid supernatants were collected and heated at 100 °C for 45 minutes in order to release thiopurine bases after hydrolysis.

Chromatographic conditions. The separation was carried out with Agilent 1100 HPLC system on Zorbax Eclipse XDB C18, 150 x 4.5mm, 5µm column using gradient elution with methanol and phosphate buffer (pH 3,5) in ratio 5:95 (*V/V*) with linear gradient to 15:85 (*V/V*) for a period of 12 minutes and flow rate of 1,2 ml/min. The temperature was set at 25 °C with UV detection at 341 nm for 6-TGN and 304 nm for 6-MMP. Method was validated following the recommendations for validation of bioanalytical methods of EMA guideline (Guideline on validation of bioanalytical methods, European Medicines Agency, 2011).

Results and discussion

The recovery values of the extraction procedure were higher than 84% for both metabolites, 6-TGN and 6-MMP. Under the proposed chromatographic conditions, no interfering peaks were observed in the retention time of analytes. The correlation coefficient from calibration curves for linearity of the method was 0.9996 for 6-TGN and 0.9989 for 6-MMP, respectively. The results for accuracy (±15% of the nominal values for the QC samples and ± 20% of the nominal value for the LLOQ) and precision (the value for coefficient of variation should not exceed 15% for QC samples and 20% for LLOQ) were within recommended limits. Stability studies indicated that no stability related problems would be expected during the routine erythrocytes sample analysis. Samples of erythrocytes spiked with 6-TGN and 6-MMP are stable after 12 hours in the autosampler, 24 hours at room temperature and can be stored for up to six months at temperature of -80 °C. The interpretation of the measured 6-TGN and 6-MMP levels, respectively is following – subtherapeutic (< 235 pmol/8*10^8 erythrocytes), therapeutic (235-400 pmol/8*10^8 erythrocytes) and high levels correlated to myelotoxicity (> 400 pmol/8*10^8 erythrocytes); below 5700 pmol/8*10^8 erythrocytes and more than 5700 pmol/8*10^8 erythrocytes – associated with hepatotoxicity (Vikingsson et al., 2013). Measured metabolite levels correlated to the clinical response from azathioprine therapy.

Conclusion

A simple, sensitive, precise and accurate HPLC method has been developed and validated for simultaneous determination of 6-TGN and 6-MMP in erythrocytes. Its main pros are: chromatographic run time less than 10 minutes (3.67 minutes for 6-TGN and 7.60 minutes for 6-MMP), facilitated pre-analytical procedure, rapid and specific determination of 6-MMP and 6-TGN intra-erythrocyte concentrations. The proposed method was successfully applied to erythrocytes samples obtained from 33 patients with inflammatory bowel disease under azathioprine therapy. The method results are in agreement with those obtained with other available methods, demonstrating its applicability for monitoring the azathioprine treatment.

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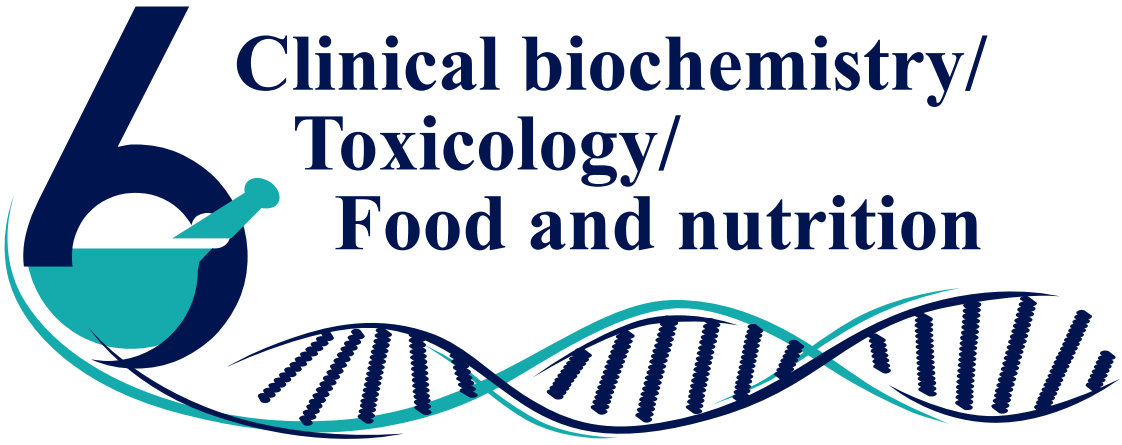
Clinical biochemistry/
Toxicology/
Food and nutrition

Macedonian pharmaceutical bulletin, **62** (suppl) 247 - 248 (2016)
ISSN 1409 - 8695
UDC: 615.322:615.726
Short communication

New *in vitro* technique for evaluation of anti-inflamatory activities of natural products and plants extracts

Neda Mimica-Dukić*, Ivana Beara and Dejan Orčić

Department of Chemistry, Biochemistry and Environmental protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia

Introduction

Lipid-derived autacoids play important roles in the inflammatory response and are a major focus of research into new anti-inflammatory drugs. These compounds include the eicosanoids such as prostaglandins, prostacyclin, leukotrienes, and thromboxane and the modified phospholipids such as platelet activating factor (PAF). Eicosanoids are synthesized from 20-carbon polyunsaturated fatty arachidonic acid, found in many cells, including activated leukocytes, mast cells, and platelets and therefore are widely distributed. Many inflammatory mediators (TNF-α, bradykinin) stimulate eicosanoid production either by direct activation of PLA2, or indirectly by increasing intracellular Ca^{2+} concentrations, which in turn activate the enzyme. Arachidonic acid can be converted to these products by three different pathways: cyclooxygenase, leading to the formation of prostanoids (prostaglandins and thromboxanes), lipooxygenase, where leukotrienes and certain mono-, di- and tri-hydroxy acids are synthesized, and epoxygenase pathway, which includes cytochrome P-450 and epoxides as final products. Accordingly, cyclooxygenases, lipooxygenases and epoxygenases are enzymes involved in these pathways (Smith, 1989). Cyclooxygenase (COX), implicated in cyclooxygenase pathway, exists in two forms, named COX-1 and COX-2. COX-1 is expressed constitutively in different tissues, blood monocytes and platelets, and transforms arachidonic acid to prostanoids, which are involved in normal cellular homeostasis. In contrast, COX-2 may be induced by a series of pro-inflammatory stimuli and its role in the progress of inflammation, fever and pain has been known (Hawkey, 1999). Furthermore, three types of lipoxygenases, termed 5-, 12- and 15-lipoxygenase are engaged in lipoxygenase pathway. Some compounds, like 12(S)-hydroxy-(5Z,8Z,10E,14Z)-eicosatetraenoic acid (12-HETE), a product of 12-lipoxygenase (12-LOX), has influence on the regulation of platelet aggregation, but is also found to be involved in the progression of several human diseases like various cancers (Nie and Honn, 2002) psoriasis (Müller, 1994) and rheumatoid arthritis (Liagre et al., 1997). Aforementioned enzymes can be found in different cell types. Thus, in human platelets, COX-1 and 12-LOX are the initial enzymes responsible for arachidonic acid metabolism leading to the formation of thromboxane B2 (TXB2), prostaglandin E2 (PGE2), 12-HHT (12(S)-hydroxy-(5Z,8E,10E)-heptadecatrienoic acid) and 12-HETE.

In vitro technique for evaluation of anti-inflamatory activities of natural products and plants extracts

Since our research is focused on biological activity of plant extracts, we aimed to evaluate and optimize an *in vitro* assay for anti-inflammatory activity, that can be easily used to determine COX-1 and 12-LOX inhibitory potential of plant extracts or natural products, using LC–MS/MS technique for quantification of metabolites and human platelets as a source of enzymes.

We used human platelets as the cell system for testing anti-inflammatory activity of numerous plant extracts and secondary metabolites. Inflammation is induced by calcymicin (calcium ionophore A23187) with subsequent addition of Ca^{2+}. Detailed procedure is described in our previous report (Beara et al., 2010; Lesjak et al., 2013). Considering quantification of formed products, our research was focused on LC–MS/MS technique in order to attain high sensitive and specific method with short analysis time. Furthermore, tandem mass spectrometry provides far better sensitivity and selectivity than traditional UV detectors

* neda.mimica-dukic@dh.uns.ac.rs

and does not depend on presence of chromophores. The method is optimized in order to get best performance for detection of PGE2, TXB2, 12-HHT (COX-1 pathway) and 12-HETE (12-LOX pathway) products, using PGB2 as internal standard. The method performances were confirmed by measuring several parameters (Beara et al., 2010).

The advantage and efficiency of this method was further confirmed through numerous subsequent studies on biological activity of natural plant products (Beara et al., 2012a, 2012b, 2014; Lesjak et al., 2011; Lesjak et al., 2013; Nadjpal et al., 2016, Simin et al., 2013). Furthermore this method provides valuable information about the metabolism of arachidonic acid under inflammation. And finally, it should be noted that one of the important advantage of applied experiment is avoidance of undesirable in vivo tests on experimental animals.

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Macedonian pharmaceutical bulletin, **62** (suppl) 249 - 250 (2016)
ISSN 1409 - 8695
UDC: 504.5:546.3/.8
Short communication

Metals specificities in environmental risk assessment

Dragana Vujanović

Department of Toxicology, Faculty of Pharmacy, University of Belgrade,
Vojvode Stepe Str 450, 11000 Belgrade, Serbia

Introduction

Metals are chemicals of natural origin and in use for a long time. All environmental media have naturally occurring mixtures of metals, and metals often are introduced into the environment as mixtures. Some of them are essential for maintaining proper health of humans, animals, plants, and microorganisms.

Metals and inorganic requiring different approaches for Environmental Hazard and Exposure assessments, compared to "typical organic contaminants". Several properties and aspects of metals – their natural occurrence, essentiality, bioavailability, homeostatic control mechanisms and acclimatization to diverse natural environments, - require specific recognition in the context of an environmental risk assessment. The basic concept of ENV-Hazard and Risk Assessment of metals consist of four stages:

- Classification & ranking (acute/ chronic aquatic toxicity, transformation /dissolution, particle sizes, ranking systems, etc…);
- Effects assessment (soil, sediment, water, data selection, data handling, effects analysis, uncertainty management, etc… sec. Poisoning...);
- Exposure assessment (data selection, data handling, local scenario, regional scenario, waste scenario, etc…) and
- Risk characterization (soil sediment water sec. Poisoning, local scenario, regional scenario, waste scenario, etc…) (USEPA, 2007).

This paper will be short introduction to challenges and key concepts specific to metals environmental assessment, through specific parts on bioavailability, effects assessment, and bioaccumulation.

* dragv@pharmacy.bg.ac.rs

Bioavailability

Bioavailability should be taken into account for hazard and risk assessment of metal compounds in order to focus on interspecies sensitivity. A tired approach can be used, ensuring that first tier assessments use effects data where bioavailability is maximized (MERAG, 2007). Knowledge of the chemistry of a compound will normally guide the need for the development of bioavailability correction models. Biotic Ligand Models (BLMs) can be used to estimate bioavailability, taking into account competition from other ions and binding of metals with natural organic matter. Many countries have developed frameworks for taking into account metal specificities in environmental risk assessment.

Bioavailability models have a specific applicability domain and have to cover soil/water parameters and background relevant for the region where they are applied; in addition it may be needed to do checks with species specific for that region. Further research is recommended to investigate to which extent commonalities between Biotic Ligand Models (BLMs) for species from different trophic levels apply amongst different metals. Further discussion is needed for developing a decision scheme as to the conditions for implementing BLMs in risk assessment.

Effects assessment

Hazard identification of metals and metal compounds is related to the toxicity of the soluble metal ions. The toxicity of the metal ions and the release of these ions from the metal-containing substances are therefore used as a basis for read-across.

Effects assessments of metals could follow the following strategy: Data compilation & read-across

- Data screening for relevancy and quality
- Database development

- Data normalization (i.e. Bioavailability correction) Data aggregation
- Species Sensitivity Distribution (SSD) construction and HCX derivation if relevant PNEC derivation (including uncertainty assessment).
- Metal-specific criteria for quality and relevancy of effects data to be used in the assessment should be developed, taking into account criteria already developed for chemicals effect data in general (MERAG,2007).

Bioaccumulation and bioconcentration

For some metals, background can be significant (relative to the PEC or PNEC) and should be integrated in the risk assessment. At the level of PEC/PNEC: the total bioavailability approach and the added risk approach could be considered; Background should be measured in unpolluted environments.

Available data for BCFs and other Accumulation Factors for metals indicate that they are inversely related to water (and sediment, and soil) concentrations. Therefore the use of a single value for assessing risks from secondary poisoning has to be considered.

Measures of tissue concentrations along the food chain provide a useful way to assess secondary poisoning.

Further discussion is needed as to how to improve comparisons/interpretations of BCFs and BAFs for metals to regulatory threshold values.

For the assessment of secondary poisoning, a tiered approach should be used, taking into account: dietary composition for consumer organisms, high BCF values could be used as a trigger to perform more detailed assessments, relevant food chains should drive the selection of BAFs and oral PNEC values, bioavailability of the metal in the food or incidentally ingested soil/sediment and the water concentration used in BCF or BAF experiments should preferably be in the same range as the PEC.

Conclusion

Presented key guiding principles is based on the unique attributes of metals. Metal speciation affects metal behavior in environmental media. PH and redox potential affect speciation. Kd values – a coefficient for mobility in soils limited use of single values. Aging of metals in media reduces bioavailability. Metal sorption behavior affects bioavailability.

The aim of this paper is describe how metals-specific attributes and principles may be applied in the context of existing risk assessment guidance and in existing human health and ecological risk assessment practices.

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Macedonian pharmaceutical bulletin, **62** (suppl) 251 - 252 (2016)
ISSN 1409 - 8695
UDC: 615.322:634.23:[616.61-099-02:632.95
Short communication

Dimethoate-induced renal toxicity in rats and the protective/ ameliorative effects of *Laurocerasus officinalis* Roem. (cherry laurel) fruit extract

Ayşe Eken[1]*, Burcu Ünlü-Endirlik[1], Elçin Özger[1], Ayşe Baldemir[2], Arzu Hanım Yay[3]

[1]*Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Merkez Kampüsü Melikgazi, Kayseri, Turkey*
[2]*Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Merkez Kampüsü Melikgazi, Kayseri, Turkey*
[3]*Erciyes University, Faculty of Medicine, Department of Basic Sciences, Merkez Kampüsü Melikgazi, Kayseri, Turkey*

Introduction

Dimethoate is one of the most important organophosphate insecticides in agriculture (Sharma et al., 2005). The toxicity of dimethoate results in deleterious effects causing oxidative stress on many organs and systems in human and animals particularly the kidney, liver, testes, brain, nervous, and reproductive system (Abou-Baker et al., 2014). The cellular antioxidant defense is reinforced by the presence of dietary antioxidants. Accordingly, there has been recently growing interest in the role and usage of natural antioxidants as a strategy to prevent oxidative damage in various health problems (Saafi et al., 2011).

Laurocerasus officinalis Roem. is known as "cherry laurel" in English. L. officinalis is grown as a native fruit crop locally called "taflan, lazkirazı, karayemiş"on the coasts of the Black Sea region of Turkey (Erdogan-Orhan and Küpeli-Akkol, 2011). The fruits and seeds of the L. officinalis are widely utilized as herbal medicine in Turkey for the treatment of stomach ulcers, digestive system problems, bronchitis (seeds), eczemas, hemorrhoids, anti-diabetic (seeds), analgesic on local pain, and as a diuretic (fruits) (Ayaz et al., 1998; Elmastas et al., 2013; Küpeli-Akkol et al., 2012; Şenaylı et al., 2012; Yaylacı-Karahalil and Şahin, 2011). By in vitro models a neuroprotective effect of the fruits of L. officinalis was found (Erdogan-Orhan and Küpeli-Akkol, 2011). *In vivo* experimental study determined the anti-inflammatory effects of *L. officinalis* leaves (Küpeli-Akkol, 2012). *L. officinalis* fruit is rich in phenolics and a good source of natural antioxidant protecting humans from several diseases caused by oxidative stress (Yaylacı-Karahalil and Şahin, 2011).

The aim of the study was to determine the nephrotoxic effects of dimethoate on biochemical indicators of kidney function tests, oxidative stress parameters, and histological examination in rats and the protective/ameliorative effects of *L. officinalis* fruit extract.

Materials and methods

60 male Wistar albino rats were divided into six experimental groups of 10 rats each and were treated daily by oral gavage for 60 days. Group I (control group) received only saline (0.9% NaCl); Group II was treated with dimethoate; Group III was given fruit extract; Group IV was treated with fruit extract and dimethoate; Group V was treated with vitamin C and dimethoate; Group VI was administrated only dimethoate for the first month. During the second month, the rats were treated with dimethoate and fruit extract. Dimethoate, *L. officinalis* fruit extract, and vitamin C were applied at 7, 4, and 100 mg/kg/day doses, respectively. At the end of the 60 days, rats were anaesthetized by ketamine and xylazine. Blood samples were taken from hearts to the sterile tubes and centrifugated at 3500 rpm for 20 min to separate serum. The serum samples were stored at -80 °C until analysis of blood urea nitrogen (BUN) andcreatine. The kidneys were removed immediately and washed with sodium phosphate buffer (pH 7.4). They are divided into two sections. One section was

* eken.ayse@gmail.com

placed in formaldehyde solution for routine histopathologic examination by light microscopy. The other half of kidney tissue section was homogenized in ten volumes of ice-cold 140 mMKCl and Tris-HCl buffer (50 mM, pH 7.6) using a homogenizer for 2 min at 13.000 rpm. The levels of malondialdehyde (MDA) for lipid peroxidation index were determined in the tissue homogenates by using spectrophotometer. The homogenates were then centrifuged at 5000 g for 60 min to remove debris. Clear supernatant was used for superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzyme activity assays.

Results and discussion

In comparison to the control group, a statistically significant increase was observed in the serum levels of blood urea nitrogen (BUN) and creatine in dimethote treated group ($p<0.05$). However, administration of L. officinalis to dimethoate treated groups (group IV and VI) provided a markedly reduction in levels of BUN and creatine ($p<0.05$). Moreover, vitamin C treated rats to dimethoate treated group showed decreased BUN and creatine levels ($p<0.05$).

Treatment with dimethoate caused an increase in the kidney tissue levels of MDA as compared with the control group ($p<0.05$). However, treatment with L. officinalis to dimethoate treated groups (group IV and VI) provided a reduction in MDA levels compared to the dimethoate treated group ($p<0.05$). The antioxidant enzymes activities of SOD and GPx in the group of only dimethote treated rats were significantly lower than those in the control group. However, L. officinalis treatment resulted in a significant increase of SOD and GPx activities ($p<0.05$). The activity of CAT enzyme was markedly decreased in dimethoate treated rats and elevated in L. officinalis treated rats as compared to control rats, but this effect was not statistically significant.

The kidneys of the control group showed normal kidney parenchym. With light microscopic examinations, histopathological changes such as mononuclear cell infiltration, glomerular atrophy, congestion and necrosis in the kidney tissues were observed in the dimethoate treated group as compared with control group. Administration of L. officinalis apparently reduced kidney tissue damage. On the other hand, there were not any differences in histologic appearance of kidneys between the control and L. officinalis treated group. Also, the kidney morphology in vitamin C treated group was similar to that of the control group.

The antioxidants may play a major role in the prevention of diseases related to oxidative stress. Therefore, there is an increasing interest in the research of antioxidant compounds such as phenolics present in the plants (Erdogan-Orhan and Küpeli-Akkol, 2011). These phenolics provide protection against toxic radicals due to their radical scavenger activities and have therapeutically beneficial effects on human health (Yaylacı-Karahaliland Şahin, 2011). It was found that L. officinalis fruit extract has strong radical scavenging activity and antioxidant effects (Erdogan-Orhan and Küpeli-Akkol, 2011; Kolaylı et al., 2003).

Conclusion

In conclusion, dimethoate exposure indicated the disruption of nephrotic architecture in kidney section as compared to control group. We observed that L. officinalis fruit extract pre- and post- administration to dimethoate treated rats significantly reduced kidney injury by preventing oxidative damage. Therefore, L. officinalis fruit may be useful for the prevention of oxidative stress-induced kidney injury.

This research was financially supported by Research Fund of the Erciyes University Scientific Research Project Unit (Project number: TCD-2013-4127).

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Macedonian pharmaceutical bulletin, **62** (suppl) 253 - 254 (2016)
ISSN 1409 - 8695
UDC: 613.278:637.146.3
Short communication

Probiotic/synbiotic enriched ayran as functional food product – quality and therapeutic benefits

Tanja Petreska Ivanovska[1]*, Zoran Zhivikj[1], Liljana Bogdanovska[1], Maja Jurhar Pavlova[2], Ivica Gjurovski[3], Trpe Ristoski[3], Kristina Mladenovska[1], Lidija Petrushevska-Tozi[1]

[1]*Faculty of Pharmacy, University "Ss. Cyril and Methodious", Mother Theresa 47, Skopje, Macedonia*
[2]*Faculty of Medicine, University "Ss. Cyril and Methodious", 50 Division 6, Skopje, Macedonia*
[3]*Faculty of Veterinary Medicine, University "Ss. Cyril and Methodious", Lazar Pop Trajkov 5, Skopje, Macedonia*

Introduction

The demand for functional foods that promote health beyond basic nutrition increases along with the increased consumer awareness for the influence of diet on their health. In respect to probiotic foods both the viability of the probiotic within foods and bioavailability within the host has to be studied (Figueroa-González et al., 2011), while at least maintaining the quality of conventional product used as a carrier. Microencapsulation may offer good protection of probiotic viability loss during manufacture, product storage and after ingestion of probiotics, especially during exposure to unfavorable GI conditions (Sánchez et al., 2012).

The aim of the study was to obtain a functional product ayran with therapeutic level of viable and metabolic active cells of L. casei 01 (a minimum of 107 log CFU per mL ayran). Quality of the prepared probiotic and synbiotic samples was examined as well as their therapeutic benefits in animal model with chemically induced colitis.

Materials and methods

Ayran samples were prepared by fortifying commercially available product (Ayran, Zdravje, Macedonia) with non-encapsulated probiotic L. casei 01 (Chr. Hansen, Denmark) and/or prebiotic oligofructose-enriched inulin (Synergy 1, Orafti-Rue L. Maréchal, Belgium) and encapsulated synbiotic. Synbiotic microparticles were produced when an overnight activated culture in MRS broth (37 °C, 24 h) with a cell load ca. 11-12 log CFU/mL in a mixture with 4% w/w alginate (Protanal LF 10/60 LS, fG 35-45%, FMC BioPolymer, IMCD, UK) and prebiotic (1.5% w/w) was infused to the spray-dryer (Büchi Mini Spray Dryer B-290, SW). Spray-drying was performed at inlet and outlet temperature of 120 °C and 58±3 °C, respectively, flow rate 6 ml/min, nozzle diameter 0.7 mm, aspirator pressure 90% and atomizer pressure 600 Nlh-1. Polyelectrolyte complexation/cross-linking of the dried powders was done under continuous stirring for at least 3 h with 0.5% w/w chitosan (Chitine, France) and 5% w/w CaCl2 (Merck, Germany) dissolved in 1% v/v acetic acid. The hardened microparticles were freeze-dried (-50 °C, 0.070 mbar, 24 h) (FreeZone Freeze Dry System, Labconco, USA) (Petreska Ivanovska et al., 2014). Analyses of the quality of ayran samples include determination of protein, fat and carbohydrate content of the ayran by Bradford, Gerber and phenol-sulphuric acid methods, respectively, while the total solids were determined using gravimetric method. Titration acidity (lactic acid,%) and pH measurements were also evaluated. AAS and HPLC was applied to quantify minerals and organic acid production, respectively. Then, to female Wistar rats (n=6, 180-250 g, 10-14 weeks old), functional ayran samples (8.5-8.9 log CFU/mL of the food product) were administered orally, once and twice daily. Plain ayran and drinking water were given to positive controls. Two weeks after starting the experiment, the rats were fasted overnight and those from the positive controls and treated groups were rendered colitic. Colitis was induced intrarectally at 8 cm proximal to the anus using TNBS (trinitrobenzenesulfonic acid) dissolved in 50% ethanol at a dose 10 and 30 mg/kg. After 6 days of continued treatment the

* tpetreska@ff.ukim.edu.mk

rats were sacrificed. Negative controls treated with 0.9% NaCl were also included in the protocol. The anti-inflammatory effects of functional ayran samples were evaluated in respect to the clinical activity/total damage score (quantified by loss on weight, consistency of feces and rectal bleeding), macroscopic and histopathological changes, colon weight/colon length and colon weight/body weight ratios, and myeloperoxidase (MPO) activity.

Results and discussion

At the day of preparation, probiotic counts of 9.43, 9.7 and 9.58 log CFU/mL were determined in ayran enriched with non-encapsulated probiotic and synbiotic, and encapsulated synbiotic, respectively. Viability loss during the shelf-life of the product was significant with viable level above the therapeutic minimum in ayran containing encapsulated synbiotic of 8.22 log CFU/mL. Total solids in the ayran samples fortified with probiotic, synbiotic and synbiotic microparticles were moderately increased due to added dry matters with no negative effect on the texture or sensory properties of the product. Significant differences of acidity and pH values were not detected between the samples, which showed no excess of acidity when probiotic was added. The content of proteins and fats in ayran samples was in accordance with the required levels (2.8 and 1 g per 100 mL of product, respectively) during the shelf-life of 15 days at 4 °C. Analyses of carbohydrates have shown increased content in samples containing non-encapsulated and encapsulated synbiotic due to the carbohydrate nature of the prebiotic, with the latter to be more significant. No significant difference for sodium content in prepared samples were observed compared to the plain ayran, while potassium and calcium content differed significantly among the samples with decreasing trend probably due to the ability of probiotic bacteria to use these minerals as nutrient sources. Exception was observed with the increased calcium content in the sample containing synbiotic microparticles which were prepared using calcium as a cross-linking agent. Increased metabolic activity of the probiotic was observed in ayran containing encapsulated synbiotic due to the higher production of lactic and acetic acid up to 81.83 mmol/L and 79.93 mmol/L, respectively. Low quantity of propionic acid (12.15-15.03 mmol/L) was determined in functional ayran samples only, but no butyric acid was detected.

In rats with induced colitis using 10 mg/kg TNBS, most significant reduction of parameters of inflammation was found in group treated by ayran containing encapsulated synbiotic. Microscopic assessment have shown ulcerations on mucosa and sub-mucosa, accompanied by extensive inflammatory infiltrate and congested blood vessels, in groups receiving plain ayran or drinking water. In the groups treated with ayran containing non-encapsulated probiotic/synbiotic visible segments of ulcerations and subepithelial polymorph nuclear infiltration were found, while higher integrity of mucosal architecture of colon tissue was seen in group treated with ayran enriched by encapsulated synbiotic. Hence, when colitis was induced using 30 mg/kg TNBS, sample containing encapsulated synbiotic was administered. The lowest value of MPO activity was observed when ayran with microparticulated synbiotic was given twice daily, but parameters of inflammation were not significantly different between groups administered once and twice daily. Dilated blood vessels in submucosal layer as well dilated intestinal glands were observed in the rats treated by ayran with microencapsulated synbiotic, regardless of the frequency of administration. These findings indicate that a single administration of ayran enriched by microencapsulated formulation successfully protect the viability of the probiotic through the upper GIT and ensure prolonged residence time of viable cells in the lower intestinum with no necessity to increase the frequency of usage.

Conclusion

Functional samples with maintained quality of the product ayran within its shelf-life were developed, while the ayran containing synbiotic microparticles has the advantage of increased probiotic viability and better profile of the bacterial metabolism end products known to maintain morphologic and functional integrity of colonic epithelium. Ayran enriched by microencapsulated synbiotic administered once a day provided efficient anti-inflammatory activity due to adhesive properties of the microparticles and their favorable interaction with the ayran as medium, thus showing potential to be used as adjuvant therapy in IBD.

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Macedonian pharmaceutical bulletin, **62** (suppl) 255 - 256 (2016)
ISSN 1409 - 8695
UDC: 613.2:577.112.388
Short communication

Approved health claims for amino acids in/as food supplements

Ermira Krasniqi*, Lidija Petrusevska Tozi

Faculty of Pharmacy, University "Ss Cyril and Methodius",
Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Food supplements in European Union (EU) are initially regulated by Directive 2002/46/EC (2002), according to which, a range of nutrients and other ingredients may be present in food supplements, including: vitamins, minerals, amino acids, essential fatty acids, fiber and various plants and herbal extracts. In 2006, EU adopted the new Regulation on nutrition and health claims made on food – Regulation 1924/2006, which applies to nutrition and health claims made in commercial communication, labeling, presentation or advertising on foods. Health claims are defined as 'any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health" (Reg. 1924/2006), currently the list of permitted health claims made on food in EU, is established by Commission Regulation (EU) No 432/2012 (Reg.432/2012).

Meanwhile, defined by the congress of Dietary Supplement Health and Education Act (DSHEA, 1994), similarly with the Directive 2002/46/EC (2002), a "dietary supplement" is a product containing a "dietary ingredient" intended to supplement the diet. According to FDA – DSHEA (1994) these products are not subject to premarket safety evaluations, allowing for products labeling claims as long as it does not diagnose, prevent, treat or cure a specific disease.

Amino acids

Amino acids are big part of cells, muscle and tissue, this way carrying out a big role in many body functions, cells structure, transport and storage of nutritious. Amino acids have been object of many scientific studies and there are many ongoing studies, consequently research over years claim for the possibility of useful of amino acids against osteoporosis, diabetes, heart trouble, metabolic disorders, erectile dysfunction, menopausal complaints etc.

This paper presents a literature review of health claims on the current state of the health claims of amino acids as dietary ingredient of dietary supplements in general, focusing in amino acids, such as: Arginine (conditionally essential amino acid in humans); Glutamine (non-essential and conditionally essential in humans); Lysine (an essential amino acid in humans).

Arginine is the precursor of nitric oxide, an endogenous messenger molecule involved in a variety of endothelium-mediated physic ological effects in the vascular system. Generally speaking, is involved in many metabolic processes and many studies so far argue for its importance in heart disease and high blood pressure, improvement of circulation, strengthening of immune system and a positive influence on male libido. Arginine supplementation is considered safe, but people with renal failure or hepatic disease may be unable to appropriately metabolize and excrete supplemental arginine, this way they should be monitored. According to Scientific opinion of EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) for Health Claims of L-arginine (EFSA FEEDAP Panel, 2015) the claimed effects "erection", "supporting spermatogenesis", effect "vascular system", "normal blood pressure" are considered of a beneficial physiological effect and L-arginine itself is considered to be sufficiently characterized but there is no cause-effect relationship established apart from the role of arginine contributing to protein synthesis.

Glutamine is the most abundant free amino acid in human blood. Glutamine is important as a constituent of proteins, means of nitrogen transport between tissues, important in acid base regulation, gluconeogenesis, as a precursor of nucleotide bases and the antioxidant glutathione. Also glutamine is considered important in proliferation of lymphocytes and other rapidly dividing cell, includ-

* ph.ermirakrasniqi@gmail.com

ing the gut mucosa and bone marrow stem cells. Many researchers suggest that glutamine may provide benefit for surgery, trauma, help support digestive system health after periods of physical stress, wound healing, helps support immune system health after periods of physical stress, helps to assist in muscle cell repair after exercise, cancer and bone marrow transplantation and it`s administration is well tolerated. Meanwhile, according to Scientific opinion of EFSA - NDA for Health Claims of glutamine such as "immune health" and "intestinal health" are considered of a beneficial physiological effect (EFSA FEEDAP Panel, 2015) and health claim referees to faster restoration of glycogen stores in skeletal muscle after strenuous exercise, "increasing cell swelling, volumization", "muscle protein metabolism" and "improves muscles metabolism" (EFSA FEEDAP Panel, 2015) are considered of a beneficial physiological effect but based on scientific data the Panel concludes the relationship of cause and efficacy of the above claimed effects has not been established.

Lysine is converted to acetyl CoA, a critical component in carbohydrate metabolism and the production of energy. Lysine is precursor of the carnitine, which is transporting long-chain fatty acids into the mitochondria for energy production. According to studies, supplemental L-lysine has putative anti-herpes simplex virus activity, also because lysine seems to have an antagonist effect with arginine, which is required for the replication of HSV. However, according to Scientific opinion of EFSA (EFSA FEEDAP Panel, 2015) for Health Claim of L-lysine maintenance of bone is considered of a beneficial physiological effect and other health claims such as "immune defense against herpes virus", "maintenance of normal blood LDL-cholesterol concentrations", "increase in appetite leading to an increase in energy intake", "contribution to normal protein synthesis" might be of a beneficial physiological effect but no cause and effect relationship has not been established. Studies have shown that L-lysine can contribute to a positive Ca balance, suggesting a potential use of L-lysine supplements for preventive and therapeutic intervention in osteoporosis.

Conclusion

Generally speaking, food supplements can be beneficial to the health of consumers, but based on a general review any health claim of food should be scientifically justified toward protection of people from any bed interpretation and information.

But still, a part of all the studies and researches, amino acids still don`t take their credit yet and are not properly recognized in medical sciences in Europe, since the health claims of food containing amino acids are still considered to be not established in terms of cause and effect relationship. Therefore, analyzing all the publications in this direction, the faster this process goes, the greater will be the chances for health benefits amongst population in relation to amino acids uses.

As it is well said by the Millward: "Few issues in nutritional science have aroused such long-standing and deep-seated controversies as protein and amino acid requirements?" (Millward et al., 1997).

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Macedonian pharmaceutical bulletin, **62** (suppl) 257 - 258 (2016)
ISSN 1409 - 8695
UDC: 615.373:616.006.6
Short communication

Preclinical studies for evaluation of antitumor effects and normal tissue toxicity of antibody conjugates

Darinka Gjorgieva Ackova*, Katarina Smilkov, Emilija Janevik-Ivanovska

Faculty of Medical Sciences, Goce Delčev University, Krste Misirkov 10-A, P.O 201, Stip 2000, R. Macedonia

Introduction

Antibody conjugates are therapeutics that function through mediating alterations in antigen or receptor function, modulating the immune system (for example, changing Fc function and T cell activation) or delivering of a specific drug conjugated to antibody that targets a specific antigen (Scott et al., 2012; Weiner et al., 2010). Four main classes using antibodies are investigated to target cytotoxic agents to cancer cells: antibody-protein toxin conjugates, antibody-chelated radionuclide conjugates, antibody–small-molecule drug conjugates, and antibody-enzyme conjugates (administered together with small-molecule prodrugs) (Teicher and Chari, 2011). Only antibody-radionuclide conjugates and antibody-drug conjugates have reached the regulatory approval stage. Radio immunotherapy, exploits the specificity of an antibody to deliver a radionuclide, and affords some potential benefits over conventional radiotherapy. Antibody-radionuclide conjugates have been successfully developed for the treatment of non-Hodgkin's lymphoma (NHL), resulting in the approval of 131I-tositumomab (Bexxar) and 90Y-ibritumomab tiuxetan (Zevalin), which are CD20-targeted agents (Maloney et al., 2010). Important steps that are necessary to transform monoclonal antibodies (mAbs) in drugs for human use must be followed to achieve success in treatment of cancer patients with antibodies. Furthermore, dealing with the challenges in the process of target selection and selection of conjugate elements including the design of antibody formulation is also imperative.

Development and evaluation of antibody conjugates

The successful development of candidate antibodies involves complex evaluations, concerning cancer biology and the properties of antibodies in vivo. Essential preclinical characterization includes identification of the physical and chemical properties of the antibody; detailed analysis of antigen expression using normal and malignant tissues; study of the immune effector functions and signaling pathway effects of the antibody; toxicity assessment; analysis of in vivo antibody localization and distribution in tumor systems; and observation of the in vivo therapeutic activity of the antibody, alone or conjugated with radioactive isotopes or other drugs.

In case of NHLs, the biodistribution studies of a radioconjugate in the tumor tissue and an assessment of whole-body toxicity and dosimetry were essential in preclinical trials leading to approval of CD20 specific radioimmunoconjugates tositumomab and ibritumomab for treatment by US Food and Drug Administration (FDA) (de Bono and Ashworth, 2010). Rituximab also has considerable success in treatment of patients with CD20 positive NHL and chronic lymphocytic leukemia. Radioimmunotherapy with rituximab labeled with suitable radioisotopes, is new opportunity after promising preliminary results where preclinical data demonstrated improved tumor response (Scott et al., 2012).

Toxicity studies

One of the most essential steps in the evaluation of a potential diagnostic/therapeutic antibody is determination of the toxicity of an antibody (often radiolabelled) and the ratio of antibody uptake in the tumor versus normal tissues. This information is essential for the rational design of antibody conjugates therapy, where uptake of antibodies by normal tissues is crucial for predicting toxicity on one side, but also is crucial for defining dose regimen where optimal tumor concentration of the antibodies will be achieved on the other.

* darinka.gorgieva@ugd.edu.mk

Normal and tumor tissue distribution can be quantified and evaluated by toxicity studies in animal models, where kinetics, distribution and induced effects in mice/rats or other nonrodent species, with or without implanted tumor are followed. These studies include administration of selected doses of radiolabelled mAbs and monitoring of tumor growth or survival of animals over time (Reilly et al., 2006). A control group of animals receives only the solvent of the formulation or non-specific radiolabelled mAbs of the same class. Generalized and gastrointestinal toxicity is followed by the weight monitoring (significant weight loss > 10-20%), while bone marrow toxicity is assessed by hematology analyses including leukocyte (WBC), erythrocyte (RBC), and platelet counts as well as hematocrit (Hct) and hemoglobin (Hb) concentrations. Biochemistry analyses included serum alanine aminotransferase (ALT) for liver toxicity and creatinine (Cr) levels for renal toxicity. In addition, samples from different tissues (liver, kidneys, etc) are obtained for hystopatological examination by light or electron microscopy. Potential radiotherapeutic agent should demonstrate specific anti-tumor effects targeted only tumor tissue with only minimal to moderate toxicity to normal tissues.

Conclusions

Varied and newly designed antibody conjugates are currently directed toward various tumor targets in clinical trials, and more are nearing clinical trial. Before reaching any possibility for patient's treatment, successfully passed preclinical phase is essential. The future development of antibody conjugates as therapeutics in cancer treatment is dependent on data from laboratory studies, on applying innovative approaches to target and antibody selection and on appropriate development strategies, leading at the end, to clinical benefit in cancer patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 259 - 260 (2016)
ISSN 1409 - 8695
UDC: 615.276.036.8
Short communication

The pro-inflammatory effects of the organic phase obtained during Cosorb process observed in different animal strains

Cristina Adriana Dehelean[1]*, Codruta Soica[2], Georgeta Simu[3], Iulia Pinzaru[1], Dorina Coricovac[1]

[1]*Department of Toxicology, "Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania*
[2]*Department of Pharmaceutical Chemistry, "Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania*
[3]*Department of Physical Chemistry, "Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania*

Introduction

Animal models are used intensively in scientific studies regarding cancer, immune or autoimmune diseases as a relevant resource for gathering data concerning the apparition, the development and the progression of these diseases. In the field of environmental toxicology, the animals, also, play important roles in establishing the degree of toxicity of the potential toxic agents.

In the scientific research the mice are preferred as animal models. The strain of mice is chosen by respecting different conditions, such as: the type of experiment that will be performed, the susceptibility of the mice to develop the pathology or the signs specifics to the model desired, the mouse genetics, the lifespan and other factors experiment-related.

The Cosorb process is known as the process used for the recovery of carbon monoxide (CO) from gas mixtures. This method requires an equimolar amount of CuCl and $AlCl_3$ that will be added to an excess of toluene and the result will be the formation of a $CuAlCl_4$-toluene complex that is capable to fix CO (Foster, 2007).

The objective of this study was to evaluate the pro-inflammatory effect of the organic phase resulted during the Cosorb process by the means of ear-inflammation mouse model using 3 different strains of mice: Balb/c, SKH-1 and C57BL/6J mice.

Materials and methods

For this experiment there were used male Balb/c, SKH-1 and C57BL/6J mice weighing between 23-35g, purchased from Charles River Labs, Budapest, Hungary and housed in standard conditions in the Animal house Facility from our University.

The organic phase composition consists of the following aromatic volatile compounds: toluene (84.46%), xylems (5.3%) and benzene (0.1%). The ear-inflammation mouse model protocol proposed in this study was performed as described in the literature (Dkhil et al., 2010; Iyadomi et al, 2000) with several modifications. In brief, different volumes of the organic phase (20, 40 and 80 µl) were applied using a micro-pipette on the skin of the front and back of the right ear and the left ear was untreated in order to be used as control. The ear swelling was monitored for 24h post-application and at different time points: 2h, 4h, 8h and 24h were taken photos and the mice were weighed. At every time point mentioned, the mice were sacrificed under anesthesia and the both ears were cut off, sized and weighed. The increase in ear's weight determined by the organic phase application which represents the intensity of ear edema was calculated by subtracting the weight of the treated ear from that of the left ear, untreated, used as control (Dkhil et al., 2010; Wang et al., 2011).

Samples from control and treated ears of mice were collected and fixed in formalin solution for histopathological analysis. The samples will be stained with hematoxylin and eosin and analyzed.

* cadehelean@umft.ro

Results and discussion

The aim of our study was to evaluate the pro-inflammatory effects of the compounds found in the organic phase: toluene, xylene and benzene as a mixture of organic solvents by using animal models of ear inflammation. We decided to use three different strains of mice (Balb/c, SKH-1 and C57Bl/6J mice) to verify if the species variability influences the effects of these compounds at skin level.

Our data indicated the presence of erythema, swelling and edema, first signs being observable earlier than 2 hours post-application of the organic phase in all three strains of mice. The weights of the right ears treated with the organic phase solution were bigger as compared to the left ears untreated used as controls, a marker of ear edema intensity. The capacity of inducing ear edema was dose dependent, the intensity of the effects being higher at the highest volumes added.

In a previous study, we showed that topical application of organic and inorganic phases induced modifications in the physiological cutaneous parameters (hydration, transepidermal waterloss and erythema) in a hairless SKH-1 mouse model (Coricovac et al., 2015; Simu et al., 2016).

The use of xylene as an inducer of ear edema mouse model was associated with acute inflammation characterized by severe vasodilation, edematous changes at skin level and infiltration of inflammatory cells (Igbe et al., 2010). Another signs of xylene toxicity at skin level after topical application were: increased vascular permeability, swelling within the dermis and proliferation of keratinocytes (Dkhil et al., 2010). Iyadomi and coworkers showed that application of toluene to the ear skin of Balb/c mice induced ear swelling and skin irritancy in a dose dependent manner (Iyadomi et al., 2000). Similar results were observed in our study, too.

Toluene is an aromatic organic solvent known as an environmental toxicant. Inhalation of toluene was associated with airway inflammation and neurotrophin production in hippocampus of mice. Benzene and xylene induce irritation of the airway and dizziness in humans. Volatile organic compounds as toluene, benzene and xylene were described as substances able to determine inflammatory responses at pulmonary level, but also to modulate neurological responses in brain (Wang et al., 2012).

According to the literature, Balb/c mice are susceptible to develop tumors, like mammary and colon cancer, to infection diseases and as animal models for ear edema studies by the dermal application of different inflammatory agents (Chen et al., 2005; Iyadomi et al., 2000).

SKH-1 hairless mice are a strain used very often in studies concerning the development of skin cancers and, in pathologies involving skin changes, due to the fact that the lack of hair offers a better observation of the changes that occur.

Conclusion

Our preliminary data indicate that the application of the organic solvents mixture induced ear inflammation in all three strains of mice used in the study. The information obtained in the present study is valuable, especially from the point of toxicity that might develop by the workers in the industry of CO recovery and, in addition, this mixture could be used as an agent for animal model of ear edema in order to test the anti-inflammatory effects of different compounds.

Acknowledgment

This research was supported by the PN-II-PT-PC-CA-2013-4-0612 grant, nr. 110/2014 (REMACAT).

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Macedonian pharmaceutical bulletin, **62** (suppl) 261 - 262 (2016)
ISSN 1409 - 8695
UDC: 615.322.015.3
Short communication

Antioxidant versus toxic capacity of selected herbal products

Blagica Jovanova*, Marija Hiljadnikova-Bajro, Tatjana Kadifkova Panovska

Institute of Applied Biochemistry, Department of Toxicology, Faculty of Pharmacy,
Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

ROS and antioxidant defense

In the past 20 years numerous achievements were made in the area of testing the cellular damage caused by the excessive production of free radicals. Reactive oxygen species (ROS) are strongly related with the onset of diseases such as cancers, atherosclerosis, neurodegenerative diseases, infections, chronic inflammatory diseases, diabetes, autoimmune diseases, hypertension, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma.

ROS are produced from molecular oxygen as a result of the normal cellular metabolism in living organisms or as a result of environmental factors. The three major types of ROS that are of physiological significance are superoxide anion, hydroxyl radical and hydrogen peroxide. They are essential for the physiological signaling pathways and other cell processes. However, these highly reactive molecules, if present at high concentrations, damage the cell structures (such as lipids, proteins, nucleic acids) and alter their functions. The disbalance between the oxidant and antioxidant effects inside a viable cell in favor of the oxidants is described with the term "oxidative stress". Oxidative stress occurs when the balance between antioxidants and ROS is disrupted because of either depletion of antioxidants or accumulation of ROS. Consequently, high concentrations of ROS result in a higher degree of oxidative stress leading to many pathological conditions.

Aerobic organisms have integrated antioxidant systems that are usually effective in blocking the harmful effects of ROS. If present in small quantities, antioxidants are powerful agents that can prevent or reduce the oxidative destruction of biomolecules. Generally, antioxidants can be divided in two categories: enzymatic and nonenzymatic. The major enzymatic antioxidants are superoxide dismutase, catalase, glutathione peroxidase, thioredoxin, peroxiredoxin, glutathione transferase. Nonenzymatic antioxidants include vitamins C and E, β-carotene, uric acid, and glutathione. However, in pathological conditions, the antioxidant systems can be overwhelmed, causing disbalance of ROS/antioxidant index. Consequently, additional source of antioxidants is essential, to enhance the metabolism against the excess of free radicals.

Natural sources of antioxidants

Beside the many benefits of the plant utilization for different reasons, herbs have been identified as sources of various phytochemicals, many of which cause antioxidant effects. For a very long time, herbs were the only medicinal therapy that people used in the treatment of various diseases. Although synthetic drugs were developed in the 19th century and replaced herbal therapy in many fields of treatment, herbs are still found in 40% of prescription drugs. Additionally, herbs are also used in the cosmetics industry, food industry and chemical industry.

Herbs and spices are reliable sources of potential bioactive compounds in human diet. Research indicates that these bioactive components may act alone or in combination with other bioactive moieties to reduce disease risk through their antimicrobial, anti-inflammatory, antirheumatic, hepatoprotective, antimutagenic, and anticancer activities. They are rich in phytochemicals that are natural antioxidants, of which phenolic diterpenes, flavonoids, flavanols, alkaloids, tannins and phenolic acids are the most prominent. Compared to other food products, herbs and spices used in diet are shown to manifest highest antioxidant capacity. Additionally, the antioxidant capacity has shown to be concentration dependent, to a degree where the naturally occurring phytochemicals with antioxida-

* blagicajovanova@hotmail.com

tive properties are not manifesting their prooxidative effects. These results are of great importance in many research areas especially for the possibility of isolation of single compounds and their utilization in medicinal purposes (Charles, 2013).

In many research papers it is pointed out that the intake of herbs is a significant contributor to the antioxidant intake in general. Dragland et al. (2003) declare a 1000-fold difference among antioxidant concentrations of various herbs, indicating the enormous diversity of their phytochemical composition. Furthermore, they recommend the use of herbs as a better source of dietary antioxidants than many other foods including fruits, berries, cereals and vegetables.

Quantification of the antioxidant capacity

Many commercially available herbs are regularly used as teas, extracts, infuses, decocts or aromatic herbs for taste improvement of food. Therefore, their antioxidant properties are already affecting the population that consumes them. The quantification of their antioxidant potential is very important, in order to evaluate their impact on the redox processes in the organisms. Various methods are used to investigate the antioxidant properties of plant samples. In general, antioxidant assays are classified as *in vitro* and *in vivo* methods, with a highlight on the first group of tests since they are relatively straightforward to perform and most of them are fast and inexpensive, compared to *in vivo* methods. Among the *in vitro* methods, the most commonly used are DPPH (1,1-diphenyl-2-picrylhydrazyl) method, FRAP (Ferric Reducing Antioxidant Power) assay, TRAP (Total radical-trapping antioxidant parameter) method, TEAC (Trolox equivalent antioxidant capacity) method, NRD and SRD (Nonsite Specific and Site Specific Hydroxyl Radical-mediated 2-deoxy-D-ribose Degradation) methods, TBA (Thiobarbituric acid) method, H_2O_2 (Hydrogen Peroxide Scavenging) assay, etc. Additionally, Folin – Ciocalteu method for the determination of total phenols and $AlCl_3$ method for the determination of flavonoids in plant samples are most commonly used assays along with the previously mentioned antioxidant assays, since many studies showed the positive correlation between antioxidant capacity of plant extracts and their total phenolic content.

Cytotoxicity testing

On the other hand, many herbs that possess antioxidant properties can also manifest toxic effects under certain conditions. According to Paracelsus' maxim: "Dosis sola facit venenum" (The dose makes a substance poisonous), any substance could be defined as a toxic substance if administered in high doses. An excellent in vivo preliminary model for the discovery of potential toxic agents is the Brine Shrimp Lethality Assay (BSLA). This is a convenient, fast and technically feasible method for the determination of LC_{50} of herbal extracts (Meyer et al., 1982). The method determines the percentage of dead *Artemia salina* nauplii after their exposure to the tested plant extract. Using probit regression analysis (Finney, 1952), the LC_{50} values are calculated and expressed for each herbal extract, which are later compared to the LC_{50} values of confirmed toxic substances (such as potassium dichromate, emetine hydrochloride etc). The classification of the examined plant extracts is achieved according to the obtained LC_{50} using different classification scales, of which the Meyer's scale (Meyer et al., 1982) and the Clarkson's scale (Clarkson et al., 2004) are most commonly used.

Plant extracts as cytotoxic agents

Many herbs which have proven toxic potential in certain doses, are promising cytotoxic agents. Their wide range of properties (antioxidant, antimicrobial, hepatoprotective, anticancer) makes them promising candidates for a formulation of chemo protective agents. This means a new opportunity for the clinical practice for the treatment of numerous diseases, either in a way of preventing their onset or reducing the extent of their progression. However, not all toxic substances possess cytotoxic properties. The precondition for a cytotoxic effect is the selectivity towards the tumorigenic cells of the substance itself. Therefore, potential cytotoxic agents are the compounds which specifically act on cancer cells, leaving out viable cells as intact. Although BSLA cannot estimatethe cell selectivity of the potential toxic herbs, it is still an excellent predictive tool that preliminary filters the potential candidates for a more detailed cytotoxicity research.

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Macedonian pharmaceutical bulletin, **62** (suppl) 263 - 264 (2016)
ISSN 1409 - 8695
UDC: 613.25:613.71/.73(437.6)
Short communication

Trend of obesity, sport and nutrition

Simona Bernátová[1]*, Zuzana Hegedusová[1], Katarína Dostálová[1], Soňa Wimmerová[1], Zora Gerová[2], Eva Horváthová[1], Štefánia Móricová[1]

[1]*Department of Preventive and Clinical Medicine, Faculty of Public Health, Slovak Medical University, Limbová 12, 833 03 Bratislava, Slovakia*
[2]*Health Promotion, Regional Public Health Institute, Ružinovská 8, 820 09 Bratislava, Slovakia*

Introduction

Lifestyle of our population with the advent of science and new technologies adversely changes, reduces physical activity and develops negative eating habits. Shift from malnutrition and infectious diseases to chronic diseases (cardio metabolic diseases, cancer, diabetes mellitus) is typical for current epidemiological situation (Riečanský, 2009). Obesity leads to a worse quality of life and shorter life expectancy. Mortality of cardio metabolic diseases is 2.5 times higher than the average in European countries. This current epidemiological situation in overweight and obesity is alarming. Compulsory school physical education is the only place for physical activity for a lot of children. And the number of these children is increasing. The research is based on project "Respect for Health". We analysed the relationship between participation in compulsory PE and cardio metabolic risk factors (blood pressure, weight and body mass index). The project "Respect For Health" is monitoring the situation in the cardiovascular health of secondary school students. Assessment of cardiovascular risk factors and physical activity (compulsory physical education (PE) and extra-curriculum sport activities) in secondary school children is followed by education in effective lifestyle measures.

Materials and methods

Target group in evaluating the relationship of participation in compulsory school physical education and risk factors for cardio metabolic diseases consisted of 760 students of which 295 boys and 465 girls. In respect of participation in extra-curricular sports activity and selected risk factors consisted of audience of 211 pupils, 119 boys and 92 girls from 55 secondary schools in Bratislava region aged 15-18. Data were collected by 2 types of questionnaire (questionnaire of parent and questionnaire of student). We measured anthropometric parameters (neckline, chestline and hipline), blood pressure and tested physical ability by Ruffier test.

Project: "Respect for Health" monitoring the situation in the cardiovascular health of secondary school students. The project was initiated by Bratislava Region in collaboration with the Regional Public Health Institute in Bratislava. The main idea of the project is based on the recognition that risk factors for cardiovascular diseases can occur at a young age. Elimination of a risk is possible thanks to prevention and change of eating habits. The project was implemented by the Regional Institute of Public Health in Bratislava with Public Health students of Slovak Medical University.

Results and discussion

For evaluation of the relationship of participation in compulsory school physical education and risk factors for cardio metabolic disease, we found out that students who regularly attend compulsory PE are more physically proficient than those who avoid it according to the Ruffier index. Students who spend more hours per a day at the computer during the week are physically less proficient than those who spend fewer hours at the computer during the week, according to the Ruffier index. All these relations were statistically significant ($p < 0.05$). We found out that smoking students avoid compulsory PE twice as much as non-smokers. The current problem is smoking in young children and the number of smokers in young age are mul-

* bernatova.simona@gmail.com

tiple (Cvopová et al., 2011). The number of smoking girls is 1.2 times higher than the number of smoking boys. This is really a good example of how one negative habit picks on another and they multiply. For other indicators, a statistically significant difference was not confirmed, but more favourable results in blood pressure, weight and body mass index (BMI) were shown for regularly participating students at the compulsory PE. In respect of participation in extra-curricular sports activity and selected risk factors, we confirmed that the active Ruffiers´ test of sport active children testified better shape of these students ($p = 0.048$). We also demonstrated a statistically significant relationship between regular supply of food and BMI values ($p = 0016$). Extra -curricular sports activity leads to better fit children. Regular diet directly affects BMI. Because of physical activity they feel their life filled with experiences and activities.

Conclusion

Lack of physical activity is one of the major risk factors of cardio metabolic diseases, with significant impact on years of disability adjusted life (DALYs) in the European region (WHO, 2006). Compulsory physical education and in the same way extra-curricular sports activities of secondary school children have a positive impact on cardiovascular risk factors. The results of our thesis confirmed the positive impact of compulsory PE for the child's body, and therefore the importance of taking measures for the development of physical activity in schools, in the family and beyond. Negative habits (avoid the PE and smoking) of lifestyle in young people often multiply. Qualified professional teachers of PE and as well as parents play a key role in creation of positive attitude towards physical activities in children."The movement is life" and in case of cardiovascular prevention this true are multiple (Farský, 2010). Parents should be an example for their children in a choice of extra – curricular sport activity. They should support children in sport activity with enthusiasm (Račková, 2011)

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Macedonian pharmaceutical bulletin, **62** (suppl) 265 - 266 (2016)
ISSN 1409 - 8695
UDC: 613.2-047.22P(560)\
Short communication

Turkey's highlights within inprofood (FP-7) project

SelenYegenoglu*, Bilge Sozen Sahne

Department of Pharmacy Management, Faculty of Pharmacy, Hacettepe University, Sihhiye, 06100 Ankara, Turkey

Introduction

As known by almost everyone in many EU countries' agenda food and health are in the first place especially in the last decade. The rationale for this trend is there is an increase both for obesity and for nutrition related disorders those cause diabetes and cardiovascular diseases (Gallus et al., 2015; van Vliet-Ostaptchou et al., 2014; WHO, 2009). Thus tailoring the nutrition system in parallel to the community's need, it's being reliable; effective and flexible are becoming more and more important each day.

Awareness rising on healthy eating has not led to significant changes in patterns of food purchase and consumption so far.

Bringing together industry, science, and the civil society in a meaningful exchange is essential to success in addressing this pivotal challenge.

Materials and methods

As a first step all the food related and patient related organizations belonging to governmental, non-governmental and business sector were tried to be determined. In the second phase the ones those will be enrolled to the European Awareness Scenario Workshops (EASWs) were selected randomly.

European Scenario Workshop, also known by the acronym EASW, is a qualitative research method which has been born in Denmark with purpose to find an agreement between the different group of actors at local level with the aim of reaching a consensual definition of city sustainable. It promotes discussion and participation. An EASW serves to foster democratic participation in decisions related to the improvement of living conditions in communities.

EASW methodology is particularly suited to:

- encourage dialogue and participation of the various components of society;
- creating a balanced relationship between environment, technology and society;
- enable a sustainable development while respecting the needs and aspirations of members of a local community (http://toolbox.climate-protection.eu/list-of-all-methodologies/).

According to its application, the EASW method could be a tool for:

- information and learning,
- understanding and participation in the decision making process, common planning for the future
- identifying responsibilities and priorities or just any combination of the above.

An EASW is built on three main activities:

a.the development of scenarios

b.stakeholder mapping

c.EASW workshop for the development of visions and ideas

The workshop typically lasts two days and goes through three phases: critical analysis phase, visionary phase and implementation phase (http://www.cipast..org/cipast.php?section=1012).

Results and discussion

In the capital of Turkey we conducted three EASWs (one in 2012 October and two in 2013 June and September). Totally 47 representatives from non-governmental,

* selen.yegen@gmail.com

governmental and business sector participated in our Scenario workshops.

In those EASWs the following were discussed:

a.How research management and policy should be on food and health?

b.In this sense should every kind of research be funded?

c.Which type of research should be supported? : Basic or practical research aiming contemporary issues?

d.How research politics will be determined and shaped on innovative food production?

Under these topics the following were found in terms of "Nutrition and Innovative Approaches on Food Production" for Turkey among NGO, business sector and government sector participants respectively:

Change in living conditions (global warming, population growth, GMO, etc.) is a significant factor. Civil society opinion is ignored while doing research on innovative food production. Resources are being used ineffectively while doing food research. There is a lack of information on healthy nutrition among the society. Scientific research outcomes are not reflected to the policies. As the inequalities become deeper, hunger; obesity; and social problems become more. The media directs the individuals in a wrong way. Use of agricultural land(s) for different aim(s) is a big problem. There is a lack of inspection in food sector. Healthy food options are expensive. Although the Consumer Protection Act exists there is a violation of consumer rights.

Resources are being wasted unwisely in researches. In order to make "healthy nutrition as a never ending process", independent authorities (i.e. EFSA) should be established. There exist unfair competitions (both internal and external). There is very easily licensing process/easy certification process on food production. Lots of misleading information are observed Consumers are unconscious in many ways. There is a deterioration of the food because of the long distances, etc. Unconsciousness exists in the steps of healthy food production.

Institutions' working together is a must. On the other hand bureaucracy is huge. Scientific data are not available or accessible. Furthermore the ones those are accessible lacks quality. There is a slow process in research and publication. Lack of national database is a big problem. Lack of National Policies for Sustainable Agriculture is another issue.

Conclusion

In the light of the conducted EASWs we concluded that the projects should be handled according to the national policies other than governmental policies. An independent institution is a must which will make healthy nutrition researches. This institution should also raise awareness among the community and make risk assessment. Innovation studies based on reliable and scientific data should be conducted. Three should be continuous dialogue/trialogue between/among NPO, government/public and academia/ research institutions.

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Macedonian pharmaceutical bulletin, **62** (suppl) 267 - 268 (2016)
ISSN 1409 - 8695
UDC: 614.876:575.224.46]:614.23(560)
Short communication

Assessment of cytogenetic damage and oxidative stress status in hospital staff occupationally exposed to ionizing radiation

Ayşe Eken[1,2]*, Ahmet Aydın[1,3], Onur Erdem[1], Cemal Akay[1], Ahmet Sayal[1], İbrahim Somuncu[4]

[1]*Gülhane Military Medical Academy, Department of Toxicology, 06018 Ankara, Turkey*
[2]*Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 38039 Kayseri, Turkey*
[3]*Yeditepe University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 34755 İstanbul, Turkey*
[4]*Gülhane Military Medical Academy, Department of Radiology, 06018 Ankara, Turkey*

Introduction

Ionizing radiation (IR) has been found to produce deleterious effects on the biological systems. IR is known to induce mutations and cell transformations, predominantly by causing single-strand and double-strand DNA breakage, thereby leading to chromosome instability and carcinogenesis (Hayata, 2005). Genetic instability can be analyzed by using cytogenetic parameters such as sister chromatid exchange (SCE), and micronuclei (MN). It is known that cytogenetic damage accumulates in humans with age, due to the prolonged exposure to oxidative damage, chemicals as well as occupational, therapeutic or accidental radiation (Ramsey et al., 1995). However, studies on genotoxic effects of low dose occupational exposure is limited and with contradicting results.

IR is known to induce oxidative stress through generation of reactive oxygen species (ROS) resulting in oxidative damage to biomolecules such as deoxyribonucleic acid (DNA), proteins, and lipids (Chi et al., 2005). Aerobic organisms possess antioxidant defense systems that deal with free radicals produced as a consequence of aerobic respiration and substrate oxidation. Cells maintain their vital functions against oxidative stress status with the help of an antioxidant defense system that includes three major classes of antioxidant enzymes: the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Sies, 1997). It has been hypothesized that chronic low-dose IR leads to oxidative stress but there are few studies to clarify oxidative stress status in radiology workers (Malekirad et al., 2005).

The aim of this study was to evaluate the genotoxic effects and oxidative stress status of occupational radiation exposure in radiology unit staff.

Materials and methods

The study population of 40 hospital workers occupationally exposed to IR was comprised of 12 physicians and 28 technicians in the units of radiology at the Gulhane Military Medical Academy in Ankara, Turkey. Radiology unit personnel were healthy volunteers occupationally exposed to IR working 5 h/day in the hospital for a period of one to 30 years. The control group consisted of 30 individuals (10 physicians and 20 administrative staff) working in the same hospital who had never been occupationally exposed to IR or chemicals. None of the study group participants had reported unusual alcohol consumption. Vegetarians and those who used vitamin supplements, antioxidants or any therapeutic drugs were excluded. No one had undertaken any medical radiological examination or any carcinogenic agent in the 6 months before blood sampling.

The occupational exposure group were routinely monitored by personal exposure measurements devices (film badges), every 40 days. The radiation dose was estimated from the official personal dosimeters based on thermoluminiscent dosimeters. This dose range represents only the dose from a single month exposure and was measured within 6 months prior to enrolment in the study.

Genotoxic effects were determined by using the cytokinesis-blocked micronucleus (CB-MN) and SCE tests in peripheral blood lymphocytes. Oxidative stress status was assessed by measuring the antioxidant enzyme activities of copper-zinc superoxide dismutase (CuZn-SOD), selenium

* eken.ayse@gmail.com

dependent glutathione peroxidase (Se-GPx), CAT and the malondialdehyde (MDA) levels as lipid peroxidation index in erythrocytes.

All statistical analyses were performed using the software SPSS 13.0 for Windows statistical package. Groups were compared with the Student t-test. The results were expressed as mean value ± standard deviation (SD). Values of $p<0.05$ and $p<0.001$ were considered to be statistically significant.

Results and discussion

The mean exposure dose of the radiological staff was 0.32±0.69 mSv ranging from 0.10 to 3.86 mSvper month according to their individual dosimeters. None of them had recorded doses above the permitted levels of 20 mSv.

It was found that the mean frequency of MN was significantly increased in radiation-exposed group compared to control group ($p<0.05$). This finding is in agreement with other studies (Maluf et al,2001; Zakeri and Assaei, 2004; Angelini et al., 2005). The mean frequency of SCE did not show any significant difference in the exposed individuals in comparison to the controls ($p>0.05$). However, Engin et al. (2005) and Mrdjanovic et al. (2005) revealed that the frequency of SCE was significantly increased in all radiation-exposed individuals compared with controls. The enzymes activities of CuZn-SOD and Se-GPx observed for the exposed group were very significantly higher than in the control group ($p<0.001$).

Oxidative stress causes various types of damage followed by cell death or an activation of the repair systems. Antioxidant enzymes are considered as a very important component of cell defense mechanisms which protect organisms from the detrimental action of ROS damaging DNA and other biomolecules (Dimova et al. 2008). The enzyme activity of CAT and MDA levels in the exposed group were found significantly lower than in the control group ($p<0.05$, $p<0.001$, respectively). Our findings may be explained in terms of chronic low-dose radiation induced radiation hormesis. Low-dose radiation has been reported to induce hormesis, which is a beneficial stimulant effect of chronic low-dose radiation and radiation-adaptive response (Prasad et al. 2004). However, its molecular mechanism or signaling pathway is not yet known and needs to be clarified (Miura et al. 2002). Additional studies will provide essential information; in particular, larger and well-designed studies may be helpful.

Conclusion

We observed that low dose chronic occupational exposure to ionizing radiation causes an increase of MN frequency in chromosomes, even though the absorbed doses were below the permissible limits. Although the small sample size prevents a definitive conclusion on the oxidative stress induced by occupational low dose IR exposure, nevertheless, our experimental evidences highlight the stimulant effect of the low dose IR on oxidative stress related parameters in hospital staff and enhanced resistance to oxidative stress.

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Macedonian pharmaceutical bulletin, **62** (suppl) 269 - 270 (2016)
ISSN 1409 - 8695
UDC: 613.929-053.6
Short communication

Dietary supplement use among adolescents

Gordana Svonja Parezanovic

Medical High School "7 April" Novi Sad, Serbia

Introduction

Dietary supplements are defined as products intended to supplement the diet. They may contain a variety of ingredients including vitamins, minerals, amino acids, herbs or other botanicals, or other dietary substances "for use by man to supplement the diet by increasing the total dietary intake". Dietary supplements are in the form of capsules, tablets, pills, and other similar pharmaceutical forms (Dietary Supplement Health and Education Act, 1994).

Medical evidence has suggested that dietary supplementation can be beneficial for a group of people, who do not have a balanced diet. In such cases, after nutritional deficiency has been detected, the increase in the intake of such nutrients is recommended, either by means of food or supplements (Gardiner et al., 2004).

Age range that constitutes youth is important in many aspects. Physical growth and mental development of young population in adolescence will affect future physical and psychological chances as adults. The use of dietary supplements among adolescents seems to be influenced by their beliefs and attitudes that dietary supplements work. The media has contributed to stimulate the use of dietary supplements by spreading, for instance, the myth of the ideal body.

Consumption of supplements in European countries ranged between 17.9% and 60% of the population (Flynn et al., 2009, Tetens et al., 2011).

During adolescence, a period when self-assurance is being developed, many adolescents do anything they can to meet that goal. Dietary supplements are sold in drugstores or gyms as over-the-counter products without advice from a pharmacist.

Based on the issues mentioned above, the objective of this study was to determine the frequency of dietary supplements consumption, types of supplements being consumed and reasons for consumption among high medical school students.

Materials and methods

The survey was conducted using an anonymous questionnaire. The total of 238 students of medical high school, of which 162 were girls and 76 boys, were surveyed. The questionnaire includes gender and grade, as well as questions concerning types and quantities of dietary supplements respondents use. The survey was conducted in April 2015. The methods of descriptive statistics were used in processing and analysing data, and then the differences of the obtained values were assessed via the chi-square test. In all analyses, differences were interpreted as statistically significant if the p-value is less than 0.05, and statistically significant if the p-value is less than 0.01.

Results and discussion

Of the total number of respondents 67.2% had used dietary supplements. Overall, 79% of participants were female. To determine the differences between gender with respect to dietary supplements consumption, chi-square test was used. Based on this test, it can be concluded that there is a statistically significant difference between the groups analysed ($p = 0.00012$, $\chi^2 = 20{,}626$). No significant association was observed between supplement use and age.

Of the total number of users of dietary supplements, 64.78% mostly bought in pharmacies, while others were purchased in supermarkets. Information on these medicines 38.8% search on the Internet, 26.12% of pharmacists, and 11.94% of the doctors. The most commonly used dietary supplements are vitamin C, then multi-vitamins and minerals (66.26%). Most of the respondents pleaded not regularly read the declaration of the purchased products (72.4%), while 73.88% thought that there was insufficient

* gsvonja@yahoo.com

information on indications and side effects

The most commonly cited reasons for usage are to maintain or improve health (68%), to increase energy (25%), to build muscle or increase weight (28%), to decrease body fat or weight loss (11%). Gender differences in types of dietary supplements are apparent. Males were more likely to consume supplements to build muscle ($p = 0.006$), and females were more likely to consume supplements to increase weight ($p = 0.002$).

The most common forms of dietary supplements being used are multivitamin, or individual vitamin/mineral preparations (particularly vitamin C). This finding is consistent with the data reported from other study (Kang et al., 2016; Moreno et al., 2014). This supplement, as the main reported supplement used among interns, indicates that they may be concerned about their nutritional adequacy in general. Thus, they selected a type of supplement providing a variety of nutrients.

Some studies shows the most commonly cited reasons for usage are to maintain or improve health, to increase energy, to build muscle or increase weight, to decrease body fat or weight loss, to increase athletic ability, to help heal injury or illness, or because of an inadequate diet (Bailey et al., 2013; Dwyer et al., 2013; Kang et al., 2016) what is similar with findings in this study.

Dietary supplement use in adolescents remains a controversial strategy to improve nutrient intakes because, even though their use is associated with lower prevalence of inadequate intakes, it is also associated with an increased risk of excessive intakes.

As the dietary supplement industry is now a multi-billion dollar industry, there is growing pressure, and a subsequent need for research to establish the efficacy and safety of these products particularly for adolescent users. The psychological and educational components of such use cannot be ignored as they play an equally important role in the health and safety of adolescents.

Conclusion

It is clear from the results of the current study that dietary supplementation is popular among adolescents.

There is need of further scientific research involving adolescents with the purpose of assessing the beneficial effects and safety of long-term dietary supplements use. More screening tests should be conducted on the consumption of dietary supplements among adolescents. Toxicity surveillance should be improved, and regulations of dietary supplements market should be based on appropriate research. Nutritional education of adolescent is highly important, especially among future medical workers.

Finally, regulations on this topic could assist the activity of health professionals and improve the education of the general population about the safe and effective use of dietary supplements.

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Macedonian pharmaceutical bulletin, **62** (suppl) 271 - 272 (2016)
ISSN 1409 - 8695
UDC: 616.61-008.64:616.155.194
616.61-008.64:616.155.294
Short communication

Acute and chronic renal failure related with anemia and thrombocytopenia

Milena Spasovska[1]* and Tatjana Kadifkova Panovska[2]

[1]*P.H.I. General Hospital - Ohrid, Sirma Vojvoda 1, 6000 Ohrid, R. Macedonia,*
[2]*Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

Renal failure (acute and chronic) is described as a decrease in glomerular filtration rate (GFR). It is a condition in which the kidneys are unable to adequately filter toxins and waste products from the blood. The cause of acute renal failure (ARF) is divided into sources of renal injury such as pre-renal, intrinsic and post-renal. In ARF there is an increase of blood urine nitrogen (BUN) and serum creatinine, and decrease urine production. It is characterized by abrupt decline in renal filtration function (Haller, 2000).

In chronic renal failure (CRF) the amount of creatinine is higher and glomerular filtration rate is lower. CRF is a progressive loss in kidney function over a period of time. In the early stages, there may be no symptoms but it gets worse gradually. The adverse outcome of CRF is kidney failure, cardiovascular disease (CVD), and premature death (Park, 2012).

Chronic and acute renal failure can be primary distinguished by different characteristics - cause and duration. CRF is a lifelong problem and it tends to get worse over months or years. Presence of disease in last few months, normochromic anemia, growth failure, and a history of nephritic or nephritic syndrome, high blood pressure, diabetes mellitus, kidney stone, glomerulonephritis and infection make chronic renal failure more likely to happen (Saucier, 2010). Acute renal failure has a sudden onset and can occur as a complication of medical conditions, surgery, or trauma.

Healthy kidneys produce a hormone - erythropoietin (EPO). The kidney cells that produce EPO are sensitive to low oxygen levels in the blood that passes through the kidney. EPO prompts the bone marrow to make red blood cells (Radtke, 1979). Red cell production due to the EPO deficiency is too low in CRF and causes development of anemia in this situation. Anemia is also seen in patients with acute renal failure, but the exact relationship between them remains unclear. In addition to anemia, platelet count also seems to be affected by renal disorder. The exact pattern of platelet count in patients with renal failure is controversial, but several studies revealed the decrease in platelet count in renal failure (Gafter, 1987; Prasad, 2012).

The aim of this paper is to evaluate the effect of acute and chronic renal failure on severity of anemia and platelet count.

Materials and methods

This case-control study was conducted on 92 patients with acute renal failure and 50 patients with chronic renal failure, and two groups of 95 and 75 individuals as controls. At first, all patients with proven renal failure were included in the study. Then, patients were divided in two groups of acute (<3 month) and chronic (>3month) based on disease duration. In order to eliminate the influences of sex and age when the observation group and the controlled group are compared, each of the two groups was selected according to the same sex and similar age. Initially, two separate blood samples were taken from each patient, 2 ml uncoagulated sample harvest for biochemical assay, and EDTA anti coagulated whole blood sample for complete blood cell count. To determine the levels of BUN and creatinine, the serums were used, and then complete blood counts were done with EDTA anti coagulated samples.

Statistical independent T- test was used to evaluate the significance of the differences between the two groups. $P<0.05$ was considered as a significant change.

* spasovskamilena@yahoo.com

Results and discussion

In groups of patients with acute (53% male, 47% female) and chronic (65% male, 35% female) renal failure, mean age was 56±15 and 58±11.5 years, respectively. In controlled groups of acute (49% male, 51% female) and chronic (73% male, 27% female) renal failure, mean age was 61±12 and 63±11 years, respectively.

The average levels of BUN in patients with acute renal failure were 31.7±7.4 mmol/L, which is above the normal value, compared to the control group (6.4±2.1 mmol/L). The average of creatinine in patients with acute renal failure is also above the normal value (256.3±79.5 mmol/L) compared to the controlled groups (88.4±35.3 mmol/L).

The observation of the group of chronic patients also shows that BUN and creatinine are high (29.9±8.9 mmol/L and 740.8±180.4 mmol/L) compared to normal ranges of the controlled group (5.3±2.4 mmol/L and 88.4±12.3 mmol/L).

The levels of Hb, HCT, MCHC and RBC counts were significantly lower in the patient group in comparison to healthy, non-renal affected people ($P < 0.05$), and anemia was substantial in patients with acute renal failure.

The study has also revealed that acute renal failure did not cause significant thrombocytopenia, and even the platelet count in these patients were slightly higher in comparison to the controlled group.

In patients with chronic renal failure, RBC count, hemoglobin, hematocrit level, MCHC, and platelet count are significantly lower than the controlled group ($P < 0.05$), but MCH and MCV levels are not significantly different between these two groups ($P > 0.05$).

Conclusion

In acute renal failure the ability of the kidney to eliminate waste products, regulate acid-base balance, and manage water homeostasis is rapidly declined. When this impairment is prolonged and has entered the chronic phase, the EPO secretion by this organ is decreasing. Low EPO level causes decrease of HCT, MCV, RBC and platelet counts. Therefore, the hematological changes in the blood count can be an early diagnostic marker for kidney failure.

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Macedonian pharmaceutical bulletin, **62** (suppl) 273 - 274 (2016)
ISSN 1409 - 8695
UDC: 615.074:543.544.3
Short communication

In vivo study of the effects of different phases of the Cosorb process on skin's intrinsic properties

Simu Georgeta-Maria[1]*, Coricovac Dorina[1], Cseh Liliana[2], Soica Codruta[1], Borcan Florin[1], Ionescu Daniela[1], Andoni Mihaiela[1], Dragos Dan[1], Dehelean Cristina[1]

[1] *"Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania*
[2] *Institute of Chemistry Timisoara of Romanian Academy, 24 Mihai Viteazul, RO-300223, Timisoara, Romania*

Introduction

The Cosorb process is a solvent extraction process which provides a low cost opportunity to a selective and almost complete recovery of high purity carbon monoxide. During the process, cuprous aluminium chloride ($CuAlCl_4$) in an aromatic solvent (by instance toluene) is used in order to form a chemical complex with carbon monoxide, which is further recovered by more than 96%. So, one could consider this process as a reliable source of carbon monoxide in the case of downstream manufacture of chemicals and pharmaceuticals. However, some down sides of this process are associated to the partially poisoning of the copper catalyst, and to some environmental risks mostly related to the disposal of the used catalyst, to the recovery of copper and aluminium, as well as to the impact on human health of the different phases resulted from the process (Chadeesingh, 2011). In this respect, toxicological studies of the different phases resulting from the Cosorb process could bring valuable information related to their potential impact onto human and environmental safety.

In this work, an *in vivo* study of the adverse effects of two phases (organic and inorganic) resulting from the Cosorb process on skin hydration and barrier level was carried-on. For this aim, murine model (C57BL/6N and SKH-1 hairless mice) was used, and the evaluation of the obtained results was performed by means of Corneometry and Tewametry.

Materials and methods

The reagents used in this study were of analytical grade and were provided by Merck and Sigma–Aldrich. The inorganic phase was evaluated by High-Resolution Continuum Source Atomic Absorption Spectrometer ContrAA 700. It was found that the main metals present in this phase are copper and aluminium, while zinc, chrome and iron are in traces. The analyze of the organic phase by means of gas chromatography/mass spectroscopy (Hewlett Packard Gas Chromatograph HP 6890 associated with a Mass Spectrometer HP 5973) showed that the main compounds are toluene (84.46%) and some of its oxidation products (10.1%) (Simu et al., 2016).

The *in vivo* study was performed on 4 months old C57BL/6N and SKH1 male mice (Charles River Laboratories, Budapest, Hungary), according to the 2010/63/EU Directive (Coricovac et a., 2015; Simu et al., 2016). Mice were fed *ad libitum* and kept under standard conditions: 12-hours light/dark cycle, constant temperature of 22.5 ± 2 °C, and 55 ± 5% humidity. Each type of mice (C57BL/6N and SKH1) were divided in 4 groups (5 mice/ group) as follows: the control group (1) - no interventions; group 2 in which the mice were treated once, topically, with 200µl of inorganic phase (on the dorsal side); group 3 in which the mice were treated once a day, 5 times/week with 100µl of inorganic phase, and group 4 in which the mice were treated once a day, 5 times/week with 100µl of organic phase. The animals were daily observed both at skin's level, and from behavior changes point of view. The evaluation of the skin's response to the inorganic and the organic phase's effect was performed by Corneometry and Tewametry. The physiological skin parameters, e.g. the hydration level of the skin surface and the transepidermal

* gsimu@yahoo.com

water loss (TEWL) were measured with the Multiprobe Adapter System (MPA5) from Courage-Khazaka Electronics (Germany), equipped with Tewameter®TM300 and Corneometer®CM 825 probes.

Results and discussion

Mice are widely used as mammalian model organism in numerous studies related to subjects within the biomedical field in order to obtain answers and new insights onto the human health and welfare, in examining skin-related gene functions, as well as in modeling skin diseases. This is due to the fact that murine skin presents a great number of similarities with human skin.

In this work, SKH-1 and C57BL/6N mice were used. SKH-1 mice are a variety of unpigmented and immunocompetent mice which are characterized by a more permeable skin than the human one. Their skin is less permeable to benzo[a]pyrene than the skin of haired mice. Moreover, a prolonged *in vitro* hydration of their skin can generate a consistent increase in permeability, particularly to polar or ionized solutes. The C57BL/6N mice were used in order to emphasize the differences between pigmented skin model and the SKH-1's ones.

Corneometry is a non-invasive technique which is able to estimate the hydration level of the skin surface (mainly at the stratum corneum level). The measurements associated to this technique offer valuable information onto the modifications of the dielectric constant, due to skin surface hydration changing. So, this method presents a great deal of interest in dermatological and cosmetic applications, but also in formulation, claim support and efficacy testing of moisturizers. Corneometry is of great importance in objective clinical diagnosis, as well as for monitoring therapies.

The obtained results from the Corneometric study showed a significant decrease of the skin's hydration level in both types of mice, as well as for both studied aggressors (inorganic and organic phases). This decrease was noticed after the first 24 hours after application of the test solution, in both animal models. The decrease of this parameter may be associated to some toxicity signs, or may indicate the apparition of different injuries at skin level. It is important to note that in the case of the mice topically treated with the inorganic phase, some skin colour changes were noticed.

Nowadays, Tewametry is used for many purposes, including dermatology (for objective clinical diagnosis) and occupational medicine, for monitoring and detecting skin damages, as well as in the testing of cosmetic products and pharmaceuticals. Through this technique, the TEWL parameter is assessed. This parameter brings information onto the skin's intrinsic properties, especially as its water barrier function. The measurements evaluate the density gradient of the water evaporation from the skin indirectly, by means of temperature and relative humidity. Great values of this parameter indicate that the water barrier function of the skin become inefficient, or even some stratum corneous deteriorations. It was found that increased TEWL values can be associated with different skin diseases, but could be also induced by different chemical compounds.

The evolution of the TEWL parameter was studied both for the inorganic phase solution and the organic one. The obtained results indicate a significant decrease of this parameter in both cases. In the case of both animal models, it was noticed that the organic phase induced cutaneous damages after only 2 weeks after exposition (2 weeks). However, the progress of lesions was slightly decreased when medical treatment was applied at this stage.

Conclusion

Modifications of skin surface hydration level, and of the TEWL parameter were induced by the topical application of inorganic and organic phase solutions resulted from the Cosorb process.

These results were more obvious in the case of the SKH1 mice model, and could be associated with the first signs of a short-term pathology at the skin's level.

Acknowledgment

This research was supported by the PN-II-PT-PC-CA-2013-4-0612 grant, nr. 110/2014 (REMACAT).

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Macedonian pharmaceutical bulletin, **62** (suppl) 275 - 276 (2016)
ISSN 1409 - 8695
UDC: 613.287.5:577.164.1
Short communication

Thiamine and riboflavin content in infant formulas available in Serbia: Level of compliance with recommended dietary intake and adequacy of nutritional needs of infants

Marko Denić[1]*, Slavica Sunarić[1], Jelena Lalić[1], Gordana Kocić[2]

[1]*Department of Pharmacy, Faculty of Medicine, University of Niš, Bulevar Zorana Đinđica 81, 18000 Niš, Serbia*
[2]*Department of Biochemistry, Faculty of Medicine, University of Niš, Bulevar Zorana Đinđica 81, 18000 Niš, Serbia*

Introduction

Breast milk is considered as an ideal source of nutrition and immunological support for infants. The World Health Organization (WHO) strongly supports breastfeeding for newborns from birth until 6 months postpartum as a global public health recommendation. Sometimes mothers are unable to make enough milk for the infant feeding and in such a case, milk-based infant formulas might be used as supplement to breast milk or may also be used as substitute if breastfeeding is impossible. Human milk provides the normative standard for infant nutrition with its unique composition that differs from infant formulas, although nutrient composition of infant formulas is generally based to have similar functional properties as breast milk.

Infant formula is a product based on milk of cows or other animals and/or other ingredients which have been proven to be suitable for infant feeding. Nowadays, a wide variety of infant formulas are available, so its nutritional safety and adequacy should be scientifically demonstrated to support normal growth and development of infants, especially during the first 6 month of age.

Infant formula is not officially a pharmaceutical product, though in many cases the manufacturers are pharmaceutical companies. Regulatory bodies around the world agree that continual nutrient analysis is crucial in infant formulas quality control. The nutritional composition of all infant formulas must fulfill the global standards recommended by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition's (ESPGHAN) international expert group that was commissioned by The Codex Alimentarius Commission. These documents contain data about minimum and maximum values of nutrient contents in infant formulas with the goal to provide safe and nutritionally adequate infant formula products that meet the nutritional requirements of healthy babies during the first months of life until the introduction of appropriate complementary feeding (Owens et al., 2012).

Thiamine (vitamin B1) and riboflavin (vitamin B2) are nutritional components present in both breast milk and infant formulas which support normal infant growth. Thiamine pyrophosphate as biologically active form of vitamin B1, is a cofactor for a number of key enzymes in Krebs cycle and in pentose phosphate pathway. Riboflavin is a precursor of the flavin coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which participate in oxidation–reduction reactions in many metabolic pathways. As FAD is part of the respiratory chain, riboflavin is central to energy production. Both vitamins are essential for development of infant tissues, function of muscles, heart, nervous system and mental activity. Also they are involved in carbohydrates, lipid and protein metabolism, red blood cell formation, respiration, antibody production and normal infant growth (Lalić et al., 2014; Sunarić et al., 2012). The levels of these vitamins in infant formulas are very important for term infants during the first 6 months of life, and especially for pre-term or low birth weight infants.

The aim of this work was to determine thiamine and riboflavin content in some infant formulas available in Serbian pharmacies, as well as to define the level to which these infant formulas comply with the ESPGHAN recommendations and with Recommended Dietary Intake (RDI) for infants.

* mdenic84farm@gmail.com

Materials and methods

Ten most commonly used standard infant formulas were purchased from a local pharmacy. All analyzed brands were initial milk-based formulas designed for infant consumption during the first 6 months of life. Powdered samples were dissolved and prepared following manufacturer's instructions, in order to attain vitamins concentrations as given on the label.

Samples preparation procedures and vitamins content analysis were done according to our previously published methods with minor modifications (Lalić et al., 2014; Sunarić et al., 2012).

The percentage of compliance has been calculated with the mean minimum and maximum levels of vitamins according to ESPGHAN recommendations using the formula: Composition in 100 ml infant formula/ ESPGHAN mean value by 100 ml × 100.

Results and discussion

Vitamins contents in analyzed samples were expressed as micrograms (μg) per 100 ml of prepared milk, as given on the label. The concentrations of thiamine and riboflavin in herein examined infant formulas were in the range of 57.5-91.3 μg/100 ml and 106.7-224.8 μg/100 ml, respectively. Also, values obtained for thiamine and riboflavin content in all samples accounted for over 88.5% of the values declared on the product label, with good precision (RSD < 1.8%, n = 5).

The amounts of both vitamins in all analyzed samples exceeded the minimum requirements according to ESPGHAN recommendations (48 μg/100 ml for thiamine and 180 μg/100 ml for riboflavin). The percentage of compliance was in the range of 39.9-63.4% for vitamin B1 and 19.8-41.6% for vitamin B2, respectively. These results were within the acceptable limits found in most of the literature and very similar compared to the data in other studies conducted according to ESPGHAN recommendations.

Recommended Dietary Intake for infants (0-6 months old) are 200 μg/day for thiamine and 300 μg/day for riboflavin. Thus, assuming that the advised daily intake of 500 ml of prepared infant formula is optimal for 0- to 6-month infants, all analyzed samples supply much higher levels than the values established by the RDI for B1 (143.7-228.2%) and B2 (177.8-374.6%).

Conclusion

In this work, thiamine and riboflavin content in different infant formulas available in Serbian pharmacies was determined. Levels of both vitamins in all samples were consistent with the values declared on the products label. Results of this study revealed that analyzed infant formulas comply with the nutritional needs of infants with regard to vitamins B1 and B2. Their content in all samples exceeds the RDI values, so there is an excess of these nutrients supplementation. Moreover, the satisfactory compliance with ESPGHAN recommendations was observed so analyzed milk-based formulas might be used as adequate breast milk supplements or substitutes.

Acknowledgment

This research was supported by grant TR 31060 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 277 - 278 (2016)
ISSN 1409 - 8695
UDC: 632.95.028:543.544
Short communication

Determination of pesticide residuals by GC-ECD

Ela Hoti[1]*, Lindita Qefalia[2], Linda Matua[1]

[1]*Faculty of Pharmacy, University of Medicine Tirana, Rruga e Dibres 371, Tirane 1005, Albania.*
[2]*Food Safety and Veterinary Institute, Rruga ''A.Moisiu" Nr.10, Tirane 1005, Albania*

Introduction

Intensive use of pesticides has resulted in their residuals being detected in all environmental segments and therefore raising concern about their monitoring throughout the world. The determination of pesticides in fruits and vegetables has always been an important issue in ensuring the quality of the products, given the fact that residues of pesticides in raw food could affect the consummator (Abbassy et al., 2014).

Neonicotinoid insecticides, which have a high affinity for insect nicotinic acetylcholine receptors (Matsuda et al., 2001) are widely used to protect against a broad range of pests, including aphids, whitefly, thrips and mealy bugs. Their physicochemical properties make them useful for a wide range of application techniques, including seed treatment, soil drench, and foliar and stem application (Tomlin, 2009). Because some pesticides can be applied to crops up to the day before harvest, the residual pesticide concentrations in crops immediately after harvest may be relatively high. Among the neonicotinoid pesticides commercially available, imidacloprid and acetamiprid are of common use in our region.

This study focuses on the residuals of acetamiprid on green peppers (*Capsicum annum* L.) one of the most important vegetable crops in our country. Various methods for neonicotinoid insecticide residue analysis have been reported, demonstrating that acetamiprid could be analyzed by both gas and liquid chromatography (Mateu-Sanchez et al., 2003; Navalon et al., 1997; Obana et al., 2002). According to the recommendation issued by Food and Agriculture Organization of the United Nations (FAO)/WHO the Maximum Residual Levels (MLR) for acetamiprid in vegetables should not exceed 0.2 mg/kg.

Materials and methods

The study was conducted in a ventilated and temperature regulated greenhouse. The surface involved approximately 250 m^2 and was divided in three main sections consisting of 30 plants each: S_1 – untreated plants; S_2 – plants treated with the minimal dose of acetamiprid (Mospilan®) 0,02%; and S_3 - treated with the maximal dose of acetamiprid (Mospilan®) 0,04%. Considering that the Pre Harvest Interval (PHI) for acetamiprid is 7 days, the residual levels have been measured up to 9 days after spraying with two different concentrations of Mospilan®. Sampling was conducted in compliance with the guidelines published by the Codex Alimentarius Commission (CCPR, 2009).

Approximately 300 g of vegetables were chopped into small pieces and blended in a homogenization apparatus so that a fine paste is formed. A portion of 5 g was accurately weighted and mixed with 10 g of hard-powdered synthetic magnesium-silica gel (Florisil®) in a mortar. Cleanup was carried out using a chromatographic column. The column (40 cm x 20 mm) was carefully prepared placing at the end glass wool and a thin layer of anhydrous sodium sulphate (Kadenezki et al., 1992). The column was then packed by transferring the mixture prepared in the mortar using a funnel. Extract was eluted with 50 ml of ethyl acetate and acetone mixture and elute was evaporated to dryness on rotary evaporator and reconstituted in 1ml hexane.

The cleaned extracts were analyzed on a Shimadzu gas chromatograph (GC) equipped with capillary column using an electron capture detector (ECD) (Chandra et al., 2010). Analysis of acetamiprid was carried out in a 30 m length, 0.32 mm internal diameter and 0.25 μm film thickness coated DB-5 column. Helium was used as the carrier gas at 1 ml min-1 flow. The injector was set at constant temperature of 260 °C and the detector temperature was

* ela_hoti@hotmail.com

300 °C. The initial oven temperature was set at 170°C (1 min isothermal) then the temperature increased with a ramp of 10 °C/ min till 280 °C and subsequently isothermal for 15 minutes. The injection volume was 1µl. All the solvent used were HPLC grade.

Results and discussion

The compound of interest was identified by comparing its retention time with respect to technical grade reference standard. The elution time for acetamiprid under the chosen chromatographic conditions was 7.29 min. The quantitative determination was carried out with the help of a calibration curve drawn from chromatographic experiments with standard solutions. The limit of quantification (LoQ) was measured by the signal to noise ratio and was 0.01 µg/ml (ppm).

The method was validated. Linear calibration curve was found between peak areas and analyte concentration in the whole range of the study. The linear regression ($y = a + bx$) parameters for method calibration were taken and the regression coefficients (R^2) was near 0.998, which allows the quantification of this compound by the method of external standardization. Accuracy and precision were calculated based on repeated injections (n=5) and the RSD was 0.51%. Recovery studies were performed to examine the efficacy of extraction and clean up. Untreated peppers were spiked with known concentration of the pure insecticide standard solution and extraction and cleanup were performed as described earlier. The recovery was 93%.

Monitoring the degradation of acetamiprid, revealed that the samples treated with the lowest dose of acetamiprid (0.02%) reached the MRL within seven days, whereas the samples treated with the highest dose (0.04%), could not reach the recommended MRL values for the same period of time.

Conclusion

Vegetable growers have been using neonicotinoid pesticides frequently to have a higher and insect free yield. Unfortunately, overdoses of systemic pesticides sprayed on to the plant, fail to dissipate and do not reach the required MRL that are advisable by the WHO directives, constituting potential health risks to consumers. The pesticides residues can be decreased only if the recommended dose is applied by the vegetable. In conclusion, a further investigation upon PHI of acetamiprid should be conducted and the data would be of great benefit to cultivators in our region.

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Macedonian pharmaceutical bulletin, **62** (suppl) 279 - 280 (2016)
ISSN 1409 - 8695
UDC: 614.878:547.533
Short communication

The toxicity of organic solvents mixtures, containing toluene and its oxidation products

Soica Codruta*, Simu Georgeta-Maria, Coricovac Dorina, Mioc Marius, Borcan Florin, Ionescu Daniela, Dragos Dan, Andoni Mihaiela, Dehelean Cristina

"Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania

Introduction

Toluene (methyl-benzene) is a colorless liquid, with high vapor pressure and an aromatic odor, mostly used in paint fabrication as a solvent, fuel industry and in chemical synthesis. Toluene also naturally occurs in the crude oil an in the tolu tree (Agency for Toxic Substances and Disease Registry - ATSDR, 2015). The main oxidation derivatives of toluene include benzaldehyde, benzoic acid, cresols or methyl-phenols, methyl-catechols and methyl-resorcinols. However, among these compounds, cresols are known to be more toxic then the others upon exposure. Cresols or methyl phenols are oxidation products of toluene, consisting of 3 isomers: o-cresol, m-cresol and p-cresol. Two of the compounds are colorless solids with low melting points (o-cresol and p-cresol), whereas the third one is a colorless thick liquid (Pubchem open chemistry database, https://pubchem.ncbi.nlm.nih.gov/).

Exposure to these substances often occurs by inhalation of contaminated air or accidental ingestion.

Toluene induced toxicity

The main pharmacological effect, correlated with toluene intoxication in humans and animals, via inhalation, is the depression of the central nervous system (CNS) (Faust, 1994). After the uptake into the blood stream, toluene crosses the blood brain barrier. In the brain, the solvent interacts with CNS neurotransmitters, especially γ-aminobutyric acid and to a lesser degree, with glycine and dopamine, inducing symptoms like: euphoria, hallucinations, delusions, tinnitus, dizziness, confusion, headache, vertigo, seizures, ataxia, stupor, and coma (McKeown, 2015). Toluene also influences locomotor activity in rats, as shown by Apawu et al. by modulating dopamine levels across the striatum. The study showed that acute and chronic exposure to toluene increased locomotor activity in rats and although dopamine levels in the striatum were increased, dopamine uptake was not affected (Apawu et al., 2015).

Other toluene exposure induced effects include cardiotoxicity, hepatotoxicity, nephrotoxicity and skeletal muscle damage and reproduction system impairment.

Symptoms related to the cardiovascular system in toluene exposure include blood pressure and heart rate fluctuations. Toluene exposed rats exhibited apoptosis in heart tissues mediated by increased caspase-3 activity (Tas et al., 2013).

Toluene is metabolized in the liver to benzoic and hippuric acid. The metabolism produces free oxygen radicals which cause liver damage through oxidative stress. A study by Tas et al. showed that rats exposed to toluene suffer severe liver damage exhibiting: increased levels of aminotransferase and malonylaldehyde (parameter for oxidative damage), balloon degeneration and fibrosis in the liver tissue (Tas et al., 2011). Liver cell apoptosis is also present, mediated by increased activity of Bax and caspase-3 enzymes (Ayan et al., 2012).

Toluene exposure affects the renal system, serum ion concentrations and locomotor activity. Patients exposed to toluene showed distal tubular renal acidosis type 1, hypokalemic acidosis, increased serum alkaline phosphatase, muscle paralysis and proteinuria due to rhabdomyolysis (Camara-Lemmaroy et al., 2015). Meydan et al. showed that rats exposed to toluene exhibited renal tissue damage. Shrinkage in the glomerular tufts, differentiation in the

* codrutasoica@umft.ro

capsule, and increase in the connective tissue in the interstitial area all occurred. Also increased levels of serum creatinine, catalase and superoxide dismutase were observed (Meydan et al., 2013).

Another study by Nakai et al., reported that toluene induces male reproductive dysfunctions in rats. The results suggested that toluene induced reproductive toxicity by direct oxidative damage of the spermatozoa was related to the formation of 8-oxo-7,8-dihydro-2'-dezoxyguanosine in the male rat testicles, which is a biological marker for DNA oxidative damage (Nakai et al., 2002).

Cresol induced toxicity

Toxic effects of cresols due to inhalation can affect respiratory functions, and produce mucosal irritation. Systemic effects on humans due to inhalation are not reported but there are several studies that report cardiotoxicity, hepatotoxicity, renal toxicity, neurotoxicity in animals exposed to cresol aerosols (Agency for Toxic Substances and Disease Registry - ATSDR, 2008). Ingestion of cresols is more severe and can cause death in humans.

The most studies regarding cresol toxicity are centered on p-cresol (4-methylphenol).

P-cresol induces hepatotoxicity in rats. The mechanism proposed for hepatotoxicity induction involves biotransformation of p-cresol to a reactive quinone methide intermediate which covalently binds to cellular macromolecules and elicits cytotoxicity (Thompson et al., 1996).

P-cresol is also a protein-bound uremic toxin (toxins which accumulate in the serum in chronic renal failure patients treated with standard dialysis) (De Smet et al., 2003). A study by Tanaka et al. focused on the effects of p-cresol on 3T3-L1 cells. The results showed that p-cresol inhibited cell proliferation, adipogenesis, glucose uptake and induced apoptosis in these cells (Tanaka et al., 2014). Another effect of p-cresol is the increase in the reactive oxygen species production, cell cycle proliferation arrest and apoptosis stimulation, as stated by Chang et al. The study carried on U937 cell lines also revealed an induced delayed effect on endothelial cell migration which can be translated in an increase in the time needed for wound closure (Chang et al., 2014).

Conclusion

Toluene and cresols are widely used in various industries. Exposure to these compounds may lead to intoxication. It is best that exposure is avoided taking in account that intoxication with toluene and cresols could be very dangerous since it can affect significant number of organs like the brain, heart, lungs, liver, kidneys, muscles and reproductive glands.

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Macedonian pharmaceutical bulletin, **62** (suppl) 281 - 282 (2016)
ISSN 1409 - 8695
UDC: 637.123:577.161.3
Short communication

Assessment of vitamin E content in bovine colostrum supplement by using solid phase extraction and HPLC method

Slavica Sunarić, Jelena Lalić, Marko Denić*, Gordana Kocić

Faculty of Medicine, University of Niš, Bulevard Zorana Đinđića 81, 18000 Niš, Serbia

Introduction

Colostrum is a pre-milk fluid secreted by mammals within the first few days after giving birth. Colostrum is a rich natural source of more than 90 essential nutrients: immune and growth factors, vitamins, minerals and amino acids. Bovine colostrum is milk produced during first few days after calving and its chemical composition is very similar to that found from humans. It contains high concentrations of immunoglobulins, cytokines, growth factors, lactoperoxidase, lactoferrin, transferrin, lysozyme, proline-rich polypeptides, lactalbumins and other proteins which play an important role for immunity and act as immunomodulators. Vitamins A, B1, B2, B6, B12, C, D, E, folic and pantothenic acid and minerals: calcium, magnesium, chromium, iron, zinc are also components of bovine colostrum (McGrath et al., 2016).

Vitamin E is a lipid soluble vitamin responsible for a variety of functions in the body. The main biological function of vitamin E is as an antioxidant which protects the polyunsaturated fatty acids of cell membranes from free-radical damage. Natural vitamin E includes two main groups of compounds: tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ). Alpha-tocopherol is the most active, whereas the activity of the other tocopherols is 70–95% less.

Colostrum is a natural food that has been used in traditional medicine for hundreds of years. It has many benefits for resistance, strength and vitality of human body. This natural product is widely considered to help slow the aging process and assist with healing the body. Aging and disease are the result of losing the natural immune and growth factors. It is considered helpful in alleviating the symptoms of influenza and the effects of some types of allergies and auto-immune diseases. It helps balance blood sugar levels and increase mental alertness and assists with weight loss. Since colostrum is an important and rich source of immune and growth factors, it is recommended regular intake, at all ages (Donovan and Odle, 1994). Pharmaceutical colostrums are based on bovine sources and they are safe and effective health supplements. They are produced as powder or capsules as dietary products for oral intake. Vitamin E is an important component of colostrum. The esterified form of vitamin E (tocopherol acetate) is commonly used in commercial pharmaceutical health supplements because of better stability in the products.

For the vitamin E determination purification of the sample is necessary in order to perform HPLC analysis, because of colostrum is very complex sample and cannot be directly injected into the HPLC column. The objective of this study was to evaluate both α-tocopherol and tocopherol acetate content in commercial pharmaceutical colostrum product by using previously developed solid phase extraction (SPE) and HPLC method.

Materials and methods

Standards of α-tocopherol and tocopherol acetate (TA) of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were analytical grade (JT Baker, Deventer, Netherlands). Chromabond C18 ec cartridges, used for purification of samples, were purchased from Macherey-Nagel, Düren, Germany.

Concentrations of α-tocopherol and tocopherol acetate in analyzed samples were determined by calibration curve method. The range of the calibration curve was 0.01-0.5 μg/mL for α-tocopherol and 0.2-2.0 μg/mL for tocopherol acetate. The stock standard solutions of α-tocopherol (1 mg/mL) and tocopherol acetate (1 mg/mL) were prepared by dissolving standards in absolute ethanol. The working

* mdenic84farm@gmail.com;
slavica.sunaric@medfak.ni.ac.rs

calibration solutions were freshly prepared for each run by diluting the stock standard with absolute ethanol.

Commercial pharmaceutical colostrum was obtained from local pharmacy. One of the bovine colostrum products, available on the Serbian market is Ekolostrum®. This product was made from the fresh bovine colostrum obtained in the first 16 hours after calving. Ekolostrum® capsule contains 480 mg high-quality 100% natural bovine colostrum powder with no additives and flavouring. The content of any form of vitamin E was not exactly declared.

Sample preparation: 500 mg of powder sample from the capsule was weighted and dissolved in 50 mL of deionized water. After complete dissolving, 1 mL of the sample was treated with 0.5 mL of 0.5% ascorbic acid and 5 mL of absolute ethanol. The mixture was vortexed vigorously for 1 min and centrifuged at 4000 rpm for 10 min at 20 °C. Finally, supernatant was purified with solid phase extraction.

Based on our previously extraction recovery evaluation, Chromabond C18 ec column was selected among five different types of cartridges. Chromabond C18 ec was preconditioned by passing through 1 mL of deonized water, followed by 1 mL of methanol and 1 mL of acetonitrile. Then, 3 mL of supernatant was slowly passed through the cartridge at a flow rate of 1 mL/min and the analyte was eluted with 2 x 1 mL of methanol.

HPLC determination was achieved at 40 °C using RESTEK Ultra IBD column (3µm, 150x3mm) with the mobile phase consisted of 100% acetonitrile and fluorescence detection (λex=295 nm, λem=330 nm) for α-tocopherol and UV detection at 220 nm for tocopherol acetate. The flow rate was kept at 0.45 mL/min.

Results and discussion

The analyzed samples of commercial colostrum supplement had average α-tocopherol and tocopherol acetate concentration of (0.2±0.01) mg/100 g and (6.5±0.3) mg/100 g, respectively. The content of α-tocopherol in the examined pharmaceutical colostrum was similar to that for the fresh bovine colostrum found by Kehoe et al. (2007), who reported that the concentration of α-tocopherol is in ranges from 0.06 to 1.04 mg/100 g, with a mean value of 0.29 mg/100 g. On the other hand, the found value was lower than for human colostrum (1.28 mg/100 g) reported in our previous work.

The high content of the synthetic form of vitamin E (tocopherol acetate) was found in the examined sample. Tocopherol acetate is not naturally present neither in bovine nor in human colostrum. TA is added to many pharmaceutical and dietary products for the fortification and enrichment, but it needs to be hydrolyzed in gut to free tocopherol which exerts its activity in vivo. Also, the different vitamin E stereoisomers obtained by hydrolysis of TA have different biopotencies.

Conclusion

This work showed that the SPE method was successfully applied for the preparation of colostrum samples for HPLC simultaneous determination of α-tocopherol and tocopherol acetate, which is important for the quality control for this type of pharmaceutical products. Examined bovine colostrum product contains appropriate amounts of vitamin E, therefore it can be considered as important nutritional source of vitamin E and good supplement for all generations.

Acknowledgments

This research was supported by grant TR 31060 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 283 - 284 (2016)
ISSN 1409 - 8695
UDC: 613.27:616.379-008.64]:599.325.1.084
Short communication

Effects of different doses zinc gluconate on copper, iron and calcium levels in experimentally induced diabetic rabbits and type 2 diabetic patients

Zorica Stanojević Ristić[1]*, Snežana Stević[1], Julijana Rasić[1], Dragana Valjarević[2], Momčilo Stanić[3]

[1]*Institute of Pharmacology and Toxicology, Faculty of Medicine, University of Pristina, Kosovska Mitrovica, Serbia*
[2]*Department of Mathematics, Faculty of Natural Sciences and Mathematics, University of Pristina, Kosovska Mitrovica, Serbia*
[3]*Biochemical-Clinical Laboratory, Medical Centre, Kosovska Mitrovica, Serbia*

Introduction

Researches with micronutrients are getting more and more important in science and also in practice. In this view zinc, chromium, copper and selenium are having a special role in preventing micro- and macro vascular diabetic complications, as integral components of antioxidant enzymes and also as cofactor of enzymes and hormones involved in the metabolism of glucose and lipid. Results of numerous studies also suggested that, because of hyperzincuria and low zinc absorption, diabetic patients are more susceptible to zinc deficiency compared to healthy persons.

On the other side, it`s known that zinc, as divalent cation, compete with cadmium, copper, lead, iron and calcium for similar binding site. The physiological effects of zinc and several other cations such as copper and calcium are due to alteration in the permeability of the cellular membrane or modulation of the activity of membrane-bound enzymes. It has been reported that zinc may suppress calcium effect by displacement of calcium ions from its cell binding sites, thus altering the membrane calcium pump resulting in a reduction in free intracellular calcium (Pathak et al., 2011). Copper deficiency might be a contributor to the glucose intolerance in diabetic patients.

There are also reports of altered metabolisms of other micronutrients such as copper (Cu) and iron (Fe) in diabetes. Moreover, previous investigation reported that Zn deficiency increases the absorption of intestinal Cu and that Cu significantly inhibits the influx of Zn across the intestinal brush border membrane (Condomina et al., 2002). It`s only unknown whether Zn supplementation can normalize the Cu/Zn ratio. Iron is capable of generating reactive oxygen species and contributes to diabetic nephropathy. Excess Fe has been implicated in the pathogenesis of diabetes and its complications.

In view of these facts, the aim of our study was to evaluate the effects of three different single doses of zinc gluconate on serum copper, iron and calcium levels in experimentally induced diabetic rabbit and type 2 diabetic patients.

Materials and methods

The experimentally study was conducted on twenty-four New Zealand rabbits, weighing 2 to 3.5 kg. Experimental diabetes was induced in rabbits by intravenous injection of alloxan (80 mg/kg BW). Two weeks after application of alloxan, animals were subjected to 16h fasting, which was determined by the concentration of glucose in the blood. The test includes only 14 rabbits with fasting glucose between 9.99 and 14.98 mmol/L, which corresponds to the degree of damage of the pancreas in patients with insulin-independent diabetes mellitus. Three weeks after induced experimental diabetes, rabbits were treated orally appropriate doses of zinc gluconate determinate by Clarc formula: first dose 5.5 mg, after the washout period

* zorica_stanojevic@yahoo.com

(10 t1/2) - second dose 9.2 mg, after the washout period (10 t1/2) - third dose 18.4 mg. Blood samples were taken at certain time intervals: before and 24h after the first, second and third dose of zinc gluconate.

The human study was conducted on a group of 12 patients with type 2 diabetes mellitus and a control group of 12 healthy subjects. All participants did not take nutritional supplements and any drugs that are known to interfere with metabolism of studied metals during the period of study and at least 2 week before sampling. Blood samples were drawn after an overnight fasting in both groups, before and 24 hour after administration of zinc gluconate in three different single doses (15, 25 and 50 mg) with washout period (10 t1/2) between treatments.

All the material used for zinc collection, separation and storage was plastic and metal free. Serum samples were frozen and stored at -20 ºC until the time of measurement. Determination of concentrations essential minerals (Zn, Cu, Fe and Ca) was performed by colorimetric method (Randox and Roche Diagnostics assays).

Each value is expressed as the mean±SD. Differences between groups were examined using the unpaired Student`s t-test, differences in the group were examined using the paired Student`s t-test and to asses possible relationship between different variables, Pearson`s correlation coefficient (r) was used.

The study protocol was approved by Ethical Committee of the University of Pristina Faculty of Medicine, in Kosovska Mitrovica (Serbia). All subjects gave written, informed consent. The animals were handled in accordance with the European Community guidelines (86/609/ EEC).

Results

Different single doses of zinc gluconate did not cause any significant change on copper, iron and calcium levels in serum of healthy rabbits. However, in diabetic rabbits the calcium concentration in serum was significantly decreased 24h after zinc gluconate administration in doses of 9.2 and 16.4 mg, compared with the baseline value (0h) marked before the treatments (p=0.041; p=0.039).

Also, different single doses of zinc gluconate did not cause any significant change on copper, iron and calcium levels in serum of healthy subjects. In patients with type 2 diabetes mellitus were found significant decreased the serum calcium concentration 24h after zinc gluconate administration in dose of 50 mg compared with the baseline value (0h) marked before the treatment (p=0.038). The Cu/Zn ratio were significantly reduced in diabetic patients 24h after zinc gluconate administration in doses of 25 and 50 mg compared with baseline values (0h) marked before the treatment (p=0.033; p=0.026).

Conclusions

Based on obtained results it can be observed that administration of zinc gluconate in single higher doses showed reduction of serum calcium level in type 2 diabetes mellitus. Considering that zinc, as divalent cation, compete with calcium for similar binding site, this could to be due to antagonistic effect of zinc on calcium.

Also, the administration of zinc gluconate in single higher doses decreased the Cu/Zn ratio only in type 2 diabetic patients. These results are very significant considering that previous studies reported that an impaired Cu/Zn ratio in serum from diabetics could be an important factor in the development of micro- and macro-vascular complications in type 2 diabetes mellitus.

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Macedonian pharmaceutical bulletin, **62** (suppl) 285 - 286 (2016)
ISSN 1409 - 8695
UDC: 637.13/.3:579.864
663.813:635.13]:579.864
Short communication

Viability and metabolic activity of *Lactobacillus casei* 01 in dairy and non-dairy products

Tanja Petreska Ivanovska*, Kristina Mladenovska, Lidija Petrushevska-Tozi

Faculty of Pharmacy, University "Ss. Cyril and Methodious", Mother Theresa 47, Skopje, Macedonia

Introduction

Probiotics are widely used as functional ingredients because they beneficially affect the host by improving its intestinal microbial balance. Accepted dose of live probiotic bacteria is required to be between 106-108 CFU per mL or g of the food product at the time of consumption (Vasiljevic and Shah, 2008). As probiotic viability is considered essential for their health benefits, viability of probiotic bacteria in foods throughout their storage is a constant challenge for the food industry. Dairy foods are the most frequently used as probiotic carriers and well accepted by the consumers, while development of non-dairy matrices has been encouraged due to the increasing demand for new probiotic products. Food matrix was found to play an important role in the beneficial health effects of probiotics on the host (Do Espírito Santo et al., 2011). Incorporation of microencapsulated probiotics is a potential strategy for manufacture of both dairy and non-dairy products at the same time improving the functional value of the product and ensuring protection of living cells from unfavorable conditions.

The aim of the present study was to examine the survival and metabolic activity of probiotic strain Lactobacillus casei 01 both in dairy product (ayran) and non-dairy matrix (carrot juice) during storage for two weeks at 4 °C.

Materials and methods

The cell suspension of probiotic *Lactobacillus casei* 01 (Chr. Hansen, Denmark) was divided into two parts: one part was used for microencapsulation and another was used as free cells for direct adding in carrot juice prepared by extraction of washed and peeled carrots and commercially available ayran (Ayran, Zdravje Radovo, Macedonia). Carrot juice samples after pasteurization at 80 °C for 20 min was fermented using a cell concentration of 7.4±0.1 log CFU/mL according to the recommendations for minimum counts of approximately 7.0 log CFU per g or mL of probiotic food to exert beneficial effects (Vasiljevic and Shah, 2008). Prebiotics fructooligosaccharides (FOS) with DP > 10 (Sigma-Aldrich, USA) and oligofructose-enriched inulin (Synergy 1) which is the mixture of oligofructose (DP 2-8) and long-chain inulin fraction (DP 10-60) (Orafti-Rue L. Maréchal, Belgium) were added to carrot juice and ayran samples containing non-encapsulated cells, respectively, at the same time with the probiotic cells. An optimal formulation of microparticles containing FOS or Synergy 1 as prebiotic with high cell viability of *L. casei* 01 (11.28-11.38 log CFU/g) was prepared using 4% w/w alginate, 0.5% w/w chitosan and 5% w/w $CaCl_2$ (Petreska Ivanovska et al., 2014). An aqueous dispersion of alginate (LF 10/60 LS, fG 35-45% Protanal, FMC Biopolymer, IMCD, UK), prebiotic at a level of 1.5 w/w and *L. casei* 01 with a cell load ca. 12 log CFU/mL was submitted to spray-drying (nozzle diameter 0.7 mm, aspirator pressure 90%, flow rate 6 mL/min, inlet and outlet temperature, 120 °C and 60 °C; Büchi Mini Spray Dryer B-290, SW) followed by subsequent cross-linking and coating in solution of $CaCl_2$ (Merck, Germany) and chitosan (Chitine, France) in 1% v/v acetic acid. The microparticles formed were cured at least 3 h, separated and freeze-dried (-50 °C, 0.070 mbar, 24 h, FreeZone Freeze Dry System, Labconco, USA). Synbiotic microparticles were added to the carrot juice and ayran, respectively, on the same day of preparation. All samples were packed into sterile flasks and stored at 4 °C. The viability of *L. casei* 01 was determined on MRS agar (Merck, Germany) after 72 h of incubation at 37 °C using a plate-count method. For enumeration of bacteria, 1 mL of the

* tpetreska@ff.ukim.edu.mk

sample was mixed with 9.0 mL of peptone water, vortexed for 15 s and serially diluted with peptone water. In the samples containing microparticles enumeration was done after removing the particles by filtration, washing with sterile saline solution and liquefying with phosphate buffer (pH 6.9). The pH values of synbiotic samples were also examined (pH meter PB 11 Sartorius, Germany). The production of lactic and acetic acid was measured using HPLC (Agilent Technologies 1200, USA) by loading supernatants of the pretreated samples with 0.5 M H2SO4 on a reverse phase column (250 mm x 4.6 mm, 5 µm, Discovery HS C 18, Supelco Park, USA) set at 40 °C and eluted with 0.005 M H2SO4 at a flow rate of 1 mL/min with detection wavelength of 210 nm.

Results and discussion

Encapsulated *L. casei* 01 added to ayran either carrot juice survived significantly better compared to non-encapsulated cells upon cold storage due to the ability of chitosan-coated alginate particles to preserve cell viability. Relatively high viable cell counts in carrot juice and ayran at the end of storage were observed, 9.11 and 8.22 log CFU/mL, respectively. The viability of free and encapsulated *L. casei* 01 in ayran samples was reduced for 3.38 and 1.21 log CFU/mL, respectively, while the corresponding cell viability loss in carrot juices was 1.49 and 0.44 log CFU/mL. Results have shown that both non-encapsulated and encapsulated *L. casei* 01 survived better in carrot juice compared to ayran. This finding may be the result of characteristics of probiotic strain or substrate as well as the oxygen content in the product. Acidity of the carrot juice as substrate was determined to be lower than ayran acidity (pH 6.25 vs. 4.55, respectively). The mild acidity of carrot juice declined to 4.23 and 5.44 after fermentation with free and encapsulated *L. casei* 01, respectively, while pH values measured in ayran samples enriched with free and encapsulated *L. casei* 01 were 4.54 and 4.58, respectively. The reduction of pH values was similar in both carrot juice or ayran samples during the storage, but ayran contains 0.4% salt which may contribute to further reduction of the cell viability. Probiotic culture *L. casei* 01 produced higher quantity of lactic acid in ayran samples compared to carrot juice samples due to the presence of lactose in dairy products. In ayran samples containing non-encapsulated and encapsulated synbiotic, the concentration of lactic acid tend to increase during storage from initial values of 51.32 and 56.94 mmoL/L to 59.14 and 72.36 mmoL/L, respectively. Production of acetic acid was lower, but with similar increasing trend to those of lactic acid. As the result of saccharolytic activity of the probiotic culture able to ferment sugars present in the carrot juice, production of lactic and acetic acid was observed. Initially measured concentration of 30.53 mmoL/L lactic acid remained constant during the storage of the carrot juice containing non-encapsulated synbiotic, while initial concentration of 18.43 mmoL/L lactic acid of the sample with encapsulated synbiotic increased to 31.53 mmoL/L at the end of storage. In the carrot juice samples both containing non-encapsulated or encapsulated synbiotic, the concentration of acetic acid was significantly lower compared to corresponding ayran samples and tend to decrease during the storage period. However, in both dairy either non-dairy product, the metabolic activity of *L. casei* 01 was better retained in samples with encapsulated synbiotic as lactic acid is the main product of lactobacilli metabolism.

Conclusion

Survival rate of the encapsulated *L. casei* 01 incorporated in carrot juice as well in ayran was found to be above the therapeutic level with retained metabolic cell activity during the investigation period. The results have shown higher probiotic viability in carrot juice compared to ayran, albeit dairy products are naturally connected with lactic acid bacteria. Further, synbiotic carrot juice may be offered to lactose intolerant individuals and to those suffering from cardiovascular diseases. Additional studies to investigate the influence of the food matrix in preserving cell viability under gastrointestinal conditions as well the ability of the matrix to deliver viable cells to the lower intestine are required.

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Macedonian pharmaceutical bulletin, **62** (suppl) 287 - 288 (2016)
ISSN 1409 - 8695
UDC: 663.236
Short communication

Effect of glucose concentration on glucose oxidase activity in a minimal model must

Verica Petkova[1], Irina Mladenoska[1]*, Tatjana Kadifkova Panovska[2]

[1] *Faculty of Technology and Metallurgy, University of Ss. „Cyril and Methodius", Rudjer Boskovic, 16, 1000 Skopje, Republic of Macedonia*
[2] *Faculty of Pharmacy, University of Ss. „Cyril and Methodius", Majka Tereza 47, 1000, Skopje, Republic of Macedonia*

Introduction

During the last couple of decades, the interest among the nutritionists and food industry for production of low alcohol and alcohol free wines has been increased. It has been reported that the consumption of low alcohol wines had a very positive effect on the consumer's health. Thus, Karatzi (Karatzi et al., 2004), showed that the consumption of 250 mL per day of alcohol free wine has improved the condition of the cardio-vascular system in patients with a progressive coronary disease. There are, also, literature data witnessing the benefit of alcohol free wine consumption and its connection with lowering the high blood pressure (Chiva-Blanch et al., 2012).

In order to produce low or alcohol free wines, food industry applies several pre-fermentative, during and post-fermentative procedures. Post-fermentative techniques are usually physical like pervaporation or utilization of spinning cone column, but all of these processes have drawbacks such are specific and expensive equipment and changes in aromatic composition (Heux et al., 2006).

Unlike post-fermentative, pre-fermentative enzymatic methods for lowering the alcohol level in wines are faster, high specific methods that minimize loss or alteration of sensory qualities and off-flavor development (Schmidke et al., 2012).

In this paper the effect of glucose concentration in the minimal model must on the enzymatic activity of several different commercial glucose oxidase preparations was examined. The first one was the commercial bakery additive Alphamalt Gloxy 5080 (glucooxidase and low catalase activity). The second one was pure glucooxidase from the mould Aspergillus niger and the third preparation was coupled mould glucooxidase and a beef liver catalase. The glucose concentration of 10 g/L and 100 g/L were used as sugar concentrations in the minimal model must. The effect of the enzyme activity on the final pH of the reaction media was also studied.

Materials and methods

Enzymatic preparations: Alphamalt Gloxy 5080 (Muhlenchemie, Germany) was a commercial enzyme preparation obtained from Aspergillus niger strain. This was a food grade bakery additive with activity of 10500 units per gram (U/g) and low catalase activity.

The pure glucose oxidase (EC 1.1.3.4) was in its lyophilized form and was also derived from Aspergillus niger (Merck, Germany). This preparation was with much lower activity than the previous one. The activity of the lyophilized glucose oxidase was 8 U/mg.

The liquid catalase (Merck, Germany) was obtained from beef liver and had an activity of 1300000 U/mL.

Media: A medium with high glucose concentration of 100 g/L was prepared. This high concentration was chosen to be of the same order of magnitude as the one measured in the real must. For comparison, the medium with lower concentration of glucose of 10 g/L was also used. The minimal model must, besides glucose, was also containing 4.0 g/L tartaric acid, 1.5 g/L malic acid and 0.5 g/L citric acid. The pH of the medium was adjusted to pH 3.5 using sodium hydroxide (10 M NaOH) to be similar as the pH of the real must.

Glucose oxidase reaction: All the three preparation used were diluted in the medium to give a certain concentration of the enzyme preparation. The reactions were car-

* irinaetf@t.mk

ried on an orbital shaker (Ceromat R, B. Brown Biotech International) with agitation rate of 150 rpm (oxygen dissolution) in 100 mL Erlenmeyer flasks containing 50 mL of medium. The reaction temperature was kept 30 °C.

Determination of glucose concentration: D-glucose was measured using DNS method for determination of reduced sugars (Miller, 1959). The absorbance was measured on a spectrophotometer Cary 50 Scan (Varian) at 540 nm wave length.

Determination of pH value: The pH value of the media was determined by utilization of Sartorius Basic pH Meter PB-11. The pH value was determined at the beginning (initial pH value) and at the end of the enzymatic reaction (final pH value).

Results and discussion

Enzymatic reaction in the minimal model must with 10 g/L glucose

The effect of the lower concentration of glucose, 10 g/L, on the enzymatic activity was evaluated by utilizing three different glucose oxidase preparations in the medium simulating a must (minimal model must). Those were Alphamalt Gloxy 5080, pure glucose oxidase and the third one was a combination of glucose oxidase and catalase. The most active of all the three was the commercial Alphamalt Gloxy 5080 preparation, showing even 71.12% conversion at the 24th hour of the reaction. Unlike the Alphamalt Gloxy 5080, the other two glucose oxidase preparations showed negligible activity towards the glucose substrate used in the concentration of 10 g/L. It was, thus, obvious, that not even the presence of catalase could increase the activity of the pure glucose oxidase, in this, not favorable conditions for the enzyme activity (pH 3.5).

Enzymatic reaction in the minimal model must with 100 g/L

In the second experiment, ten times higher concentration of glucose was used, as it was actually necessary to simulate the concentration of glucose present in the real must (approx. 100 g/L glucose+100 g/L fructose). None of the three enzymatic preparations used was active enough at this high concentration of glucose. However, the Alphamalt Gloxy 5080 showed certain activity. Namely, during the reaction period of 120 hours it converted only 7.37% of the substrate. It can be assumed that for the conversion of glucose into gluconic acid in media with high glucose concentration, at these unfavorable conditions for glucose oxidase activity, the acidic environment, high concentrations of enzyme preparation, preferably Alphamalt Gloxy 5080, should be used.

The pH value of the media and its difference between the initial and the final value (after the reaction), is also a good indirect indicator of the enzymatic activity. Thus, the very logical results were obtained when the pH value of the medium containing 100 g/L was used as a minimal model must. Only a very slight difference in the pH value (from 3.55 to 3.50) was measured in the medium with coupled glucose oxidase/ catalase enzymatic system used as a catalyst. It was interesting that the Alphamalt Gloxy 5080 has changed the pH value of the medium from 3.50 to pH 3.00, and it was the most active enzymatic preparation among the three examined.

Conclusion

The effect of glucose concentration on the enzyme activity was evaluated by utilization of three different enzymatic preparations. It was found out that the commercial bakery additive Alphamalt Gloxy 5080 was the most suitable enzymatic preparation for glucose oxidation, at as low pH of the medium as pH 3.5, although even this enzymatic preparation was not very active at the higher glucose concentration (100 g/L). This paper is only a part of the complex research process for development of a sophisticated method for pre-fermentative lowering of the sugar concentration in the must, with a final aim- production of low alcohol wine.

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Macedonian pharmaceutical bulletin, **62** (suppl) 289 - 290 (2016)
ISSN 1409 - 8695
UDC: 613.2:546.4/.8]:543.421
Short communication

Determination of lead and cadmium in foods by Graphite Furnace Atomic Absorption Spectroscopy

Suzana Angelova[1]*, Biljana Mladenovski[1] and Tatjana Kadifkova Panovska[2]

[1]*PHI Center for Public Health Kumanovo, 11th Octomvri bb, 1300 Kumanovo, R. Macedonia*
[2]*Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

Humans can be exposed to heavy metals through various ways, including consumption of contaminated food. Although heavy metals are usually present in foods at very low levels, long-term exposure can have negative health impacts. According to the Macedonian regulations for general requirements for food safety, determination of cadmium (Cd) and lead (Pb) are obligatory. It can enter food either through environmental processes or through contamination in processing and/or packaging. Therefore, it is very important to measure levels of Cd and Pb in a variety of food matrices. A major challenge in the analysis of food samples is the extremely low analyte levels versus the very high matrix levels. The most commonly used technique for the determination of Pb and Cd is a graphite furnace atomic absorption spectroscopy (GFAAS) after microwave digestion (Kenneth, 2014; Belc et al., 2014).

This article will present the use of GFAAS for the determination of Pb and Cd in a variety food samples.

Materials and methods

Chemicals

All solutions were prepared in polypropylene volumetric flasks using ultra pure deionized water. All the plastic and glassware were cleaned by soaking in dilute HNO_3 (1+9), were rinsed with distilled water and air dried before use. All reagents used (nitric acid 65%, hydrochloric acid 30%, hydrogen peroxide 30%) were of Suprapure quality (Merck, Darmstadt, Germany).

Lead and cadmium stock solutions, 1000 mg/L (Merck, Darmstadt, Germany) are commercially available. Cadmium and lead working solutions were prepared fresh daily by dilution of the cadmium and lead stock solutions with 0.5% (v/v) nitric acid.

Mixtures of palladium [$(Pd(NO_3)_2$] and magnesium nitrate [$Mg(NO_3)_2x6H_2O$] in deionized water was used for Cd analysis. Ammonia phosphate [$NH_4H_2PO_4$] and magnesium nitrate [$Mg(NO_3)_2x6H_2O$] was used for Pb analysis. Matrix modifiers were added automatically to each blank and samples.

Samples

Different samples were bought from local markets and/or were brought to our laboratory by border health inspectors: 160 cereal samples and their products (113 wheat, 40 wheat flour, 4 barley; 3 oat flakes); 13 piper samples; 5 onion samples, 3 garlic samples; 2 cabbage samples; 2 beetroot samples; 4 mushrooms samples and 7 potato samples.

Sample preparation

The samples were dried at 105 ˚C for 24 h. Dried samples were homogenized using an agate pestle and stored in pre cleaned polyethylene bottles until analysis.

Microwave digestion system Multiwave 3000 Anton Paar equipped with quartz vessels Q80, was used for sample preparation. The samples were rapidly digested in a microwave oven. Samples of 300 mg each were accurately weighed into the digestion vessels, followed by the addition of nitric acid (65%), and/or hydrochloric acid (30%) and/or hydrogen peroxide (30%), all suprapure quality. A blank digest was carried out in the same way. Than vessels were placed into the rotor and heated in the microwave oven, according to the temperature program recommended by the manufacturer. The rotor was removed from the mi-

* suzanaangelova@yahoo.com

crowave oven and allowed to cool to room temperature. The vessels were carefully opened in a fume cupboard and the inner walls rinsed with ultra pure deionized water. The final volume of each sample was made up to 20 mL with. All sample solutions were clear.

GF AAS conditions

All samples were analyzed using a GFAAS. The wavelength and GFAAS instrument parameters for the determination of lead and cadmium are according to the MKC EN 14083:2010. A Perkin Elmer A Analyst 600 was used for heavy metals analyses, equipped with THGA Graphite Furnace and Zeeman background correction, AS-800 Autosampler. Winlab 32 software for the acquisition and processing of data was used.

Methods

Calibrations were performed using external standards. The limits of detection (LOD) were calculated as triplicate, and the limits of quantification (LOQ) were calculated as six times the standard deviation of repeated measurement of a blank solution.

The accuracy of the method was tested by lead and cadmium reference materials with certified known lead and cadmium content and expressed through z-scores results ($-2 \leq z \leq 2$). Recovery checks were carried out using spikes.

Repeatability was estimated for lead and cadmium, based on standard deviation and relative standard deviation, using the data from the QC sample analyses.

Results and discussion

Three working standard solutions were used for the linearity testing. Lead was calibrated at 10 µg/l, 20 µg/l and 30 µg/l, resulting in a calibration correlation coefficient (R^2) of > 0.999. Cadmium was calibrated at 1 µg/l, 2 µg/l and 3 µg/l, resulting in a calibration correlation coefficient (R^2) of > 0.999.

The limits of detection (LOD) were 0.2 µg/l for Pb and 0.1 µg/l for Cd. The limits of quantification (LOQ) were 0.5 µg/l for Pb and 0.3 µg/l for Cd. The values obtained matched closely with the certified values (z- score for Cd was 0.1 and for Pb - 0.3). The obtained results were satisfactory, showing levels of Pb and Cd close to the allowed levels of concentration. The recoveries of the trace metals were in the range from 95% for Cd and 103% for Pb. The standard deviations were less than 5%.

Lead content of almost all cereal samples is bellow 0.2 mg/kg limit according to national regulation (Official Gazette of Republic of Macedonia, 102/2013), except for the six samples where the concentration of lead was 0.574 mg/kg, 0.820 mg/kg, 0.901 mg/kg, 0.721 mg/kg, 0.255 mg/kg and 0.385 mg/kg. These concentrations exceed the maximum allowed concentration. Cadmium content of all cereal samples is bellow 0.2 mg/kg according to the national regulations.

Lead and cadmium content of all vegetables samples (onion, garlic, beetroot, and potato) is bellow 0.1 mg/kg (maximum permitted limits by national regulation).

Lead content of piper samples is bellow 0.1 mg/kg and for cabbage and mushrooms is bellow 0.3 mg/kg, (maximum permitted limits by national regulation). Cadmium content is bellow 0.05 mg/kg for piper and 0.2 mg/kg for cabbage and mushrooms.

Conclusion

The proposed method for trace heavy metal determination in food samples showed satisfactory value for recovery, detection limits and standard deviation. Therefore, it can be recommended as suitable procedure for routine analysis of heavy metals in variety food matrices.

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Macedonian pharmaceutical bulletin, **62** (suppl) 291 - 292 (2016)
ISSN 1409 - 8695
UDC: 579.67:582.282.123.4]:543.544.5.068.7
Short communication

Determination of aflatoxins in some foodstuffs by HPLC

Suzana Angelova[1]*, Valide Sabani[1] and Tatjana Kadifkova Panovska[2]

[1]*PHI Center for Public Health Kumanovo, 11th Octomvri bb, 1300 Kumanovo, R. Macedonia*
[2]*Faculty of Pharmacy, Ss.Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

Aflatoxins are toxic secondary metabolites produced mostly by *Aspergillus flavus* and *Aspergillus parasiticus*. Among them, aflatoxin B_1 exhibits the highest toxicity and carcinogenetic and it can be found in many commodities (groundnuts, nuts, cereals and their products, dried fruits, herbs) (Soleimany et al., 2012). The International Agency for Research on Cancer (IARC) classified naturally occurring aflatoxin B_1 as carcinogenic to humans - Group 1.

Total aflatoxin content in food is regulated by legislation worldwide - Commission Regulation 466/2001, 2174/2003 and 1881/2006. Our country has adopted the EU regulations since December 2005 - 118/2005.

Different analytical methods are used for aflatoxins analysis. The HPLC methods for mycotoxin analysis have gained more attention due to their efficiency and high sensitivity, especially when fluorescence detection is used since it provides high selectivity, low LOQ and accurate analysis. Since only the aflatoxins B_2 and G_2 show natural fluorescence, B_1 and G_1 must be derivatized prior to detection. This can be done photo chemically using irradiation with UV light at 254 nm. The aflatoxins B_1 and G_1 are thus hydroxylated, leading to stable and measureable fluorescence (Ibáñez –Vea et al., 2011; Ren et al., 2007). Very important aspect concerning mycotoxin analysis is sample preparation and clean-up procedure. Application of immunoaffinity column (IAC) provides clean extracts due to the specificity of the antibody, applicability to complex matrices, good precision, accuracy and sensitivity of analytical methods (Scott et al., 1997).

The aim of this article was determination of B_1 afflatoxin and total aflatoxins in some foodstuffs collected from local supermarkets, using immunoaffinity column clean-up procedure and liquid chromatography with fluorescence detection.

Materials and methods

Chemicals

HPLC grade reagents (methanol, acetonitril, water) and chemicals were purchased from Merck (Darmstadt, Germany). For clean-up purification immunoaffinity columns Afla-OtaCLEAN LCTech, Germany was used. Aflatoxins mix from Supelco, with concentrations of B_1 1.026 µg/ml, B_2 0.311 µg/ml, G_1 1.046 µg/ml and G_2 0.322 µg/ml were used as standards.

Samples

Many different commodities, cereal samples and their products (94), nuts (17), dried fruits (55), spices (33) and sunflowers seeds (12) were brought to our laboratory by border health inspectors or food operators during 2015 year.

The samples were kept in their original packages in dark, dry and cool place before analyzed.

Sample preparation

The extraction and purification of aflatoxins was performed according to LCTech sample extraction and clean-up procedure. According to the matrix, the appropriate method was selected. For non fatty matrices e.g. wheat and maize, 20 g of sample was extracted with 100 ml methanol: water (8:2 V/V) in a blender jar at a high speed for five minutes. For fatty matrices (nuts, paprika, and chilli) as well as for spices (black pepper, coriander, cumin, ginger), 20 g of tested samples with addition of 2 g NaCl, was ex-

* suzanaangelova@yahoo.com

tracted with 100 ml methanol: water (8:2 V/V) and 50 ml of n-hexane. The mixture was filtered through a fluted filter paper or centrifuged. The purified extract (14 ml) was added to an 86 ml PBS buffer (pH 7.2) and filtered again through 0.2 µm microfiber filter paper. 50 ml of the second filtrate (for spices maximum 14 ml) was quantitatively passed through the immunoaffinity column. The column was washed with 10 ml of water. Then, eluted with at 2 ml of methanol and measured directly by HPLC (ISO 16050, 2012).

HPLC conditions

HPLC analysis was performed with Agilent Technologies 1260 Series chromatographic system equipped with vacuum degasser G4225A, Binary Pump G1312B, Auto sampler G1329E, Column Compartment G1316C. Fluorescence Detector G1321B and UVE System for Photochemical derivatization LC Tech The UVE™ (LCTech, Germany). Post-column photochemical derivatization was used to increase the sensitivity of detection of Aflatoxins B_1 and G_1. Aflatoxins were separated on ZORBAX Eclipse plus C_{18} Column 4.6 x 100 mm, 3.5 µm at room temperature. The mobile phase was a mixture of water:acetonitril:methanol (63:11:26, V/V/V). The flow rate was 1 ml/min and the injection volume was 10 µl. The detection was carried out at and data were acquired using Agilent life Sciences OpenLAB CDS ChemStation software.

Methods

The limit of detection (LOD) was 3:1, calculated as S/N. The limit of quantification (LOQ) was 10: 1, S/N. Recovery, as a part of method performance evaluation, was determined according to the method of standard addition. Repeatability was estimated for B_1 and B_2, based on standard deviation and relative standard deviation, using the data from the QC sample analyses.

Results and discussion

Three-point calibration curves were linear in the proposed concentration range for all four aflatoxins and they had satisfactory coefficient of correlation (R^2) in the range of 0.9991- 0.9996.

Limits of detection were in the range 3 - 8 µg/kg for all four aflatoxins and limits of quantification were in the range of 9 – 23 µg/kg.

The following fortified concentration levels were applied: for B_1 (5.0 µg/kg), for B_2 (1.5 µg/kg), for G_1 (5.0 µg/kg), for G_2 (1.5 µg/kg), using an aflatoxin-free Trilogy reference material. For B_1 the recovery was 93.93%, for B_2 92.14%, for G_1 100.96% and for G_2 the recovery was 78.87%.

Repeatability was 0.16% for B_1 and 3.7% for B_2.

Laboratory for toxicological chemistry at PHI Center for Public Health Kumanovo took part in FAPAS Food Chemistry proficiency Test 04238 Aflatoxins B and G in Maize. Proficiency testing aims to provide an independent assessment of the competence of participating laboratories and is an essential element of laboratory quality assurance. The performance of the laboratory is shown with z-scores results ($-2 \leq z \leq 2$). Z- scores results for B_1 was - 0.8, for B_2 -1.1, for G_1 -0.5, for G_2 -1.5 and 0.9 for total aflatoxins. They were statistical satisfactory.

Most of the cereals, nuts and dried fruits samples are below LOD. Two samples of red pepper powder had 1.486 ng/g and 1.612 ng/g total aflatoxins, respectively, which was below the permitted MRL of 10.0 ng/g. Four samples of sunflowers seeds had 28.7 ng/g, 19.4 ng/g/ 22.6 ng/g and 8.3 ng/g total aflatoxins, respectively. The values exceeding the maximum permissible limits of 4 ng/g set by national regulative.

Conclusion

Liquid chromatography linked to fluorescence detection (HPLC/FD) and system for photochemical derivatization is suitable for routine analysis of aflatoxins in many commodities. Limits of quantification, precision, and recovery were satisfactory allowing it to be used also as a method for aflatoxins confirmatory analysis.

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Macedonian pharmaceutical bulletin, **62** (suppl) 293 - 294 (2016)
ISSN 1409 - 8695
UDC: 615.322:615.272
Short communication

Screening of some plant species for their antioxidant and antibacterial activity

Eljona Chilku[1]*, Blagica Jovanova[3], Snezana Ivic Kolevska[2] and Tatjana Kadifkova Panovska[3]

[1]*Department of Human Medicines, Agency for Medicines and Medical Devices, Skopje, Republic of Macedonia*
[2]*PZU Re-Medika, Skopje, Republic of Macedonia*
[3]*Institute of Applied Biochemistry, Faculty of Pharmacy, Ss. Cyril and Methodius University, 1000 Skopje, Republic of Macedonia*

Introduction

Biochemical reactions in the body generate reactive oxygen species, which can damage important bio-molecules, leading to several disease conditions (Bhatt and Negi, 2012). Antioxidants are compounds which have the ability to trap the free radicals. In recent years, the interest in finding naturally occurring antioxidants has increased considerably for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Zheng and Wang, 2001). Majority of the antioxidants from plants are secondary metabolites like phenolic and flavonoids that have been reported to be potent free radical scavengers (Baba and Malik, 2014).

Resistance to available antibiotics is increasing at a very alarming stage globally. The antibacterial activity of plants is continuously attracting global attention. Plants, as the source of medicine, have been playing an important role in the health services around the world (Subedi et al., 2012). All crude extracts from plants represent a valuable source of antioxidant and antibacterial agents, as well (Hossain et al., 2014).

Recently, there have been great efforts to find safe and potent natural antioxidants and antibacterial agents from various plant sources. Therefore, the aim of the present study was to evaluate the *in vitro* antioxidant and antibacterial activity of ethanol extracts of leaves of *Betula pendula* L., *Tusilago farfara* L., *Plantago major* L., *Urtica dioica* L., *Rosmarinus officinalis* L. and *Melissa officinalis* L.

Materials and methods

Dried leaves of *Betula pendula* L., *Tusilago farfara* L., *Plantago major* L., *Urtica dioica* L., *Rosmarinus officinalis* L. and *Melissa officinalis* L. were purchased from the botanical departments of Macedonian manufacturers.

The crude extracts were prepared by extraction of grounded dried leaves of plants with ethanol. The total phenolic content was quantified by Folin-Ciocalteu method and was expressed as Gallic acid equivalents (GAE), milligram's per 1 gram of dry weight. The total flavonoids content was quantified by Aluminum chloride method, which was expressed as milligrams of quercetin equivalents (QE) per gram of dry extracts. *In vitro* antioxidant activity was carried out by DPPH assay and expressed as IC_{50} (Gyamfi et al., 1999). The antibacterial activity was carried out by well diffusion method. This activity was qualitatively assessed by presence or absence of inhibition zone (Perez et al., 1999).

Results and discussion

The total phenolic content was ranged as followed: *Melissa officinalis* L.(181.98±0.6016 GAE mg/g)>*Rosmarinus officinalis* L. (179.03±0.4171 GAE mg/g)>*Tusilago farfara* L.(169.59±0.5840 GAE mg/g)>*Betula pendula* L. (169.12±0.0834 GAE mg/g)>*Plantago major* L. (128.9±0.8343 GAE mg/g)>*Urtica dioica* L. (70.71±0.6516 GAE mg/g). The total flavonoid content was 154.52±2.5534 QE mg/g, 115.13±0.1178 QE mg/g,142.3±5.0675 QE mg/g, 58.47±0.6123 QE mg/g,

* ecilku@yahoo.com

75.97±0.4249 QE mg/g and 25.63±1.0606 QE mg/g, respectively. Ranking based on the antioxidant activity was: *Melissa officinalis* L. (2.6 µg/ml)>*Rosmarinus officinalis* L. (78.58 µg/ml)>*Tusilago farfara* L. (140.9 µg/ml)>*Betula pendula* L. (244.45 µg/ml)>*Plantago major* L. (295.9 µg/ml)>*Urtica dioica* L. (458.7 µg/ml).

Sensitivity of test strains, in decreasing order, was as follows: *Pseudomonas aeruginosa* ATCC 9027>*Enterococcus faecalis* ATCC 19433>*Escherichia coli* ATCC 25922 >*Escherichia coli* ATCC 8739 >*Klebsiella pneumoniae* ATCC 700603>*Staphylococcus aureus* ATCC 25923.

Melissa officinalis L. showed the highest total phenolic and flavonoids content and the strongest DPPH radical scavenging activity, followed by *Rosmarinus officinalis* L. and *Tusilago farfara* L. It is known that phenolic compounds in plant extracts contribute significantly to their antioxidant potential because of their unique structure (Bhatt and Negi, 2012). *Betula pendula* L. showed moderate antioxidant properties beside the high phenolic content. *Plantago major* L. showed moderate antioxidant properties. *Urtica dioica* L. showed the lowest total phenolic and flavonoids content and the weakest DPPH radical scavenging activity.

According to the results of antimicrobial screening, *Rosmarinus officinalis* L. showed strong antibacterial activity against all six bacterial stains. *Tusilago farfara* L., showed strong antibacterial activity towards the investigated bacteria except the weak antibacterial activity against *Escherichia coli* ATCC 8739 and *Klebsiella pneumoniae* ATCC 700603. *Betula pendula* L. has been shown to possess the strongest antimicrobial activities against *Enterococcus faecalis* ATCC 19433 and *Pseudomonus aeruginosa* ATCC 9027. *Plantago major* L. and *Urtica dioica* L. exhibited strong antimicrobial activity towards *Pseudomonas aeruginosa* ATCC 9027, weak activity against *Escherichia coli* ATCC 8739 and *Klebsiella pneumonia* ATCC 700603. *Melissa officinalis* L. shows moderate antibacterial activity against gram (+) bacteria and very weak or no activity against gram (-) bacteria.

Conclusion

The results indicated that all of the plant extracts tested in this study could be considered promising antioxidant sources and great potential as antibacterial compounds against gram-positive and gram-negative bacteria.

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Macedonian pharmaceutical bulletin, **62** (suppl) 295 - 296 (2016)
ISSN 1409 - 8695
UDC: 581.151.044:546.4/.8
Short communication

Approach to detect possible genotoxic effects of metals in plants

Darinka Gjorgieva Ackova[1]*, Tatjana Kadifkova Panovska[2], Katerina Bačeva Andonovska[3] and Trajče Stafilov[4]

[1] *Faculty of Medical Sciences, Goce Delčev University, 2000 Štip, R. Macedonia*
[2] *Faculty of Pharmacy, Ss. Cyril and Methodius University, 1000 Skopje, R. Macedonia*
[3] *Research Center for Environment and Materials, Macedonian Academy of Sciences and Arts, 1000 Skopje, R. Macedonia*
[4] *Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, 1000 Skopje, R. Macedonia*

Introduction

During the intensive research in past decades, the scientific community and regulatory agencies also, focused their interest on measuring contaminant levels in tissues and environmental samples and on understanding the mechanisms of toxicity of different and pervasive contaminants. Among them, metals were always in focus because of possible detrimental and long lasting effects on living organisms (Gjorgieva et al., 2011; Stafilov et al., 2010). DNA damage and genotoxic stress are important parameters that are followed in a case of chemicals exposition as an indication of carcinogenicity (Aydin et al., 2015; Kekec et al., 2010; Villatoro-Pulido et al., 2013). Plants are unique systems in their ability to serve as in situ monitors for environmental genotoxins. Molecular assays related to DNA-based techniques, like Random Amplified Polymorphic DNA (RAPD), are very sensitive method of screening for nucleotide sequence polymorphisms that are randomly distributed throughout the investigated genome (Gjorgieva et al., 2012).

The current study was designed to assess the effects of long term, high metal exposition (cadmium, lead, copper, nickel and zinc) on DNA damage.

Materials and methods

Four different plants [*Taraxacum officinale* (Asteraceae), *Matricaria recutita* L. (Asteraceae), *Robinia pseudoacacia* L. (Fabaceae), and *Urtica dioica* (Urticaceae)] were chosen as model systems for genotoxicity assessment in a case of metal stressors. Plant samples were collected from two different areas, metal polluted (area around city of Veles, known for its lead and zinc industrial activity in the nearest past) and referent area (without metal exposition, Plačkovica Mountain). Element analysis (analyzed by Inductively Coupled Plasma – Atomic Emission Spectrometer (ICP-AES)), DNA extraction (frozen plant samples were used for DNA isolation by using REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich)) and RAPD-PCR analysis were performed with samples included in this study. Amplifications were performed in a DNA thermo cycler according to protocol of Enan, 2006. Seven different primers (with 60-70% GC content) were used. Sequences (5'→3') from Primer 1 to 7 were: Primer 1 - GGTGCGGGAA; Primer 2 – GTTTCGCTCC; Primer 3 – GTAGACCCGT; Primer 4 – AAGAGCCCGT; Primer 5 – AACGCGCAAC; Primer 6 – CCCGTCAGCA; Primer 7 - GGCACTGAGG. All amplifications were repeated twice in order to confirm the reproducible amplification of scored fragments. Only reproducible and clear bands were scored for the construction of the data matrix.

Results and discussion

RAPD technique was used to quantify DNA sequence changes. Agarose-gel electrophoresis of extracted DNA from all samples, revealed total of 37 bands with different molecular weights ranging from 1250 to 5000 bp. It generated distinctive polymorphism value of 72.97% (27 bands) total in four plant species investigated. Polymorphism in the RAPD profiles is scored as the presence or absence

* darinka.gorgieva@ugd.edu.mk

of DNA bands in comparison to the control. The numerical analysis based on the banding pattern obtained from the samples exposed to metals was compared with that of the control sample via hierarchical cluster analysis. RAPD profiles generated by samples exposed to metals were different from those obtained using control DNA. Samples which were close to the pollution source (high metal-exposition area) showed considerable polymorphism. The diagnostic analysis (considering band intensity differences or the disappearance and/or appearance of RAPD bands) and the phenetic numerical analysis are important parameters in the RAPD method (Bhaduri and Fulekar, 2015; Gjorgieva et al., 2012; Zhiyi and Haowen, 2004). Amplification with primer 1 and primer 2 yielded not enough significant polymorphism, so we find that this primer sequences are not suitable for fingerprinting genome of this four plant species. When amplification is performed with primer 3 and 5, the highest number of new bands is obtained (9 and 8 bands, respectively), with exception of primer 5 for T. officinale, where probably there is no suitable sequence in the genome of this plant for annealing of above mentioned primer. The dendrogram constructed using NTSYSpc programme (Rohlf, 1994) is statistical presentation of the obtained date and showed that there is grouping in separate clusters of the same plant species collected from two different areas, according to modifications in the RAPD "fingerprints" following metal exposure. In accordance with literature data that considered concentration of 40 mg L-1 of lead and 30 mg L-1 of cadmium (Aydin et al., 2013; Aydin et al., 2015) as the point of maximum appearance and disappearance of new bands in RAPD assay, we assume that high metal content in all samples from Veles area, determined in present study, was directly involved in DNA damage detected which can lead to initiation of mutations.

Conclusion

RAPD assay is a valuable tool to evaluate the effects of toxicants on organisms under optimized conditions and for genotoxicity studies. RAPD markers can be used as diagnostic markers. In this regard, induction of DNA band changes with one kind of a stressor (found in areas with metal pollution) might provide evidence of its genotoxic potential.

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Macedonian pharmaceutical bulletin, **62** (suppl) 297 - 298 (2016)
ISSN 1409 - 8695
UDC: 615.277.035.1
Short communication

Biochemical pathways in cancer progression as pharmacological targets

Milena Prculovska*, Ivana Angelovska, Tatjana Kadifkova Panovska, Marija Hiljadnikova-Bajro

Institute for Applied Biochemistry, Faculty of Pharmacy, University SS Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Background

Despite the public awareness and technological advancement in early cancer detection as well as the discovery of many novel therapeutic treatments, cancer takes away many lives worldwide each year. The limited therapeutic effects of the traditional treatments and their numerous side effects which compromise patients' compliance, urge the need for development of new medicinal anticancer agents. Therefore, numerous new research studies have been directed towards development of more specific targeted therapy.

The most common reason for mortality among cancer patients is the tumor progression and formation of metastasis within organs distant to the location of the primary tumor. The purpose of this paper is to review the most relevant biomolecular factors that enable the metastatic process, and their potential for employment as targets for future development of new therapeutic anticancer agents.

Angiogenesis

The access to the host's vascular system and the development of a blood network around and within the tumor, a process known as angiogenesis, is of crucial importance for tumor's supply with oxygen and nutritional substances and subsequent increase of the tumor mass, invasion and cancer dissemination. A lot of experimental and clinical data evidence that tumors can't grow beyond a diameter of 2-3 mm until the blood network is created, i.e. they can't progress until they acquire angiogenic ability. Hence, it has been proposed that the inhibition of angiogenesis could suppress the tumor's growth and its targeting has an important clinical validity for development of new approaches for cancer treatment.

The vascular endothelial growth factor (VEGF) and its receptor vascular endothelial growth factor receptor(VEGFR) have major roles in the process of angiogenesis and they can often be overexpressed in tumorous cells. Having in regard the role of VEGF as a potent angiogenic factor, many therapeutic strategies that target the angiogenesis via inhibition of VEGF signal pathway have been developed. These therapeutic strategies include use of: small molecule tyrosine kinase inhibitors of VEGF receptors, neutralizing monoclonal antibodies against VEGF or its receptor, soluble VEGF receptors which act as VEGF traps and ribozymes which specifically target VEGF mRNA (Cardones and Banez, 2006).

Other important biochemical mediators are the fibroblast growth factors (FGFs), which belong to the family of heparin binding proteins. The fibroblast growth factors and their receptors participate in the proliferation and migration of endothelial cells, production of proteases and angiogenesis. Recent studies show that FGFs synergize with VEGF during neovascularization and that FGFs could be involved in the mechanism for resistance towards anti-VEGF agents. Therefore, the new plausible therapeutic approaches are focused either on the combined or the consecutive inhibition of these two crucial angiogenetic signal pathways (Lieu et al., 2011).

Angiopoietins are growth factors that also participate in the formation of blood vessels in several tumor types. Angiopoietin-1 and -2 are the best characterized cytokines which accomplish their biological function by binding to the Tie-2 receptor. Angiopoietin-1 enables survival of endothelial cells, formation and stabilization of the vascular network. On the other hand, the positive regulation

* milena.prculovska@gmail.com

of angiopoietin-2 disrupts the interaction between angiopoietin-1 with its receptor leading to destabilization of the blood vessels and subsequent enabling of VEGF-inducted angiogenesis. Because of this, the targeting of angiopoetin-2 has a promising potential for cancer treatment. A novel therapeutic strategy involves development of agents that target the angiopoietin/Tie-2 signaling pathway. The first such agent which has reached advanced stages of clinical trials is AMG-386. AMG-386 is an anti-angiopoietin peptibody which inhibits the interaction between the ligands angiopoietin-1 and angiopoietin-2 with Tie-2 receptor (Neal and Wakelee, 2010).

Cell detachment and extracellular matrix degradation

Apart from its relation to the angiogenesis, the acquisition of metastatic potential is also related to the detachment of the malignant cell from the primary tumor and degradation of proteins which comprise the surrounding extracellular matrix (ECM). The loss of cell-cell adhesion capacity enables the malignant cells to dissociate from the primary tumor, while the changes in the cell-matrix interaction enable the cells to invade the surrounding stroma and disseminate. The most important adhesive molecules which have been considered as potential therapeutic anticancer targets are E-cadherin and integrin.

E-cadherin is type-1 transmembrane protein that mediates the cell-cell interaction. The reduced expression of E-cadherin is associated with an increase of the metastatic potential. One of the possible approaches for reduction of the invasive capacity of cancer cells implies enhancement of the inter-cell adhesive capacity through overexpression of E-cadherin.

The integrins are surface receptors which mediate the cell-ECM interaction. The integrin's role in the metastasis of malignant cells, their localization on the cell's surface and sensitivity to a pharmacological blockade, makes them attractive for pharmacological targeting. Small molecule integrin antagonists, including the αvβ3 and αvβ5 inhibitor Cilengitide, have shown encouraging activity in controlling cell proliferation and metastasis. Beside from the small molecule antagonists, monoclonal antibodies against integrins, such as CNTO95, etaracizumab (MEDI-522), and volociximab are being subjected to clinical trials (Xiangming, 2015).

ECM is composed of a thick network of proteins such as collagen and fibronectin. It must be invaded by the invasive cancer to enable itself space for migration. The most important proteolytic enzymes are the matrix metalloproteinases (MMPs) which belong to the group of zinc-dependent endopeptidases. These enzymes have an important role in tumor invasion, metastasis, and angiogenesis and are considered the major mediators of changes in the new microenvironment at the time of progression of the cancer. As such, they are an attractive target for its treatment.

The Epithelial-mesenchymal transition pathway is a dynamic process that enables the polarized epithelial cells to lose the epithelial differentiation and acquire mesenchymal phenotype via numerous biochemical and morphological changes. Thus, they gain higher migratory ability and Felicity's group suggests the Epithelial-mesenchymal transition pathway as promising target for development of therapeutic agents for prevention of tumor dissemination and metastasis (Felicity et al., 2014).

Conclusion

Understanding the biomolecular mechanisms underlying the process of malignant progression and identification of the factors involved in this process should enable new therapeutic agents that would target the key molecular pathways of cancer growth and dissemination to be developed in future. In that way, selective action, higher efficiency and minimization of side effects of anticancer drugs would be accomplished.

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Macedonian pharmaceutical bulletin, **62** (suppl) 299 - 300
(2016)
ISSN 1409 - 8695
UDC: 615.277.035.1
Short communication

Biomolecular mechanisms of cancer initiation as targets for therapeutic intervention

Ivana Angelovska*, Milena Prculovska, Tatjana Kadifkova Panovska, Marija Hiljadnikova-Bajro

Institute for Applied Biochemistry, Faculty of Pharmacy, University SS Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

The term cancer is broadly used to describe over a hundred malignant diseases in the human population. The incidence and prevalence of cancer are constantly increasing. It is presumed that by 2030 the annual number of newly diagnosed cases will reach 23 600 000 which is an increase of 68% compared to the reported cases in 2012 (Bray et al., 2012). This fact points out the expansive tendency of cancer worldwide and emphasizes the need for dedicating higher attention both to prevention and treatment of this disease.

The traditional methods for cancer treatment have limited efficiency and high toxicity, exerting inevitable aggressiveness towards healthy tissues. Hence, medical practitioners and scientists are faced with the need for considering the options of targeted action of the therapeutic agents and for focusing further research efforts on their development. The targeted therapy would be directed towards normalization of what is changed or cessation of some of the active cascades that initiate tumor development. There are already established molecular targets which can contribute to the inhibition of tumor's growth and restoration of normal functions. The aim of this study is to review currently available data on the biomolecular processes involved in the early steps of cancer initiation and the possibility of their use as therapeutic targets in order to achieve maximal efficacy and minimal toxicity of the anticancer treatments.

Cyclin and cyclin dependent kinases

Cyclin and cyclin dependent kinases (CK/CDK) are responsible for the normal function and control over the cell cycle. They are check-up points which control the possible irregularities during the cell cycle. It is known that, in certain tumors, the levels of CK/CDK are constitutively increased, continuously encouraging the cell cycle and the creation of new cells. As such, CK/CDK are serious target candidates towards which new therapeutic agents can be directed in order to reduce their expression and normalize the cell division. Studies have been done on cyclin E which is important for controlling the cell cycle in G1 phase and its activation is important for postponing the malignant processes. It is believed that the new medicaments should act at the level of inhibiting distinct phases of the cell cycle. Recently, several preclinical and phase I/II clinical studies have been conducted using a novel, reversible CDK4/6 inhibitor named Palbociclib. It was shown that Palbociclib has the role of CDK4/6 as a potential target in estrogen receptor-positive (ER+) breast cancers (Finn et al., 2016).

Oncogenes and tumor suppressor genes

The activation of oncogenes and inactivation of tumor suppressive genes have an important role in the process of malignant cell transformation. One of the cancer treatment modalities has the opposite goal. The suppression of the RAS-pathway is a significant target regarding the mechanism of oncogene inactivation. This can be achieved by several mechanisms. One of them is disabling the binding of the Epidermal Growth Factor (EGF) to its receptor (EGFR), so that the dimerization of the intercellular part of the receptor, which associates with the adapter protein Son of Sevenless (SoS) and the receptor of the growth-binding protein 2 (Grb-2), is prevented. It is very important to attain this blockade, as the SoS-Grb-2 complex directly ac-

* angelovska_i@yahoo.com

tivates the RAS-oncogene. RAS-induces intracellular cascade of kinases for promotion of proliferation. On the other hand, with activation of the RAS-oncogene, an activation of RAF-oncogene happens, which encourages RAF-Mitogen-activated protein kinases and direct signals are sent to the core for starting cell division. The function of the RAS-oncogene is related to the proliferation, genetic expression, differentiation, emphasizing the cell survival and apoptosis. Because of the fact that RAF-MEK-ERK cascade is very complex and it is difficult to find RAF and MEK inhibitors or inhibitors for RAS genes, the new strategies for inhibition of the RAS-oncogene are directed towards mutations of RNA and RAS-oncogene (Baines et al., 2011).

A significant representative of the group of tyrosine kinases is the BCR-ABL oncogene. This oncogene is related to the Philadelphia chromosome-positive leukemia. The kinase activity of BCR-ABL is the primary factor for stimulation of myeloid cells proliferation. As such, BCR-ABL oncogene is considered as a potential target, during treatment of chronically myeloid leukemia, for Imatinib, which inhibits both the ABL and BCR-ABL tyrosine kinases. However it was discovered that in some patients, white blood cells become resistant to Imatinib. One newer approach presents BMS-354825 developed by Bristol-Myers Squibb, that binds to the active form of ABL and overcomes 14 of 15 Imatinib-resistant mutants (Scholar et al., 2005)

When it comes to the tumor suppressive genes, a method should be developed for their activation in order to delay cell's growth and proliferation and to re-establish the processes of DNA reparation and apoptosis. The most studied gene is p53 which is also most frequently inactivated in cancer processes. Research directed towards new targets should take into consideration the suppression, the possible induction of p53 in normal, healthy cells, as well as the fact that increased levels of MDM2 are expected. The increased levels of MDM2 arise from the activation of p53 with its potential target and are expected to cause suppression of p53 as negative regulator (Wang et al., 2010).

Apoptosis

Apoptosis is a genetically controlled form of cell death with a huge importance for physiological tissue remodeling during embryogenesis and maintaining homeostasis later in cell's life. The process of apoptosis is regulated via extrinsic and intrinsic pathways. The extrinsic pathways are activated through cell surface receptors such as the tumor-necrosis factor receptor (TNFR), death receptors and the CD95/FAS/APO1 receptor. The intrinsic pathways are activated through intracellular stimuli caused by cytotoxic medicaments, hypoxia, DNA damage. These paths are especially interesting for investigation as potential targets for anti-cancer agents. One of the possible targets are Smac peptidomimetics which act as mimetic agents but the poor pharmacokinetic properties prevents their clinical application (Elsayed et al., 2015).

Conclusion

The above mentioned pathways involved in initiation and development of malignant neoplasm, as well as those which are to be identified in future, will have a significant contribution in the development of a suitable therapy for this disease, by specifically and selectively targeting malignant cells and aberrant signaling pathways without harming the physiologically healthy cell processes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 301 - 302 (2016)
ISSN 1409 - 8695
UDC: 616.33-022.7:579.835.1]:547.495.2:543.5
Short communication

Biochemical identification of *Helicobacter pylori* using the urea breath test

Irena Smokvarska*, Tatjana Kadifkova Panovska, Marija Hiljadnikova-Bajro

Institute for Applied Biochemistry, Faculty of Pharmacy, University SS Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

The infection with the bacterium H*elicobacter pylori* (*H. pylori*) is one of the major causative factors for the development of gastric cancer. The presence of *H. pylori* bacteria in the human gastrointestinal tract is determined by different invasive and noninvasive diagnostic methods. The invasive strategies are based on endoscopy which can be complemented by histology, urease test or tissue culture to detect bacteria. Non-invasive tests include: antibody tests (using serum, saliva or whole blood samples) which do not directly detect the presence of *H. pylori* but rather the presence of an immune response to *H. pylori* in the form of antibodies. These antibodies may persist even after successful eradication. The antigen tests are performed on stool, saliva or urine samples, current interest focuses mainly on the stool antigen test for the detection of *H. pylori* antigens by an enzyme immunoassay technique. One of the recent advances regarding methodological approaches for diagnosis and monitoring of patients with suspected gastric infection with *H. pylori* is the urease test also termed urea breath test.

The aim of this paper is to provide an up-to-date comprehensive review on the urea breath test as a noninvasive method, its application and expansive usage for early detection of infections caused by the *H. pylori* bacteria, as well as prevention of other progressive illnesses by enabling prompt eradication of the infection.

Studies on the urea breath test

The urea breath test is an accurate, practical and readily available test which becomes known as a golden standard when compared to other tests for identification and quantification of *H. pylori*. Being both specific and sensitive, the urea breath test is based on testing the expired air samples collected before and after ingestion of urea containing specifically labeled carbon. *H. pylori* bacteria produce an enzyme called urease that converts urea into carbon dioxide and ammonia. The carbon dioxide is excreted with the expired air from the lungs and the amount of the labeled carbon dioxide can be measured in the expired air sample, in order to determine the presence of the active *H. pylori* infection in the stomach and the level of the infection. In order to postpone stomach draining during the examination, a citric acid solution is consumed prior to the test. The lemon acid induces imminent relaxation of the gastric fundus as well as inhibition of the antral motility through biliary reflux. It has been reported that best results can be obtained with expired air samples collected within 10-15 minutes after the urea is consumed (Berger, 2002). The collected samples of expired air can be easily stored for a couple of days at different temperatures.

A substantial number of research projects have studied the influence of several factors on the sensitivity and accuracy of the test. The first studies were focused on the labeling of the urea used in the breath test.Generally, two methods are being used for labeling the urea-carbon, the first one uses a steady and heavy isotope ^{13}C while the other one uses radioactive isotope ^{14}C (Berger, 2002). It is also possible to label the urea with both ^{13}C and^{14}C but this approach has relative strengths and flows. The radioactive ^{14}C urea breath test can be performed only in hospitals that have nuclear wards and for some patients a few attempts might be needed in order to produce sufficient amounts of CO_2 to change the color of the solution. On the contrary, ^{13}C urea breath test can be used in non-nuclear laboratory settings and the samples can be sent for mass spectrometry analysis elsewhere which is a great advantage in compari-

* smokvarska.irena@gmail.com

son with the radioactive ^{14}C urea breath test. Furthermore, the small amount of expired air which is needed for analysis, makes ^{13}C urea breath test ideal for usage (Desroches, 2001). Even though the dose of ^{14}C used in the urease test is very low, it is contraindicated for application in pregnant women and small children.On the contrary, ^{13}C – urea breath test can be used for the above mentioned groups of patients and is especially useful for testing the pediatric population (Jones et al., 2005).

The hydrolysis of the ^{13}C – labeled urea by *H. pylori* urease in the stomach produces $^{13}C0_2$ which is then transported to the lungs, whereby ^{13}C in the expired air is revealed by the usage of special equipment. Currently several types of equipment are suggested for evaluation of the ^{13}C expired in air samples: Mass Spectrometry – Isotope Relationship (MSIR), non – dispersive isotope – selective IR spectroscopy (NDIRS) and laser analyzer equipment (LAE) (Parente et al., 2001).

The influence of the amount of consumed urea on the accuracy of the urea breath tests has been extensively studied and debated. The oldest tests were performed with 350 mg of urea, later the dose was decreased to 125 mg or 100mg and all tests turned out to be successful.Recently, it was suggested that 75 mg of urea might be sufficient for obtaining reliable results, but later on, it has been confirmed that an urea dose as low as 50 mg can be used for obtaining more precise urea breath test results.

Urease – producing oropharyngeal bacteria can cause falsely positive results of the urea breath test. This problem has been solved by encapsulating the urea for its oral administration. Considering the advantages, the development of a capsular formulation with ^{13}C urea is an outstanding progress and the most appreciated system for labeled urea delivery. A 50 mg capsule with ^{13}C urea for detection of *H. pylori* in expired air manifests high sensitivity and distinctiveness in clinical conditions (Mattar et al., 2014).

Several types of drug treatments are associated with falsely-negative results when using urea breath test. Therefore, antibiotics, bismuth concoctions and proton pump inhibitors should not be taken within one month prior to the urea breath testing. Also, according to some studies H_2–antagonists cause falsely-negative results if administered in high doses. This should be taken into consideration, especially because patients are frequently treated with H_2–antagonists before urea breath test is performed.

Conclusions

The UBT is based on the simple principle that a solution of isotopically labelled urea is rapidly hydrolyzed by the abundantly expressed urease of *H. pylori*. The UBT is a non-invasive, simple and safe test, providing excellent accuracy both for the initial diagnosis of *H. pylori* infection, confirmation of its eradication after treatment and patients' follow-up.

Although definitive standardization of the protocol for UBT isn't currently available, several recommendations could be suggested. It seems prudent to perform UBT in fasting conditions until new data definitively clarify this issue. Citric acid should be used as a test meal, amounts of 75 mg or even 50 mg of urea seem to be sufficient to perform highly accurate UBT. The test can be carried out by different types of equipment, such as IRMS, NDIRS or LAE with recommendation to collect and test two breath samples, one collected before and another collected 10–30 min after urea ingestion. Further studies should be performed to justify the cost-benefit ratio for routine application of this method in the biochemical laboratories.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 303 - 304 (2016)
ISSN 1409 - 8695
UDC: 664.854:579.67]:543.544.5.068.7
Short communication

Determination of Ochratoxin A in some dried fruits by liquid chromatography

Suzana Angelova[1]*, Valide Sabani[1] and Tatjana Kadifkova Panovska[2]

[1]*PHI Center for Public Health Kumanovo, 11th October bb, 1300 Kumanovo, R. Macedonia*
[2]*Faculty of Pharmacy, Ss.Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

Ochratoxin A (OTA) is the most common mycotoxin that can affect human health. The International Agency for Research on cancer (IARC) classifies it as possibly carcinogenic to humans (group 2B). This mycotoxin is produced by toxigenic *Aspergillus* and *Penicillium* species (Pfohl-Leszkowicz et al., 2007). OTA has been extensively documented as a contaminant of a wide variety of foods including cereals, green coffee, spices, nuts, dried fruits, beer, wine, grapes, and grape juice (Bellí et al., 2005). The target organ of OTA is the kidney. However, it has a wide range of other toxic effects, including hepatotoxicity, immunotoxicity, teratogenicity and neuorotoxicity (Pfohl-Leszkowicz et al., 2007; Sava et al., 2006).

Many countries and international organizations have regulated the OTA content in several commodities. The European Commission (EC) has enforced the limits of OTA in cereals and cereal products with the following levels: 5.0 ng/g for raw cereals grains, 3.0 ng/g for cereals and cereal products intended for human consumption, 0.5 ng/g for baby food and cereal- based food intended for young children. For the dried vine fruits, soluble coffee and some dried fruits, the EC has set a maximal permissible limit for Ochratoxin A at 10.0 ng/g. Liquid chromatography linked to fluorescence detection (HPLC/FD) was extensively used for OTA confirmatory analysis (Ghali et al., 2009).

The aim of this article was to determine concentration of OTA in some foodstuffs collected from supermarkets by liquid chromatography.

Materials and methods

Chemicals

HPLC grade reagents (methanol, acetonitril, glacial acetic acid) and chemicals were purchased from Merck (Darmstadt, Germany). For clean-up purification immunoaffinity columns Ochraprep (R-Biopharm Rhone, Glasgow, Scotland), were used. Ochratoxin A Trylogy, with concentrations of 10.0 µg/ml in methanol was used as a standard.

Commercially available Ochratoxin A, with concentrations of 10.0 µg/ml in methanol, was used as a stock solution. Ochratoxin A spiking solution contains 2.5 µg/ml Ochratoxin A and Ochratoxin A standard solution (0.25 µg/ml Ochratoxin A). Ochratoxin A working solutions, in concentration ranging from 2.0 ng/ml to 10.0 ng/ml, were prepared by serial dilution of the standard solution.

Samples

During 2015 year, 57 dried fruits samples including raisins (8), figs (8), dates (5), apricots (5), plumes (10), cranberries (3), cherries (3), pineapples (3), strawberries (2), goji (1), and papaya (1) were randomly collected from local supermarkets. Samples were stored in plastic bags at -200C until the analysis.

Sample preparation

To ensure homogeneity before analysis, laboratory samples are normally slurred with water in the ratio of five parts fruit to four parts water, and test materials in this form were used.

A test portion, 45 g of fruit slurry, is extracted with a mixture of 50 ml methanol and 5 ml phosphoric acid solution 0.1 mol/L. The extract is filtered, diluted with phos-

* suzanaangelova@yahoo.com

phate buffered saline, and applied into an immunoaffinity column containing antibodies specific for Ochratoxin A. The Ochratoxin A is isolated, purified and concentrated on the column and then released with elution solvent. Ochratoxin A is quantified by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection (MKC EN 15829:2011).

HPLC conditions

HPLC analysis was performed with Agilent Technologies 1260 Series chromatographic system equipped with vacuum degasser G4225A, Binary Pump G1312B, Auto sampler G1329E, Column Compartment G1316C and Fluorescence Detector G1321B. Ochratoxin A was separated on ZORBAX Eclipse Plus C_{18} Column 4.6 x 100 mm, 3.5 µm at room temperature. The mobile phase was a mixture of water:acetonitril:glacial acetic acid (99:99:2, V/V/V). The flow rate was 1 ml/min and the injection volume was 10 µl. Total running time was four minutes. The detection was carried out at and Data were acquired using Agilent life Sciences Open LAB CDS ChemStation software.

Results and discussion

The described analytical methods effectively separated the Ochratoxin A of all analyzed products. It was observed that under these optimized chromatographic conditions, Ochratotoxin A eluted in less than 5 minutes. The retention time was 3.131 minutes. The calibration curve was set in the range from 2.0 to 10.0 ng/ml using four-point calibration curve. The regression equation was y=0.02198191x +0.0013271 with R^2 - 0.99988. The accuracy was 4.7%. The limit of quantification was 1 ng/g. The mean recovery for Ochratoxin A was 86.4%.

Laboratory for toxicological chemistry at PHI Center for Public Health Kumanovo took part in FAPAS Food Chemistry proficiency Test 17150 Ochratoxin A in dried Vine Fruit. Proficiency testing aims to provide an independent assessment of the competence of participating laboratories and it is an essential element of laboratory quality assurance. The performance of the laboratory is shown with z-scores results ($-2 \leq z \leq 2$). The result for z-score was at satisfactory level of 1.8.

Ochratoxin A was found in two of all analyzed products. Two samples of raisins contain 2.01 ng/g and 1.89 ng/g, respectively. None of the samples exceeded maximum permissible limits of 10 ng/g set by national regulative (Official Journal of the R Macedonia No.102/2013).

Conclusion

The described method is suitable for routine analysis of Ochratoxin A in dried fruits. Limits of quantification, precision, and recovery were satisfactory. The benefit from short time analysis is not only in the increased sample throughput, but also in dramatically decreased solvent consumption. Therefore, the liquid chromatography linked to fluorescence detection (HPLC/FD) was extensively used for OTA confirmatory analysis.

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Macedonian pharmaceutical bulletin, **62** (suppl) 305 - 306 (2016)
ISSN 1409 - 8695
UDC: 615.322.074:615.918
Short communication

Determination of the toxic bioactivity of methanol extracts of selected commercial herbal teas

Blagica Jovanova* and Tatjana Kadifkova Panovska

Institute of Applied Biochemistry, Department of Toxicology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Herbal medicine has been used since ancient times for prevention and treatment of numerous diseases. Different cultures utilize numerous plant species which are usually characteristic for the geographical region where they are natively growing. The repeated use of herbal preparations for various purposes is commonly based on the positive experience and the beneficial outcome of their usage as part of traditional medicine of the population.

According to the World Health Organization statistics, 80% of the population relies on traditional herbal medicine as a primary healthcare system. Furthermore, more than 50% of the population in developed countries such as Germany (90%), France (75%) and Canada (70%) has at least once in their lifetime relied on herbal products as a primary treatment. Beside the common practice of traditional herbal medicine, commercially available herbal products are also widely used in the treatment of cardiovascular diseases, allergies, asthma, arthritis and many other diseases. On the other hand, the increasing usage of herbal products is becoming major concern of health regulatory bodies and the general public, mainly because of the high availability of the herbal products on the market (Ekor, 2014). Moreover, a great portion of these products are classified as food or dietary supplements in some countries, making them easily accessible for the general public. The common perception of herbal products being generally safe regardless of the administered dose is often misleading. Therefore, toxicity evaluation of commercially available herbal products is mandatory, considering the possibility of using their toxic potential in cytotoxicity studies.

Materials and methods

Plant samples of 9 different species were tested for their toxic potential: 2 samples of bark (*Rhamnus frangula* and *Quercus pedunculata*), and 7 samples of whole herbal parts (*Capsella bursa-pastoris, Epilobium parviflorum, Hypericum perforatum, Polygonum aviculare, Teucrium chamaedrys, Viscum album* and *Galega officinalis*). These samples are commercially available as one-component herbal teas and are commonly used for the treatment of various pathological conditions. The tested plant samples were prepared as methanol extracts, lyophilized, freeze dried and later reconstituted with DMSO (dimethyl sulfoxide).

For the evaluation of potential toxicity among the selected herbal teas, the Brine Shrimp Lethality Assay (BSLA) was the most suitable method. BSLA is based on counting dead *Artemia salina* nauplii after their exposure to plant extracts in a certain time frame. The most relevant time period for the determination of LC_{50} is after 24 hours. After this period, dead nauplii were counted and the mortality of *Artemia salina* was calculated and expressed for each concentration of tested plant extract. The obtained results for the mortality of the brine shrimps were further analyzed with regression analysis and LC_{50} values were obtained by application of probit regression analysis.

Results and discussion

According to the obtained LC_{50} values for the tested methanol extracts, the toxicological bioactivity is decreasing in the following order: *Polygonum aviculare* > *Teucrium chamaedrys* > *Capsella bursa-pastoris* > *Epilobium parviflorum* > *Galega officinalis* > *Rhamnus frangula* > *Quercus pedunculata* > *Hypericum perforatum* > *Viscum album*.

* blagicajovanova@hotmail.com

Out of the tested plant samples, highest toxic potential manifested *Polygonum aviculare* (LC_{50} 185 μg/mL) and the lowest toxic potential was obtained for *Viscum album* (LC_{50} 590 μg/mL). The classification of the samples was done according to 2 scales: Meyer's scale and Clarkson's scale (Meyer at al., 1982; Clarkson et al., 2004). Meyer's scale classifies plant samples as toxic (LC_{50}< 1000 μg/mL) and non-toxic (LC_{50}> 1000 μg/mL). Clarkson's scale classifies plant samples in several subcategories: plant samples with high (0 - 100 μg/mL), medium (100 – 500 μg/mL), low (500 – 1000 μg/mL) and no toxic potential (>1000 μg/mL). All tested samples were classified as toxic according to both scales, since the obtained LC_{50} values for each sample is below 1000 μg/mL. However, the tested plant extracts manifested a different level of toxicity. In accordance with the Clarkson's scale, *Viscum album* was classified as a plant sample with low toxic potential, while the rest were classified as plant samples with medium toxic potential. The obtained results for the toxic potential of the selected herbs are in accordance with the literature results. Thus, the anticancer effects of *Polygonum aviculare* were investigated against MCF-7 cells (human breast cancer cell line). It has been shown that the methanol extract of *Polygonum aviculare* induced cytotoxicity in MCF-7 cell line, which confirmed the cytotoxic (anticancer) potential of this plant species (Habibi Roudkenar et al, 2011). Furthermore, the cytotoxic potential of the methanol extract of *Teucrium chamaedrys* against HCT-116 cells (human colon carcinoma cells) was evaluated. The obtained results have shown that *Teucrium chamaedrys* exhibited a significant cytotoxic activity against the HCT-116 cells after 24 hour exposure (Stankovic et al., 2011).

Conclusion

All tested herbal teas manifested medium toxic potential, except for *Viscum album* which manifested low toxic potential according to Clarkson's scale of toxicity, but the mechanism and origin of this activity need to be further examined and clarified. The Brine Shrimp Lethality Assay is a reliable preliminary tool to evaluate the toxic potential of plant samples. This is also supported by the good correlation between BSLA and other *in vivo* toxicity models. Therefore, the Brine Shrimp Lethality Assay is a convenient approach for a pre-selection of promising toxic plant samples that could further be examined for their cytotoxic potential in a more detailed research.

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Macedonian pharmaceutical bulletin, **62** (suppl) 307 - 308 (2016)
ISSN 1409 - 8695
UDC: 615.277.035.1
Short communication

The cancer metabolism and associated therapeutic interventions

Iva Antova*, Tatjana Kadifkova Panovska, Marija Hiljadnikova-Bajro

Institute for Applied Biochemistry, Faculty of Pharmacy, University SS Cyril and Methodius, MajkaTereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Studies of the cancer metabolism attract a growing scientific interest and targeting the metabolism should be one of the first choices of treatment. The aim of this study is to summarize the current extensive knowledge of the metabolic aberrations in cancer and propose plausible molecules and pathways that could be targeted for specific treatment of this disease.

Key concepts and clinical potential of the Warburg effect

The important discovery that highlighted the area of cancer metabolism is the Warburg effect, an anti-physiological way for energy generation used by the cancer cells for their proliferation. This effect explains the alteration of the normal process of oxidative phosphorylation following the process of glycolysis and excessive lactate production despite adequate oxygenation of the tissue, providing an acidic environment that encourages the proliferation and tumor invasion. Several studies have contributed to the discovery of key biochemical pathways and their regulators, which can shed light on the field of cancer metabolism and can serve as new targets for cancer therapy (Semenza, 2011).

One of the main metabolic features of all cancer cells is an increased utilization of glucose that offers several advantages: the survival in an environment with unstable oxygen concentration which is otherwise fatal to normal cells referring predominantly to the oxidative phosphorylation as a source of adenosine triphosphate (ATP), secondly the production of lactate as an end product of aerobic glycolysis makes acidic environment, which favors invasion of cancer and also suppresses the activity of anti-cancer immune effectors. Additionally, cancer cells utilize glycolytic intermediates for anabolic reactions necessary for rapid proliferation, and, finally, the production of pyruvate and nicotinamide adenine dinucleotide phosphate (NADPH), the two main products of glycolysis and the pentose phosphate cycle, are used by the cancer cells in order to deal with oxidative stress. Pyruvate catches hyperoxides while NADPH participates in the glutathione peroxidase mediated destruction of hydrogen peroxide (Singleterry et al., 2014).

The essential role of glycolysis as a necessary process for cell proliferation is based on the fact that this process provides compounds with hydrocarbon skeleton, which are required for the biosynthesis of various biomolecules such as nucleic, amino and fatty acids. In spite of the fact that the process of oxidative phosphorylation provides greater amounts of ATP, the process of glycolysis is a much faster process of energy supply, and therefore is a more appropriate process to meet the large energy needs of cancer cells during active division. There are various types of deviation regarding the process of oxidative phosphorylation and activation of glycolysis used by cancer cells. One of them is activation of the enzyme phosphofructokinase, a key enzyme for the process of glycolysis, by reduced production of ATP. Another approach implies changing the pH of the environment by reactive oxygen radicals and hydrogen peroxide. These radicals are known to cause intracellular alkalization which can activate the enzyme and thereby induce the process of glycolysis. Additionally, hydrogen peroxide can also activate HIF 1 and the oncogenic RAS, SRC and MYC that are involved in the expression of enzymes and transporters participating in the process of glycolysis.

Another major catabolic way that links the glucose metabolism to nucleotide biosynthesis and production of NADPH is the pentose-phosphate cycle. This process is necessary for the antioxidative surveillance and reduc-

* iva_antova@yahoo.com

tive biosynthesis. It is controlled by the enzyme glucose-6-phosphate- dehydrogenase (G6PD), which activity is mediated by several signals and it serves as a sensor for the cellular NADP+ level.

As glycolysis has a crucial role in the development of cancer, the Warburg effect can be used for therapeutic targeting of cancer metabolism. One approach is inhibition of the glycolytic enzymes involved in certain limiting steps of glycolysis, such as hexokinase 2 which ionizes the molecule of glucose and as such is retained inside the cell. A molecule that affects this enzyme is 2 deoxyglucose, a competitive inhibitor that cooperates with glucose for the active site of hexokinase 2, whereby with phosphorylation, this compound can't be further metabolized and accumulated inside the cell. The activity of the enzyme phosphofructokinase is also a potential target. The fact that this enzyme is very sensitive to small changes in pH and its action is induced by high pH values, the inactivation of the Na/H+ pump NHE1 which excretes H+ ions from the cell, may lead to decreased intracellular pH, decreased activity of phosphofructokinase and inhibition of the process of glycolysis, endangering the survival of cancer cells. Molecules that inhibit the activity of the Na/H+ pump NHE1 are amiloride and 5,5-dimetilamiloride. Lactate dehydrogenase is another enzyme that contributes for the Warburg effect which may serve as a target for aerobic glycolysis in the heart.

Another approach for reducing the glycolysis level in cancer cells is by inhibiting the synthesis of glucose transporters and glycolytic enzymes. HIF1 is a transcription factor that controls the activity and its inhibition is assessed in multiple studies. As a result, several Food and Drug Administration approved anticancer drugs such as topotecan, imatinib, celecoxib and ibuprofen have proven to inhibit the activity of this factor. The role of G6PD as an oncogene makes this enzyme a potential therapeutic target. Hense, the combination of oxythiamine and dehydroepiandrosterone, has an additive inhibitory effect over cancer cells. This indicates that the combinatory targeting of the pentose phosphate cycle and other metabolic pathways may be an effective approach to selective suppression of cell growth.

Targeting the glutamine metabolism

The dependability of the cancer cells survival on the aerobic glycolysis solely has been discredited by research showing that glutamine metabolism is also essential for certain types of cancer. Recent studies demonstrate the vital need for this amino acid, its buffering effect against oxidative stress, the role of a mediator in intracellular signaling, and as a quality control of macromolecules and organelles (Shanware et al., 2011). Crucial enzymes involved in the glutamine metabolism such as isocitrate dehydrogenase, are found to be mutated in certain types of cancer, such as gliomas and acute myeloid leukemia, hense targeting this enzyme may be an alternative anti-cancer therapy. Other two important enzymes that control the glutamine metabolism are glutamate dehydrogenase and glutamate aminotransferase. Glutamate dehydrogenase can be inhibited by a component of the green tea named epigallocatechin-3-gallate, while the aminotransferase can be inhibited by aminooxyacetate.

There are various other potential factors that play an important role in maintaining the high glycolytic phenotype of the cancerous tissue that can be used as potential targets such as: oxidative stress which causes apoptosis in cancer cells, transporters that facilitate the entry of glucose, the facilitated lactate efflux and the active transport of protons in the tumor microenvironment.

Conclusion

The growing awareness of the difficulty for therapeutic targeting signal transduction in tumors, and the side effects that accompany this approach, shift the therapeutic strategies towards targeting the aberrant tumor metabolism. Further research and discoveries in the field of cancer metabolism may lead to a better understanding of the disease and enable development of efficient anti-cancer drugs.

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Macedonian pharmaceutical bulletin, **62** (suppl) 309 - 310 (2016)
ISSN 1409 - 8695
UDC: 615.918:582.475
Short communication

Evaluation of the toxic potential of *Pinus* species natively growing on the territory of Republic of Macedonia

Blagica Jovanova[1]*, Marija Karapandzova[2], Tatjana Kadifkova Panovska[1], Svetlana Kulevanova[2]

[1] *Institute of Applied Biochemistry, Department of Toxicology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia*
[2] *Institute of Pharmacognosy, Department of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia*

Introduction

Pinus species is a diverse and widely distributed group of plants that are harvested and used in folk medicine for thousands of years. They grow natively in Macedonia forming pure and mixed stands in subalpine forests (Kałucka et al., 2013). The common used parts of the plant are root, stem, bark, branch, resin, tar, leaf, pollen, cone, seed, needles (Kızılarslan and Sevg, 2013). They are used as antiseptics, tonics, expectorants, analgesics, and especially in the treatment of respiratory (common cold, asthma, bronchitis) and urinary system disorders, skin diseases (wounds) and digestive system diseases. The bark and resin obtained from the Pinus trees are the primarily used parts of the plant, mainly because of the antimicrobial and antiseptic properties. Additionally, diverse benefits have been claimed for pine needles as well, such as antibacterial, cholesterol lowering, antioxidant and antitumorial effects.

In this research, needles and bark of selected *Pinus* species were tested for their toxicological bioactivity, as a starting point for the selection of promising toxic agents that could be further investigated in more detailed studies of cytotoxicity.

Materials and methods

Needles and bark of *Pinus peuce, Pinus sylvestris, Pinus nigra* and *Pinus mugo* were collected at five different localities in the Republic of Macedonia: Pelister, Kozhuf, Karadzica, Berovo, Nidze. The plant material was harvested in the year of 2008, 2009 and 2010. The collected plant materials weredried at room temperature for two weeks, and then milled to fine powder. A stock solution from each sample was prepared as a methanol extract, followed by lyophilization and freeze drying. The final extracts were prepared by reconstitution of the lyophilized powders with DMSO (dimethylsulfoxide). The toxicity of each plant extract was tested using the Brine Shrimp Lethality Assay (BSLA) (Meyer et al., 1982). The toxic potential was determined by counting the number of dead *Artemiasalina*nauplii after 24 hours exposure to the tested plant extracts. Furthermore, based on the calculated percentage of mortality of the brine shrimps, LC_{50} values were calculated using probit regression analysis (Finney, 1952). According to the obtained LC_{50}values, the tested plant samples were classified using two scales of toxicity: Meyer's scale and Clarkson's scale (Clarkson et al., 2004; Meyer et al., 1982).

Results and discussion

The obtained LC_{50}values represent the toxic potential of each*Pinus* species selected for toxicity testing in this research. According to the obtained data, every sample of needles was classified as toxic according to Meyer's scale and Clarkson's scale of toxicity. *Pinuspeuce* needles collected from the locality of Pelister have the lowest LC_{50} value (LC_{50}83 mg/mL). According to this result, it is classified as a sample with high toxic potential (0-100 mg/mL) using the Clarkson's scale of toxicity. The toxic bioactivityof the needles declines in the following order: *Pinus peuce* (Pelister)>*Pinus mugo* (Karadzica)>*Pinus sylvestris*

* blagicajovanova@hotmail.com

(Kozhuf)>*Pinus nigra* (Kozhuf).

Among the tested samples of bark, the highest toxic potential was detected for *Pinus peuce* collected from Nidze(LC_{50}135 mg/mL) and was classified as a sample with medium toxic potential (100-500 mg/mL) using Clarkson's scale.The results vary among the samplesand an important factor appears to be the locality of their origin: the samples of bark collected from the locality of Nidze (*Pinus peuce* and *Pinus sylvestris*) manifested greater toxicity compared to the same species collected from the localities of Berovo and Pelister. The toxic bioactivity of the tested bark samples declines in the following order: *Pinus peuce* (Nidze) >*Pinus sylvestris* (Nidze) >*Pinus sylvestris* (Berovo) >*Pinus peuce* (Pelister) >*Pinus nigra* (Berovo).

However, the prominent toxicity detected for the tested samples is probably a result of certain bioactive compounds with toxic characteristics that are naturally found in the selected *Pinus* species, since the samples were collected from localities which are characterized with a clean and healthy environment for plant growth. According to literature data, α-pinene is the dominant compound in most *Pinus* species. Additionally, this compound is a confirmed toxic agent (Leite et al., 2009), which suggests the origin of the toxic potential for the tested plant samples in this research.

Conclusion

All tested *Pinus* needles and bark were classified as samples with a toxic potential according toMeyer's scale and Clarkson's scale. Moreover, Clarkson's scale classified the examined samples in subcategories, according to which most of the tested extracts were samples with medium toxic potential. This gives a future perspective for further analysis of their cytotoxic potential and determination of the components responsible for the toxic effects they manifest. Although BSLA cannot determine the compounds responsible for a toxic effect, it is still a useful tool for the selection of potential cytotoxic candidates based on the extent of toxicity they manifest against *Artemia salina* nauplii.

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Macedonian pharmaceutical bulletin, **62** (suppl) 311 - 312 (2016)
ISSN 1409 - 8695
UDC: 616-009-02:632.95
Short communication

Exposure to organophosphates: cholinergic and non-cholinergic targets

Biljana Antonijevic[1]*, Evica Antonijevic[1], Danijela Djukic-Cosic[1], Marijana Curcic[1], Nina Umicevic[2]

[1]*Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe Street,11000 Belgrade, Serbia*
[2]*Faculty of Medicine, University of Banja Luka, 14 Save Mrkalja, 78000 Banja Luka, Republic of Srpska*

Introduction

The primary molecular mechanism of action of the organophosphorus (OP) pesticides is inhibition of acetylcholinesterase (AChE) producing excessive acetylcholine (ACh) accumulation and overstimulation of cholinergic neurons. The organophosphates (OPs) or their active metabolites are electrophilic compounds with moderate to high potency for phosphorylating the serine hydroxyl group located in the active site of AChE. The reactivity of OP compounds varies depending upon the chemical structure. OPs may also interact (inhibit) with other serine esterases: neurotoxic esterase (NTE), butyrylcholinesterase (BuChE), carboxylesterases (CarbE), phosphorylphosphatases (A-esterases), fatty acid amide hydrolase (FAAH), arylformamidase (AFMID), acylpeptide hydrolase (APH) (Casida and Quistad, 2004).

Materials and methods

In order to explore cholinergic and non-cholinergic targets of OPs, literature review was undertaken.

Results and discussion

The half-life of recovery of inhibited brain AChE is about 1 week (approximately 5% per day) in experimental animals and is believed to be the same in man. BuChE activity takes approximately 4-6 weeks to return to pre-exposure levels whereas erythrocyte AChE requires 5-7 weeks. BuChE is generally more sensitive than AChE to OP esters. Some OPs (tri-o-cresyl phosphate, mipafox, leptophos) considered to be extremely potent to inhibit NTE producing organophosphate-induced delayed polyneuropathy that is associated with inhibition of at least 70% of the activity sometimes followed by irreversible transformation to its non-reactivatable form.

The endocannabinoid arachidonyl ethanolamide (anandamide) and the endogenous sleep-inducing agent oleamide are hydrolyzed by FAAH. Anandamide also binds to the CB1 receptor in brain that is the target for marijuana and its principal psychoactive ingredient Δ9-tetrahydrocannabinol. Particularly potent inhibitors of FAAH *in vivo* in mice are chlorpyrifos and tribufos.

The OPs may create a nicotinic acid deficiency by blocking its biosynthetic pathway. Tryptophan is the metabolic precursor for N-formyl-L-kynurenine, L-kynurenine, nicotinic acid, NAD(H), and NADP(H) in mammals. AFMID (formerly known as kynurenine formamidase), the second enzyme in this pathway, is very sensitive in birds and mammals especially to OPs with nitrogen-containing hetero cyclic or aliphatic leaving groups (diazinon, diazoxon, monocrotophos).

APH hydrolyses the N-terminal acetylated amino acid residue on peptides, thereby removing one form of protection from proteolysis. It also hydrolyses oxidized proteins in human erythrocytes with a possible physiological function of removing oxidatively damaged components in cells. APH activity has been potently inhibited by chlorpyrifosmethyl oxon, dichlorvos and diisopropyl fluorophosphates with resultant IC_{50} values of 18.3, 118.7 and 22.5 nM, respectively. Malathion and malaoxon inhibit lysyl oxidase (LyO) (IC_{50} = 1-9 nM) as well as proline hydroxylase (IC_{50}= 50-58 nM) in homogenates of Xenopus embryos, suggesting that they alter posttranslational modification of collagen with resultant morphological defects in

* abiljana@pharmacy.bg.ac.rs

connective tissue.

Additionally, OPs may have a direct action on muscarinic and nicotinic receptors, binding to (with high/low affinity) and modulating the function of these receptors (as indicated by clinical effects). Specific binding of an OP compound to muscarinic receptors does not necessarily produce predictable effect (some OPs bind to muscarinic receptors and activate them, while others may bind to and inhibit the action of muscarinic receptors). OPs may bind to allosteric sites on nicotinic receptors stimulating receptor desensitization. The OP concentrations required to act directly on nicotinic receptors are much higher than those on muscarinic receptors, suggested that muscarinic receptors are more important as secondary targets in OP action. Cannabinoid system is also sensitive to OPs and their metabolites. Chlorpyrifos oxon is a potent in vitro inhibitor of cannabinoid CB_1 receptor binding (IC_{50} = 14 nM) but is effective in vivo only at near lethal levels. Combination of possible interactions will produce resultant toxic effect(s) for the certain OP. The spectrum of effects is modulated by various toxicokinetic factors.

Exposure to OPs can induce four different clinical syndromes: 1. acute cholinergic crisis as a result of AChE inhibition (in CNS and PNS), 2. intermediate syndrome whose underlying mechanism(s) is still unclear(excessive stimulation of cholinergic receptors etc.), 3. organophosphate induced delayed neuropathy (OPIDN) that has been explained by the inhibition of NTE, and 4. chronic organophosphate induced neuropsychiatric disorder (COPIND) due to long-term low-level exposure (LTLL) (Antonijević and Stojiljković, 2007). Chronic exposure to OP substances is a controversial area of toxicology: a number of authors propose that prolonged exposure at low levels of organophosphates can produce adverse effects in man and in animals, while others have reported no such effects. Several studies have reported long-term, persistent, chronic neurotoxicity symptoms in individuals as a result of acute exposure to high doses that cause acute cholinergic toxicity, or from long-term low-level, subclinical doses of these chemicals with or without previous history of acute episode. Available data on neurotoxicity showed/suggested that: animals treated chronically with OPs exhibit reduced sensitivity (adaptive responses), probably due to down-regulation of ACh receptors and reduced synthesis of ACh, so that the presynaptic release is restricted; sublethal or subclinical doses of OPs can produce apoptotic neuronal cell death and involve oxidative stress due to an increase of the excitatory neurotransmitter, glutamate and subsequent increase in intracellular calcium; genetic susceptibility (genetic variation in paraoxonase) could be related to OPs neurotoxicity; inhibition of brain CarbE, the activity of which appears to be unrelated to the liver and plasma type, and/or even other non-target enzymes such as carboxyamidase, whose endogenous function is unknown although it has been suggested that it has a role in xenobiotic metabolism, may play a role in explaining the mechanism of cognitive dysfunction; behavioral changes could be induced by the effects of OP compounds on neuropeptide metabolism due to inhibition of acylpeptide hydrolase, the enzyme which is responsible for the removal of N-acetylated amino acids from the N-terminus of short peptides.

Several low dose studies as well as some epidemiological studies did not identify any link between neurological disorder and erythrocyte and/or plasma cholinesterase activity. The mechanisms of OPs – induced chronic neurotoxicity have yet to be established. An important consequence of this is that non-cholinergic mechanism can be expected to show different structure-activity relationship to those established for conventional end points such as acute toxicity (Vučinić at al., 2014).

Conclusion

In conclusion, based on the available data, their quality and quantity, and the fact that inhibition of AChE is both the most specific and one of the most sensitive mechanism of OPs action, it seems that inhibition of AChE is still the most reliable parameter to predict absolute and relative potency with regard to long-term low-level exposure and neurodevelopmental toxicity produced by OPs.

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Macedonian pharmaceutical bulletin, **62** (suppl) 313 - 314
(2016)
ISSN 1409 - 8695
UDC: 616.12-009.72-074
Short communication

The role of cardiac markers in the diagnosis of acute myocardial infarction and angina pectoris

Emilija Kostoska[1]*, Aleksandra Crvenpanova[1], Tanja Angjuseva[1], Zan Mitrev[1], Tatjana Kadifkova Panovska[2]

[1]*Special Hospital for Surgical Diseases Filip Vtori, 1000 Skopje, R. Macedonia*
[2]*Faculty of Pharmacy, Ss. Cyril and Methodius University,Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

Acute myocardial infarction (AMI) is a life-threatening condition which diagnosis must be established right away and to be currently treated. Early identification and confirmation of acute myocardial infarction is quite important for the patient care and making the right decisions. The acute myocardial infarction belongs within the spectrum of acute coronary syndromes, including unstable angina pectoris, acute myocardial infarction without elevation of ST-segment (NSTEMI) and acute myocardial infarction with elevation of ST-segment (STEMI). Acute myocardial infarction is a major cause of morbidity and mortality worldwide (Atman et al., 2000).

Angina pectoris is the medical term for chest pain or discomfort caused when the heart muscle doesn't get enough oxygen-rich blood. But, angina is not a disease. It is a symptom of an underlying heart problem, usually coronary heart disease (CHD). There are many types of angina, including stable and unstable angina. In fact, unstable angina is a pre-infarction syndrome (Braunwald, 1990).

Diagnosis on an electrocardiogram (ECG) is not an absolute criterion, particularly in the case of small infarctions. Cardiac markers are used in the diagnosis and risk stratification of patients with chest pain and suspected acute coronary syndrome (ACS) (Higgins et al., 1999). The measurement of cardiac markers in the blood is the basis of the diagnosis of acute myocardial infarction for more than 50 years. The release of biomarkers in the bloodstream in higher than normal amounts, suggests a pathological process. Thus, the detection of elevated concentrations of cardiac biomarkers in the blood is a sign of cardiac injury that may be due to toxic effects or hemodynamic stress. Traditional cardiac markers (CK-NAC, LDH) have many disadvantages: they secrete into the circulation very late (12 hours after necrosis of the heart muscle), thus preventing the early diagnosis. To overcome this situation, for detection of cardiac necrosis, there are introduced new biochemical markers from the circulation, CK-MB and troponin (Rajappa and Sharma, 2005).

CK-MB rise twice higher than normal after 6 hours of the infarction, to reach its maximum after 24-48 hours, when its concentration decreases, therefore it can't be diagnosed cardiac necrosis quickly. Troponin I appears in the serum relatively early after the infarction (approximately 4 hours), reaches its maximum 12-48 hours after necrosis, and remains in the circulation up to 14 days later. The cardiac troponins, in particular, have become the cardiac markers of choice for patients with acute coronary syndrome (Thygesen et al., 2010).

The aim of the study was to evaluate the clinical value of cardiac Troponin I over CK-MB as essential marker in early assessing myocardial cell damage.

Materials and methods

Blood samples (serum and heparinized plasma) were taken from 38 patients (29 males and 9 females) with complain on chest pain at the Special Hospital for Surgical Diseases "Filip Vtori" Skopje. According to their age, the patients were divided into 3 groups (from 31-50; 51-70 and over 70 years old) in order to prove which will be the most risk group in regards of cardiovascular diseases.

Daily measurements of Troponin I and CK-MB levels were made in all patients with no history of coronary heart disease. Regardless the values, the measurements were re-

* emilija.kostoska@filafarm.com.mk

peated every 4 hours in order to conceive the trend line of the markers. Also, on the receipt day of the patients, the ECG tests were made, respectively.

Determination of these markers was made with Immunoassay System –Advia Centaur XP by of chemiluminometric immunoassay method. Minimum concentration limit ("cut off" value) for Troponin I is 0.78 ng/ml and the referent values for CK-MB are from 0-15 ng/ml. Therefore, any increase of the concentration of these markers, indicates the condition of acute myocardial infarction. If there is no increasing above the baseline value of Troponin I and if ECG tests are normal, it's a question of angina pectoris.

Results and discussion

In regards of age group, the obtained results indicated that the risk group in terms of cardiovascular diseases (in this case AMI) is the group from 51-70 years (29 patients).

As expected, serum Troponin I levels increased in 30 from all 38 patients (79%). Thus, these increased concentrations of cardiac troponin (cTnI) which deviate from normal values, indicate of acute myocardial infarction. Therefore, 20 patients of them (67%) had elevation of ST-segment (STEMI) on ECG test which can confirm the diagnosis. There were no signs of myocardial infarction on ECG test (NSTEMI) on the rest 10 patients (33%), only the concentration of Troponin I was elevated. In this case, Troponin I is the leading biochemical parameter which affirms that these patients had AMI, which is very important.

CK-MB was elevated only in 9 of the 38 patients and according to literature data (that this marker stays elevated in the blood up to 72 hours), it appears in blood after 6-9 hours after the onset of chest pain, unlike the Troponin which was already increased. Just to confirm the lower sensitivity of CK-MB compared with Troponin I, when the second tests were made (after next 4 hours), only on 5 patients the concentration of CK-MB started to elevate. On the other side, the elevated CK-MB levels on those 9 patients, appeared to be decreasing next day (the concentrations were in the referent values), unlike Troponin I which concentration was still over the "cut off" value.

In the rest 8 patients (21%) the ECG findings were normal and the results for Troponin were in the referent values, which indicate that these patients were with angina pectoris. If their symptoms become worse over time, test for Troponin must be ordered, because it can lead to a heart attack.

Conclusion

Cardiac Troponin I appears to be diagnostically superior to CK-MB due to its high sensitivity (appears very early after infarction) and specificity.

Therefore, Troponin I is considered as "gold standard" in the early detection of myocardial infarction and is necessary in setting the correct diagnosis. Thus, the use of test for Troponin enables early noninvasive diagnosis of acute myocardial infarction.

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Macedonian pharmaceutical bulletin, **62** (suppl) 315 - 316 (2016)
ISSN 1409 - 8695
UDC: 615.212.7.074:340.66
Short communication

Challenges in interpretation of forensic toxicological findings for opiates: case report and a literature review

Marija Bujaroska*, Nataša Bitoljanu, Ljupčo Čakar, Renata Jankova-Ajanovska, Zlatko Jakjovski, Verica Poposka, Aleksej Duma

Institute of Forensic Medicine, Criminology and Medical Deontology, Medical Faculty, Ss. Cyril and Methodius University, Skopje, Macedonia

Introduction

The routine screening in the Laboratory of Forensic Toxicology encompasses assays for determination of psychoactive substances, commonly used drugs and presence of ethanol. The positive finding on opiates does not always imply the presence of heroin (HER) and/or its metabolites because various drug formulations contain an opiate as an active substance. The detection of HER and its main metabolite 6-monoacetylmorphine (6-MAM) as a general marker of HER use, poses some analytical challenges, mainly due to their short half-lives (Goldberger et al.,1994; Paterson and Cordero, 2006; Rook et al., 2006). The scope of this study includes 3 forensic cases with positive findings on opiates, where the presence of HER and 6-MAM could not be detected, but some additional markers of HER use were identified.

Materials and methods

The analyses which are subject of this study were conducted by routine screening for the presence of psychoactive substances, using fluorescence polarization immunoassay and/or biochip array technology for urine and blood samples, respectively. The positive results were confirmed by gas chromatography-mass spectrometry (GC-MS), after previous sample preparation using ion exchange solid phase extraction (SPE) columns. In the case of urine samples, acid hydrolysis was performed prior to SPE, in order to determine free opioid alkaloids.

Results

Case 1: The routine screening of the postmortem urine samples did not show positive results for the presence of psychoactive substances. However, further analyses using GC-MS were conducted due to the information on the presence of a syringe and an unknown powdered substance near the body of the deceased. The GC-MS analysis of the serum revealed the presence of the following substances: caffeine, morphine (MOR), acetaminophen and traces of noscapine, papaverine and hydrocodone. In the unknown powder sample and the syringe, acetaminophen, caffeine, codeine (COD), 6-MAM, papaverine, MOR and noscapine were detected.

Case 2: Positive results on the presence of opiates and methadone in postmortem blood and urine samples were obtained during the routine screening. Further GC-MS analyses of the biological samples were conducted and the following substances were identified in the serum: nicotine, cotinine, caffeine, theophylline, theobromine, paracetamol, methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), metamizol, MOR, meconine and traces of COD. The same substances were identified in the urine sample, along with noscapine, hydrocotarnine and desmethylpapaverine.

Case 3: Screening of postmortem urine samples showed positive finding on opiates, whereas postmortem blood screening showed presence of opiates, methadone, benzodiazepines and cannabinoids. The GC-MS analysis confirmed the presence of caffeine, nicotine, tramadol, methadone and meconin in serum. The same substances were detected in the urine sample, along with COD, MOR, papaverine, hydrocotarnine and paracetamol.

* bujaroska_m@yahoo.com

Discussion

HER is quite unstable in aqueous medium and is rapidly converted to 6-MAM due to spontaneous hydrolysis (Knight et al., 2014; Rook et al., 2006). This can be noticed in the results of the first case presented, where only 6-MAM was detected in the extracts from the syringe and the unknown powdered substance. The same hydrolysis occurs in the serum, resulting in the deacetylation of HER to 6-MAM, which is further metabolized to MOR (Rook et al., 2006). Different data on the half-lives of HER and 6-MAM have been reported in the literature. It is thought that HER can be detected in blood 10-40 min after intravenous administration, while 6-MAM 1-3 h, reaching maximum plasma concentration immediately after injection (Rook et al., 2006). In our cases, larger number of opioid alkaloids and their metabolites were identified in urine rather than in blood. Several studies conducted using postmortem biological samples suggest that the most suitable medium for detection of 6-MAM is the cerebrospinal fluid (CSF), while others consider the vitreous humor to be the sample with the greatest number of positive findings on 6-MAM compared to other fluids and tissue samples from the same forensic cases, with CSF having the second greatest number of positive results (Goldberger et al., 1994; Pragst et al., 1999; Wyman and Bultman, 2004). 6-MAM was not detected in any available biological sample from the three forensic cases, thus further analysis is needed to determine the origin of the opiates. Even though MOR is an end product of HER metabolism, it can also be a metabolite of COD, which is an active substance in some formulations. Some studies have compared MOR/COD concentration ratio, suggesting that MOR/COD concentration ratio >1 indicates HER use (Bogusz et al., 2001; Ceder and Jones, 2001; Konstantinova et al., 2012). Other studies suggest the use of acetylcodeine (AC), a by-product of HER synthesis, as a marker of illicit HER use. AC is considered as the only definite marker of illicit HER use, along with 6-MAM, but its short half-life aggravates its usefulness in forensic applications (Bogusz et al., 2001; Goldberger et al., 1994; Musshoff et al., 2010). This is also confirmed by the results of our study, where AC was not detected in any death case. Though some authors propose the presence of papaverine, especially its metabolites as a reliable marker of HER use, several studies have shown that papaverine metabolites can be detected in urine after the consumption of poppy seeds (Musshoff et al., 2010; Paterson and Cordero, 2006). COD and noscapine (and its metabolites meconin and cotarnine) are also considered as markers of HER use (Bogusz et al., 2001; Paterson and Cordero, 2006). However, they can also be detected in biological samples after food consumption as papaverine (Bogusz et al., 2001; Musshoff et al., 2010; Paterson and Cordero, 2006). In our cases, almost all opium alkaloids were identified, but 6-MAM was not detected. Pharmaceutically prepared HER is not available as a treatment option in Republic of Macedonia, therefore it can be concluded that the deceased had used illicit HER.

Conclusion

The absence of 6-MAM in postmortem biological samples is not an indicator of HER non-use. When interpreting the results for the opiate presence due to the use of HER or different opioids, the results of numerous researches can be utilized, such as MOR/COD ratio as the most relevant marker or the presence of AC as a definite marker of HER use. The determination of other opioid alkaloids such as papaverine, noscapine and its metabolites, together with additional forensic evidences can be useful in autopsy cases.

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Macedonian pharmaceutical bulletin, **62** (suppl) 317 - 318 (2016)
ISSN 1409 - 8695
UDC: 613.83:616-036.88(497.7)"2011/2015"
Short communication

Drug-related deaths linked with concomitant use of methadone and benzodiazepines in the period between 2011 and 2015 in the Republic of Macedonia

Marija Bujaroska*, Nadica Sibinovska, Klimentina Trajkova, Verica Poposka, Goran Pavlovski, Viktorija Belokaposka Srpanova, Biljana Janeska

Institute of Forensic Medicine, Criminology and Medical Deontology, Medical Faculty, Ss. Cyril and Methodius University, st.MajkaTereza 19, 1000 Skopje, R. Macedonia

Introduction

Opioid dependence is a complex socio-medical state and a serious problem for the society, linked with a high morbidity and mortality rate (WHO, 2004). Even though several pharmacological agents are available, in the Republic of Macedonia, the maintenance therapy includes methadone (METH) and buprenorphine, with METH being the first agent used in the opioid substitution treatment (OST) and it still remains the main prescribed medication (EMCDDA, 2013). There are indications that alongside with the general decrease in drug seizures between 2009 and 2012 in Republic of Macedonia, there was some shortage in supply, encouraging users to replace heroin with other substances such as METH, benzodiazepines (BZDs) and tramadol (EMCDDA, 2013). Despite the prescription of BZDs for therapeutic purposes, the prevalence of their misuse among OST patients is reported to be high in other countries and is related to severe consequences such as non-fatal and fatal overdoses (EMCDDA, 2015).

The aim of this report is to assess the number and nature of drug-related deaths (DRDs) in the Republic of Macedonia over the period 2011-2015, with the emphasis on death cases involving positive findings for METH and BZDs.

Materials and methods

All toxicological analyses from autopsies performed at the Institute of Forensic Medicine in Skopje during a 5-year-period were reviewed. Postmortem toxicological analysis of blood and urine samples was conducted using biochip array technology and fluorescence polarization immunoassay, respectively. Whenever possible, the positive results from the screening were confirmed by gas chromatography-mass spectrometry (GC-MS), after previous sample preparation by ion-exchange solid-phase extraction (SPE). Acid hydrolysis was performed prior to SPE for the urine samples showing positive results for BZDs tested with screening methods. The concentration of ethanol in the samples was determined by headspace gas chromatography-flame ionization detection.

Results and discussion

The postmortem toxicological analyses were performed on a total of 1251 cases in the 5 year period being reviewed. A total of 89 cases showed positive results for drugs of abuse, whereas combined use of psychoactive substances was noticed in 57 cases (64.05% of all DRDs). Concomitant use of METH and BZDs was implicated in 39 DRDs over the time period studied (43.82% of the total DRDs), with the highest number of fatalities involving their combined use in 2013 (14 cases). The percentage of METH and BZDs DRDs gradually increases starting from 2011, with the highest peak seen in 2012 and 2013 (50% of all DRDs). After that, the percentage declines in 2014, reaching the same level as in 2011. A rise in the trend of DRDs is seen again in 2015. No information on the possible involvement of deceased in OST was available. In all cases analyzed by GC-MS diazepam was the only BZD identified. The presence of ethanol was detected in only 9 cases of all studied DRDs. Other substances (cannabi-

* bujaroska_m@yahoo.com

noids, cocaine, tricyclic antidepressants and opiates) were detected in addition to the combination of METH and BZDs in 16 cases.

The number of deaths in which METH is either the cause of death or a positive toxicological finding has significantly increased (Mijatović at al., 2014). In some studies it has been reported that alcohol and BZDs are most commonly detected concomitants together with METH (Laberke and Bartsch, 2010; Mijatović at al., 2014). Nonetheless, regarding alcohol presence, this was not confirmed in this report where ethanol was detected in only 9 cases of all DRDs. Concomitant use of METH and BZDs was implicated in 43.82% of the total DRDs, with diazepam being the only BZD identified in all analyzed cases by GC-MS, similar to other studies (Iwersen-Bergmann at al., 2014; Mijatović at al. 2014). Furthermore, this correlates with the data from EMCDDA stating that BZDs with a more rapid onset of action (diazepam, alprazolam) appear to be more frequently used by opioid users than those with a slower onset (EMCDDA, 2015). Much of the BZDs misuse consists of self-medication for treatment of psychiatric and mood disorders, alleviation of withdrawal symptoms and increase of the rewarding and reinforcing effects of the opioids (Eiroa-Orosa at al., 2010; EMCDDA, 2015).

The potential for significant morbidity and mortality with METH, either alone or in combination with BZDs, is widely reported (Lee at al., 2014). METH's inhibitory effect on the brain's respiratory center can lead to respiratory depression, hypoventilation and pulmonary edema (Bernard, 2013). It is suggested that μ-opioid agonists suppress respiration by acting on the respiratory centers in the brainstem, decreasing the ventilatory response to CO_2 (Pattinson, 2008). In this way, the inspiration is prolonged, while changes in tidal volume can be observed at higher opioid doses (Lalley, 2003) The concurrent use of opioids with BZDs and other central nervous system depressants is considered to be an important element in the mechanism of death (Bernard, 2013). Some authors suggest that BZDs compete with METH for μ-opioid receptors, resulting in higher concentrations of METH in acute intoxications with METH and BZDs, while in chronic abuse situations, BZDs inhibit hepatic enzymes that metabolize METH, also leading to increased METH concentrations (Mikolaenko at al., 2002). Moreover, it had been postulated that BZDs may also have effects on signal transduction and second messenger systems involved with μ-opioid receptor regulation (Poisnel at al., 2009). Other possibly fatal METH-related effects in the body, such as pulmonary oedema and arrhythmias secondary to QT-interval prolongation must be also borne in mind (Bernard, 2013).

The high frequency of positive findings on METH and BZDs in postmortem analysis of DRDs implies possible METH diversion and widespread availability of BZDs among METH users.

Conclusion

It is complicated to establish the role of BZDs in DRDs and their misuse has been shown to contribute to morbidity and mortality rate among METH users. Much still needs to be done regarding the monitoring of METH and educating the patients involved in OST about the risks associated with multiple drug use.

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Macedonian pharmaceutical bulletin, **62** (suppl) 319 - 320 (2016)
ISSN 1409 - 8695
UDC: 615.216.24.099:616.89-008.441.44(497.7)
Short communication

Unusual case of suicide with pentobarbital

Natasa Bitoljanu[1]*, Verica Poposka[1], Elena TrajcovaKovacovska[1], Aleksandar Stankov[1], Iskra Trencevska Ivanovska [2], Zdravko Cakar[1]

[1] Institute of Forensic Medicine, Criminology and Medical deontology, Medical Faculty, "St. Cyril and Methodius" University, st. Majka Tereza 19, 1000 Skopje, R. Macedonia
[2]Psychiatric Hospital Skopje, Medical Faculty, "St. Cyril and Methodius" University, st. Aco Shopov б.б. (Proleterska), 1000 Skopje, Republic of Macedonia

Introduction

According to World Health Organization, the suicide death rate per population of 100000 in the Republic of Macedonia is 5.42 suicides (WHO, 2014). The most common suicide methods in our country are hanging, intoxication with corrosive substances and gunshot wounds. Rarest way of suicide is intoxication with drugs especially with barbiturates. We have only two cases of intoxication with barbiturates in the last five years.

Materials and methods

Case hystory: Female, twenty-five years was found dead lying on the bed in a hotel room, where she stayed. During the external examination of the body on the crime scene we found complete development of rigor mortis. On the bedside table, were found a box of Diazepam, gel tube of Lidocaine, a plastic bag with white powder contents and two suicide letters (one designated for the mother, and one for the police). The suicide letters for the police contained the whole process of the suicide, with mentioning of the drug types used. In the letter was described Pentobarbital used because causes coma and respiratory depression, Lidocaine used for decrease the unpleasant bitter taste and Metoclopramide used to prevent vomiting.

Postmortem blood and urine samples were collected for toxicological investigation. Toxicological qualitative analysis of blood using gas chromatography mass spectrometry (GC-MS) was performed. Determination of ethanol was performed using headspace gas chromatography. Quantification of urine samples was performed with fluorescence polarization immunoassay (FPIA, AxSym system analyser– Abbott, USA).

Results

Autopsy findings

Postmortem lividity was developed, distributed on the dorsal portion of the body and disappeared on blunt pressure. Medico-legal autopsy revealed no external changes. No findings of natural disease were observed. During the internal examination, we observed non-specific signs of asphyxia. The brain was slightly oedematous. The lungs were congested and oedematous, with subserous petechial hemorrhages.

Toxicology findings

GC-MS toxicological analysis confirmed the presence of Pentobarbital, Diazepam, Lidocain-M-(desethyl) and Metoclopramide. No ethanol was detected in the samples. FPIA results from the urine samples indicated a level of pentobarbital (more than 2000 ng/ml) and 498 ng/ml of diazepam. According to urine cut–off levels for reporting positive blood level or limit of quantitation (CLR,2016), both urine pentobarbital and diazepam are over the cut off. No other drugs were found in the blood and urine of the victim.

Psychoautopsy

The suicide letter to her mother contained the reason of the suicide explaining the lack of reason and motivation for life and fatigue, mentioning unsuccessful treatment at

* nbitoljanu@gmail.com

physiatrist. In the letter to her mother she stated that she was tired from being depressed all the time and that she had had nothing to live for, because she had no intention to work, get married or have a child. She had one previous suicide attempt with barbiturates and benzodiazepines.

Discussion

Suicide is an act or an instance of taking one's own life voluntarily and intentionally. Suicide is a behavior that differs from one person to another and from one time to another and has different motivations and anticipated gains. There are differences in the suicide method used in different countries. Three methods – hanging, pesticide poisoning and firearm are dominated. Jumping from a height and other methods of poisoning (i.e. mainly poisoning by drugs) occasionally appears as important alternative methods. The suicide method with poisoning is more popular in women than in men. In general, underlying suicide patterns tell us more about the availability and acceptability of suicide methods than about other disparities (Ajdacic-Gross et al., 2008). Factors that affect the risk of suicide include genetic vulnerability and psychiatric, psychological, familial, social, and cultural factors. The effects of media are also important; the spread of information about suicide methods affects the choices that people make when attempting to kill themselves. In this case during forensics analyses and psychoautopsy of the suicide letters we concluded depressed mood, lack of interest in pleasure, fillings of worthlessness, guilt, suicide statements and attempts all point out to major depressive disorder. Detailed description of suicide, the information of drug effects used direct on preparation process and internet investigation, which suggest awareness of the consequences.

The suicide letter mentioned alleged ingestion of Pentobarbital, Diazepam, Lidocaine and Reglan. The toxicology department of our institute confirmed the presence of these compounds in the blood and urine of the victim. Pentobarbital is a short-acting barbiturate that acts like a non-selective central nervous system depressant, and is primarily used as sedative hypnotic, as an anticonvulsant in sub hypnotic doses, medically induced coma and euthanasia in veterinary medicine (Charney et al., 2006; Greenblatt et al., 1979). In cases of intoxications, suppression of the central nervous system, hypotension, hypothermia, coordination disorders, respiratory failure and coma are the major clinical symptoms (Fell et al., 1968). Diazepam is a derivative of benzodiazepine that is widely prescribed as an antianxiety agent. Diazepam is sometimes used with other medications to treat seizures. When barbiturates are combined with other central nervous system (CNS) depressants, such as alcohol, opiates, or benzodiazepines, overdose is even more dangerous due to additive depressant effects on the CNS and respiratory system (Mactier et al., 2014). The clinical effects of barbiturates and benzodiazepines are similar and result as sequelae to hyperpolarizing the neuron, there are subtle differences in terms of receptor binding. Barbiturates increase the duration of Cl ion channel opening at the gamma-aminobutyric acid (GABA) receptor, which, in turn, increases the efficacy of GABA. Benzodiazepines, on the other hand, increase the frequency of Cl ion channel openings at the GABA receptor, which, in turn, increases the potency of GABA (Sharma and Hoffman, 2011).

Conclusion

Suicides with intoxication with these types of drugs are rare because of the unavailability of drugs, because they are prescription drugs, or are intended for clinical practice only. This case, suggest on detailed planned and determination to end her life. The combination of Pentobarbital, Diazepam, antiemetic drugs and the content of the suicide letter stating when, and how she will die, suggest that the victim was familiar with drug effects and knew where to get them. We should point that among all classical forensic methods, we include psychoautopsy as a good method which helps us to finalize all investigation and make scientific based conclusions for motivation and way of taking her own life.

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Macedonian pharmaceutical bulletin, **62** (suppl) 321 - 322 (2016)
ISSN 1409 - 8695
UDC: 582.477-147:615.272(497.7)
Short communication

Evaluation of antioxidant activity of berries of *Juniperus excelsa, Juniperus communis* and *Juniperus oxycedrus* from Macedonian flora

Leonard Kurti*, Blagica Jovanova, Ariana Kelmendi, Tatjana Kadifkova Panovska and Svetlana Kulevanova

Faculty of Pharmacy, Ss. Cyril and Methodius University,Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

For centuries, juniper berries and leaves extracts and their essential oils have been used in the folk medicine due to their therapeutic properties, such as antimicrobial, anti-inflammatory, diuretic, hypoglycemic, hypotensive and analgesic effect. Due to the exclusive aroma, juniper cones are utilized in the food industry as a spice and in the production of alcoholic beverages as flavor. Today, there is an increased interest in the identification of natural and safe sources of antioxidants, in order to replace synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary-butyl hydroquinone (TBHQ) which have a limited use due to their toxicity and carcinogenicity.

The purpose of this study is to determine total phenols, total flavonoids and the antioxidant activity of ethanol extracts from berries of *Juniperus excelsa, Juniperus communis* and *Juniperus foetidissima* from different localities of the R. of Macedonia.

Materials and methods

The plant samples were collected in late autumn in 2011 and 2012. The berries (4 samples) of *J. communis* (JC) were collected from Skopje (sk), Velestovo (ve), Kicevo (ki), and Resen (re), the berries (2 samples) of J. excelsa (JE) from Dojran (do) and Velestovo (ve) and the berries (2 samples) of *J. foetidissima* from Valandovo (va) and Velestovo (ve). Botanical identification was made at the Institute of Pharmacognosy, Faculty of Pharmacy - Skopje. The plant material was properly dried, stored in paper bags and kept in dark and cool place until testing.

The plant material (0.5 g) was milled to a fine powder and extracted with 70% ethanol. The extraction procedure for sample preparation was performed with 10 mL of 70% ethanol for 30 min in the ultrasonic bath. After filtration of the extracts, the volume was adjusted to 10 mL.

Estimation of the total phenolic content (TPC) was done using Folin-Ciocalteu reagent according to the Singleton's method with minor modifications.

The total flavonoid content (TFC) was estimated using the aluminium chloride colorimetric method described by Talari (Talari, 2012).

The antioxidant activity was determined using three methods: DPPH (1,1-diphenyl-2-picrylhydrazyl) method, FRAP (Ferric Reducing Antioxidant Power) method and H_2O_2 (Hydrogen peroxide) method.

The ability of the tested extracts to reduce the DPPH radical was determined from the color bleaching of the purple ethanol solution of DPPH. The antioxidant activity of the extracts is expressed as IC_{50} (mg/mL), indicating the concentration that is required to cause 50% reduction of the DPPH radical. The FRAP activity of the extracts was determined according to the procedure described by Oyaizu. The scavenging capacity of the extracts for hydrogen peroxide was determined according to the method of Ruch with minor modifications.

* leonardkurti@hotmail.com

Results and discussion

The content of total phenolic compounds in all investigated samples vary from 41.32±0.20 mg GAE/g dw to 96.83±0.41mg GAE/g dw in the samples JF(va) and JE(ve), respectively. The total flavonoid content in the plant samples was in the range from 14.03±1.20 mg QE/g dw to 34.01±3.87 mg QE/g dw in the samples of JF(va) and JC(ki), respectively.

All the extracts showed antioxidant activity using the DPPH method. The DPPH radical scavenging activity of the samples increased with an increase in extract concentration. The ability of the corresponding extract for reduction of DPPH free radicals expressed as IC_{50} values was obtained in the range from 0.006 mg/mL for the sample JF(ve) to 0.713 mg/mL for the sample JC(ki). The obtained reducing ability against DPPH radical was compared to the IC50 values for Quercetin and BHA (1.0 µg/mL and 6.0 µg/mL, respectively). All tested samples manifested good scavenging potential for H_2O_2. The lowest inhibitory activity against H_2O_2 was found in JE(do):17.68%, 16.29% and 15.24% for the extract concentrations 25, 10, and 5 mg/mL, respectively, while the highest inhibitory activity was found in JF(va): 52.73%; 49.47% and 44.2%, for the corresponding extract concentrations.

The ferric reducing antioxidant potential was determined by evaluating the capacity of the extract to reduce Fe^{3+} to Fe^{2+}. Very similar to the DPPH test, the reducing antioxidant potential of every extract showed a positive correlation with their concentrations. The JF(ve) sample showed the strongest reduction of iron 51.08%, 25.19%, and 16.33% for the corresponding concentrations of the extract (0.71; 0.28 and 0.14 mg/mL), while the weakest activity is detected in the JC(re) sample with 18.85%, 11.13% and 6.88% for the corresponding extract concentrations. The activity of extract was compared to the reducing capacity of two standard substances - ascorbic acid and quercetin in concentrations of 0.007 mg/mL, with 27.71% and 21.37% FRAP capacity, respectively.

The berries of the Macedonian wild grown *J. communis, J. excelsa* and *J. foetidissima* are a rich source of polyphenolic compounds. These results are corresponding to those obtained by other authors (Miceli et al., 2009, Elmastas M., 2006). Additionally, the obtained values for the antioxidant potential of the tested samples are also in accordance with the results found in numerous research papers in the field of interest, suggesting that the selected plant samples are a rich source of phenolic and flavonoid components, and a potential source of antioxidant compounds (Stassi et al., 1998).

Conclusion

According to the obtained results for total phenols, flavonoids and the antioxidant potential of the selected plant samples, it may be concluded that the ethanol extracts of berries of *J. communis, J. excelsa* and *J. foetidissima* represent potential sources of natural antioxidants. Although a positive correlation was obtained between the total phenol content and the antioxidant activity, further studies are required to clarify which of the components are responsible for the antioxidant properties. In addition, screening with *in vitro* assays has little meaning if there is no clear evidence of the effectiveness of the extracts in vivo. Therefore, it is suggested that further studies are to be performed in order to evaluate the in vivo effects of these extracts.

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Macedonian pharmaceutical bulletin, **62** (suppl) 323 - 324 (2016)
ISSN 1409 - 8695
UDC: 616.153.915
616.153.922
Short communication

Biological variation of serum cholesterol and triglycerides

Biserka Simonovska[1]*, Nikola Simonovski[2], Elizabeta Popovska[2]

[1]*Dijagnostika FARMAHEM, 4ti Juli 2/3, 1000 Skopje, Macedonia*
[2]*Alkaloid AD, Bld. A. Makedonski 12, 1000 Skopje, Macedonia*

Introduction

Many analytes of interest in the clinical laboratory can vary over an individual's lifetime, simply because of natural biological factors involved in the aging process. These variations may occur rapidly at critical points in the life cycle, such as during the neonatal period, childhood, puberty, menopause, or old age. Biological variation has an important effect on the interpretation of all laboratory investigations, including triglycerides and cholesterol (Fraser, 2001).

Biological variation, i.e., the normal day-to-day variation of cholesterol is within the range of 3-5%. Many studies have also documented a seasonal variation. Although there is a discordance between the studies, cholesterol levels tend to be higher in winter and lower during the summer months independent of the country of origin, ethnicity, age, sex, and baseline lipids. The seasonal variation has been reported to be as high as 12% (Deeg, 2006).

Triglycerides are the main constituent of body fat in humans and animals, as well as vegetable fat. They are also present in the blood to enable the bidirectional transfer of adipose fat and blood glucose from the liver, and are a major component of human skin oils.

Cholesterol is a sterol (or modified steroid), a lipid molecule that is biosynthesized by all animal cells because it is an essential structural component of all cell membranes required to maintain both membrane structural integrity and fluidity. It enables animal cells to dispense with a cell wall to protect membrane integrity and cell viability.

Cholesterol and triglycerides, like many other essential components of the body, attract clinical attention when present in abnormal concentrations. For the diagnosis of different lipoprotein disorders, one can usually rely on simple plasma values of total cholesterol and triglycerides. Obtained lipid values will be interpreted in relation to age and gender according to reference values (Mula-Abed et al, 2008).

Methods

Analysis of serum total cholesterol and triglycerides is usually performed on blood specimens collected by the standard procedure. Fasting venous blood was collected at 8-10 am following an overnight fast, from apparently healthy participants (men and women, aged 15-67 years). The specimens were collected in 1-year period. Serum TG and total cholesterol were measured by enzymatic methods using kits from Biosystems (Spain) and biochemistry analyzer Mindray BS-200. Cholesterol was assayed by the cholesterol esterase/cholesterol oxidase/4-aminophenazone/phenol method and the triglycerides by glycerol phosphate oxidase/peroxidase method (Biosystems, 2012)

Standard statistical methods were used or the analysis of data. The mean and SD were calculated for each parameter of serum triglycerides and cholesterol from each participant. The data was inspected for any outlier (defined as values outside ± 3 SD from the mean). There was no outlier and all results were within the mean value ± 2 SD.

Results

The study was conducted with cholesterol samples from 210 apparently healthy participants with mean of 5.22±1.85 (Coefficient of variation (CV) 17.73%) and triglycerides samples from 204 participants with mean of 1.56±1.54 (CV 49.46%). These results were divided for observation in groups by age and gender.

* bsimonovska@gmail.com

There are 10 groups with number of participants: Group 1: 15-29 years male, Group 2: 15-29 years female, Group 3: 30-39 years male, Group 4: 30-39 years female, Group 5: 40-49 years male, Group 6: 40-49 years female, Group 7: 50-59 years male, Group 8: 50-59 years female, Group 9: 60+ years male and Group 10: 60+ years female. From these groups 5 groups were made for observation by gender. The obtained results from the participants were compared with the reference values provided by the used kits manufacturer (Biosystems, 2012).

The results from cholesterol measurements follows:

Group 1, N=26, x=5.11±1.76 (CV 17.19%); Group 2, N=21, x=4.87±1.6 (CV 16.46%); Group 3, N=23, x=5.37±1.84 (CV 17.2%); Group 4, N=21, x=5.00±1.76 (CV 17.67%); Group 5, N=20, x=5.58±1.92 (CV 17.24%); Group 6, N=20, x=5.25±1.06 (CV 20.19%); Group 7, N=21, x=5.27±2.16 (CV 20.5%); Group 8, N=20, x=5.57±1.12 (CV 10.1%); Group 9, N=21, x=5.15±2.22 (CV 21.53%); Group 10, N=17, x=4.99±1.5 (CV 14.97%).

The results from triglycerides measurements follows:

Group 1, N=24, x=1.57±1,7 (CV 54.12%); Group 2, N=22, x=1.16±1.0 (CV 43.37%); Group 3, N=22, x=1.94±1.06 (CV 27.53%); Group 4, N=21, x=1.20±1.7 (CV 70.71%); Group 5, N=17, x=1.96±1.58 (CV 40.64%); Group 6, N=18, x=1.40±1.8 (CV 64.65%); Group 7, N=20, x=1.72±1.86 (CV 54.13%); Group 8, N=21, x=1.73±1.26 (CV 36.36%); Group 9, N=20, x=1.33±1.08 (CV 40.38%); Group 10, N=19, x=1.66±1.48 (CV 44.39%).

Discussion

Biological variation has an important effect on the interpretation of different laboratory investigations, including triglycerides and cholesterol. There is also a growing interest in evaluating the cut-off limits for the desirable thresholds of serum cholesterol and triglycerides according to different recommendations and clinical trials, with a trend is towards lowering these limits.

The inter-individual variations for cholesterol are 17.73% and for triglycerides are 49.46%. The study by Ford (1989) had reported an inter-individual variation 17.3% for cholesterol, which is in agreement with the values observed in our study. The range of variability in serum lipid profile between individuals in this study for cholesterol is 4.3-6.14 mmol/L, but according to the kit documentation cholesterol level should be lower than 5.2 mmol/L and for triglycerides this range is between 0.79-2.33 mmol/L (lower than 1.7 mmol/L declared in the kit documentation).

Conclusion

From the data for cholesterol, with the usage of t-test, we can conclude that there are no significant differences in the results between different age groups and gender, but there is a significance in results for triglycerides ($p<0,05$) between participants with age of 15-49 in both male and female groups. The study should be widened with implementing analytical variation and intra-individual data.

Extensive medical research has identified hyperlipidemia as a major risk factor for heart disease with an established clinical correlation between hyperlipidemia and the incidence of Coronary Heart Disease (CHD). The importance of this study is detection of sudden changes in lipid values that may indicate a change in diet, medications, or onset of a new disease state.

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Macedonian pharmaceutical bulletin, **62** (suppl) 325 - 326 (2016)
ISSN 1409 - 8695
UDC: 616.153.495.2
616.153.495.9
Short communication

Biological variation of serum creatinine and urea

Biserka Simonovska[1]*, Nikola Simonovski[2], Elizabeta Popovska[2]

[1]*Dijagnostika FARMAHEM, 4ti Juli 2/3, 1000 Skopje, Macedonia*
[2]*Alkaloid AD, Bld. A. Makedonski 12, 1000 Skopje, Macedonia*

Introduction

Many analytes in biochemistry laboratories may vary during the life of an individual, simply because there is a factor involved in its aging process. Also gender in various analytes can play an important role. In our study we made a comparison between different age groups, gender and reference range for creatinine and urea in serum (Cvitkovic and Mesic,1999).

Creatinine is a chemical waste molecule that is produced when muscles use creatine, another naturally occurring product in the body, to create energy. About 2% of creatine is converted to creatinine each day. Most of the waste creatinine is expelled from the body in the urine (Hosten, 1990). The creatinine level measured for an individual should be relatively constant over time and should be within a "normal" range. The creatinine level is one of the main indicators of kidney function. If the creatinine level is elevated, the patient should be further evaluated for other signs of kidney disease and renal problems. Normal creatinine level ranges will depend on age, gender and other health factors (Taylor, 1989).

Blood urea nitrogen, known by the simple initials BUN is the measurement of urea nitrogen levels in the blood. Urea nitrogen is naturally produced by the liver as a waste product of digested protein. After production, urea is transported to the kidneys where it is excreted from the blood stream. The BUN test is therefore conducted to determine how much urea nitrogen is still present in the blood, and this test mainly determines if the kidneys renal functions are working as they should. There are many factors that could bring about high or low levels of BUN.

BUN and creatinine tests are often used together to determine the ratio of blood urea nitrogen to creatinine. The BUN to creatinine ratio ultimately helps the physician to find the cause of the poor kidney function and the causes may vary from dehydration to malnutrition or kidney failure.

Methods

Analysis of serum creatinine and urea was performed on blood specimens collected by the standard procedure. Fasting venous blood was collected at 8-10 am following an overnight fast, from apparently healthy participants (men and women, aged 15-65 years). The specimens were collected in a period of one year. Serum creatinine and urea were measured by enzymatic methods using kits and from Biosystems (Spain) and the biochemistry analyzer Mindray BS-200. Creatinine was assayed by picrate in alkaline medium forming colored complex, Jaffé method (Fabiny and Ertingshausen, 1971) and urea by urease/glutamate dehydrogenase method (Gutmann and Bergmeyer, 1974).

Standard statistical methods were used or the analysis of data. The mean and SD were calculated for each parameter of serum creatinine and urea from each participant. The data were inspected for any outlier (defined as values outside ± 3 SD from the mean). There was no outlier and all results were within the mean ± 2 SD.

Results

The study was conducted with creatinine samples from 220 apparently healthy participants with mean value of 74.19±23.22 (coefficient of variation (CV) 15.64%) and urea samples from 217 participants with mean of 4,88±1,31 (CV 26,94%). These samples results were divided for observation in groups by age and gender.

There are 10 groups with number of participants: Group 1 – 15-29 years male, Group 2 – 15-29 years female, Group

* bsimonovska@gmail.com

3 – 30-39 years male, Group 4 – 30-39 years female, Group 5 – 40-49 years male, Group 6 – 40-49 years female, Group 7 – 50-59 years male, Group 8 – 50-59 years female, Group 9 – 60-65 years male and Group 10 – 60-65 years female. From these groups 5 groups were made for observation by gender. The obtained results from the participants were compared with the reference values provided by the used kits manufacturer (Biosystems, 2014).

The results from creatinine measurements were as follows:

Group 1, N=24, x=79.63±9.67 (CV 12.14%); Group 2, N=22, x=64.457±9.85 (CV 15.28%); Group 3, N=24 x=80.42±8.3 (CV 10.32%); Group 4, N=23, x=65.10±7.26 (CV 11.15%); Group 5, N=22, x=80.23±9.09 (CV 11.33%); Group 6, N=21, x=63.28±6.75 (CV 10.67%); Group 7, N=24, x=81.37±10.68 (CV 13.12%); Group 8, N=21, x=68.28±6.02 (CV 8.81%); Group 9, N=21, x=83.28±10.832 (CV 13.0%); Group 10, N=18, x=72.78±9.81 (CV 13.48%).

The results from urea measurements were as follows:

Group 1, N=24, x=4.56±0.88 (CV 19.3%); Group 2, N=22, x=3.8±1.16 (CV 30.53%); Group 3, N=24, x=5.364±1.21 (CV 22.57%); Group 4, N=21, x=3.91±1.33 (CV 34.01%); Group 5, N=22, x=5.3±1.16 (CV 21.89%); Group 6, N=20, x=3.97±0.74 (CV 18.64%); Group 7, N=23, x=5.35±1.39 (CV 25.98%); Group 8, N=23, x=5.19±1.49 (CV 28.71%); Group 9, N=20, x=5.59±1.58 (CV 28.26%); Group 10, N=18, x=5.56±1.29(CV 23.20%).

Discussion

Blood and urine analytes concentrations vary due to age factors from infancy to old age. From infancy to puberty, serum creatinine concentration uniformly increases, depending on the skeletal muscle development.

In the elderly, the kidney concentrating ability is decreased, which results in reduced creatinine clearance. This is due to a decreased urinary creatinine excretion as a result of reduced lean body mass rather than to renal dysfunction. Plasma urea concentration and urinary protein excretion increase with age.

In addition to differences in gender specific hormones, differences also exist in hematology and clinical chemistry parameters. Plasma concentrations of amino acids, urea, uric acid and creatinine are higher in males than in females. Clinical chemistry analytes are greatly influenced by diet and drinking. A high-protein diet increases plasma urea, serum cholesterol, and phosphate concentrations. In contrast, starvation, fasting and malnutrition also induce clinically significant changes in analytes concentrations. Long-term starvation entails decreased concentrations of blood protein, cholesterol, triglycerides, apolipoproteins, and urea. On the other hand, the concentrations of creatinine and uric acid increase. Urinary excretion of ammonia and creatinine is increased, whereas the excretion of urea, calcium and phosphate is reduced. Due to the decreased skeletal mass, serum concentrations of urea and creatinine as well as creatinine clearance are reduced. Hemolysis can cause an interference factor for the analysis of creatinine. Also, bilirubin can cause false positive results in creatinine determination.

Conclusion

From this data for creatinine, with the usage of the t-test, we can conclude that there are significant differences in results between different age groups and gender, but there is no significance in results for urea for participants with age of 50-65 in both male and female groups.

The inter-individual variations for creatinine are 15,64% and for urea are 26,94%. The range of variability in serum creatinine between individuals in this study is between 61,57-100,4 µmol/L for male participants and 50,9-82,66 µmol/L for female, but according to the kit documentation creatinine levels should be 62-106 µmol/L for male and 44-80 µmol/L for female. For urea this range is between 2,08-7,64 mmol/L (2,6-6,5 mmol/L by the kit documentation).

It is of great importance that physicians be aware of these factors, because the final interpretation of the analyses and decisions on therapeutic procedures based on this interpretation actually lead to the physician's final judgment. In this process, the laboratory should act as a reliable and consistent collaborator.

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Macedonian pharmaceutical bulletin, **62** (suppl) 327 - 328 (2016)
ISSN 1409 - 8695
UDC: 613.83:633.75
Short communication

How long are opiates present in urine after consumption of product which contains poppy seeds?

Danijela Đukić-Ćosić[1], Katarina Baralić[1], Milka Kostadinović[1], Marko Antunović[2], Snežana Đorđević[2], Zorica Bulat[1], Marijana Ćurčić[1], Evica Antonijević[1], Aleksandra Buha[1], Biljana Antonijević[1]*, Vesna Matović[1]

[1]*Department of Toxicology "Akademik Danilo Soldatović", University of Belgrade – Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*
[2]*National Poison Control Centre, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Serbia*

Introduction

Opiates are one of the most frequently analyzed psychoactive substances due to their high abuse potential. Worldwide urine screening tests use for preliminary results of opiate abuse for employees in the workplace, athletes in doping control and etc. (Lachenmeier et al., 2010; Lum and Mushlin, 2004). However, existing problem in interpreting the results of opiate urine analysis arises because immunoassays screening tests have high sensitivity but lower specificity because of cross-reactivity with substances other than the drugs in question (Lum and Mushlin, 2004). Therefore, it is well known that positive opiate results may be obtained during drugs of abuse screening related to certain drugs and food intake (EFSA, 2011; Jankovičová et al., 2009; Lachenmeier et al., 2010; Lum and Mushlin, 2004).

Seeds of the opium poppy plant are legally sold and widely consumed as food. Morphine and codeine are naturally occurring substituent of the poppy plant, *Papaver somniferum*. The opiate content of poppy seeds varies greatly and is dependent on the seed origin and method processing (EFSA, 2011; Samano et al., 2015). Due to contamination during harvesting, the seeds can contain morphine and other opiate alkaloids (Lachenmeier et al., 2010). Literature data and our previous case study indicate that consummation of both, raw poppy seeds and baking mixtures, induce positive immunoassay test for opiates (Đukić-Ćosić et al. 2014; Jankovičová et al., 2009; Samano et al., 2015; Smith et al., 2014). However, the time period during which it is possible to obtain false positive opiates results is not yet sufficiently understood.

The purpose of this case study was to determine time period of presence of opiates in urine after the consumption of product which contain poppy seed using immunoassay test and liquid chromatography-mass spectrometry (LC/MS) technique.

Materials and methods

Female participant age between 20 and 25 years without previous known history of substance abuse provided written informed consent to participate in this case study. Participant consumed a piece of strudel with poppy seeds from a local bakery in the morning on the first day and collected *ad libitum* each urine sample during next three days. Urine samples (n=8) were collected 5, 12, 24, 30, 36, 48, 60 and 72 hours after strudel ingestion. Specimens were analyzed with the Opiates Test Card (MP Biomedicals, LLC, California) immunoassay at 300 ng/mL cutoffs, and all positive samples were quantified for morphine and codeine by LC/MS. Urine specimen was prepared using liquid/liquid extraction of opiate by mixture of chloroform and isopropanol (9 : 1; v/v). Extracts were analyzed by LC/MS technique: separation column Waters Spherisorb 5 µm, ODS2, 4.6 x 100 mm; mobile phase: ammonium acetate:acetonitrile (80 : 20; v/v), mobile phase flow rate 0.3 mL/min; mass detection range: 100-400 m/z.

* abiljana@pharmacy.bg.ac.rs

Results and discussion

The screening immunoassay test for opiates (cutoff 300 ng/mL) was positive for all investigated urine samples. These results in accordance with previous studies that poppy-seed food products, such as poppy-seed strudels, may cause a true-positive immunoassay screening test for opiates due to the presence of low, but still detectible, levels of opium alkaloids (Lum and Mushlin, 2004). The morphine content in poppy seeds from around the world is variable (0.1-294 mg/g), and exposure concentrations depend on poppy seed origin, harvesting procedure and the method of poppy seed foodstuffs preparation (Samano et al., 2015; Thevis et al., 2003). Significant reductions in opiate content have been documented after food preparative processes, with decreased drug concentration shown after washing, soaking, grinding and baking (Lachenmeier et al., 2010; Samano et al., 2015). To minimize the number of positive opiate tests resulting from poppy seed consumption, cutoff concentration for morphine and codeine raised from 300 to 2,000 ng/mL (Lachenmeier et al., 2010; Samano et al., 2015). However, only screening immunoassay test for opiates with lower cutoff (300 ng/mL) are available in our country.

In all investigated urine samples, morphine and codeine concentrations were determined by LC/MS. The following values were obtained for morphine: 0.25, 0.06, 0.05, 0.04, 0.02, 0.02, 0.01 and 0.01 mg/L, and codeine 0.07, 0.09, 0.06, 0.02, 0.009, 0.008, 0.001, 0.001 mg/L, respectively. The highest measured concentration of morphine and codeine was obtained at 5 and 12 hours, respectively. This is in agreement with literature data that peak concentrations of morphine and codeine appeared 4-12 hours after consumption of poppy seed (Lachenmeier et al., 2010). However, for interpreting urine opiate results need to know that measured concentrations of morphine and codeine after the consumption of products which contain poppy seed are noticeably lower then concentrations of opiates which can be measured in urine of opiate abusers. Furthermore, presence of heroin metabolite 6-monoacetylmorphine can be detected in urine of opiate abusers using LC/MS or GC/MS techniques (Lachenmeier et al., 2010; Samano et al., 2015).

In contrast to results of this case, the most studies with intake of poppy seeds, raw and cakes, shows that urine samples were negative by 20 hours after consumption (Jankovičová et al., 2009; Lachenmeier et al., 2010; Smith et al., 2014). Present data show that, even 72 hours after the oral intake of strudel containing poppy seeds, the urinary level of opiate alkaloids can be detectible. Namely, approximate detection time of opiates of abuse in urine is up to three days and these data demonstrated that opiates can be detected in urine after consumption poppy seed strudel during the same time period as in users of psychoactive substances. Thevis et al. (2003) also demonstrated positive opiate urine at 48 h after consumption a typical poppy seed cake with high morphine content (151.6 mg/kg). The consumption of poppy seed products in those out-of-competition time spaces might be considered as non problematic, but the long length of stay of opiates in urine samples can lead to positive doping results in competition tests up to three days after oral intake of cakes with poppy seeds (Thevis et al., 2003; Lachenmeier et al., 2010).

Conclusion

The results of the present case study support previous findings and confirm that opiates can be detected in urine after consumption popular poppy seed strudel. However, these data indicates that time period of presence of opiates in urine can be prolonged after consumption of this product. This finding should be taken into account in routine testing of employees in the workplace, members of the military, students in schools, as well as athletes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 329 - 330 (2016)
ISSN 1409 - 8695
UDC: 635.34-152.75:613.262
Short communication

Nutritional properties of two hybrids of dried and fresh cabbage

Ljubica Karakashova[1], Frosina Babanovska - Milenkovska[1],
Biljana Chuleva[2]*, Silvija Nakova[1]

[1] *Faculty of Agricultural Sciences and Food, Ss Cyril and Methodius University, bul Aleksandar Makedonski bb, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Public Health, ul. 50 Divizija, bb, 1000 Skopje, Republic of Macedonia*

Introduction

Cabbage (*Brassica oleracea var. Capitata*) is a vegetable crop, economically important in Republic of Macedonia. In nutrition, it is used in fresh or processed state.

Dobrićević et al. (2006), have determined the chemical composition of 100 g of cabbage, where the amount of vitamin C ranges from 20 to 100 mg/100 g, dry matter content from 5% to 9%, fiber 1.0% to 1.7%. According to Rumeza et al. (2006), the water content in cabbage was 92%, protein 1.6%, fat 0.2%, carbohydrates 4.8%, ash 0.6%, Ca 52 mg/100g and P 44 mg/100g. The energy value is 24 kcal/100 g. Vitamin C which is contained in the cabbage has antioxidant effect, it protects the human body from stress, and it is a cofactor in several vital enzymatic reactions (Cauniietal, 2010). According to Gjorgjev et al. (2008), the content of Ca and P in cabbage is in proportion that contributes to a better use of these micronutrients for growth and development of the organism. There are also the following elements: K, Na and Cl, and the trace elements: Cu, Zn, Co and Mn (Lambaša, 2006). Cabbage contains a significant amount of aromatic substances and organic acids, which give the typical taste and smell (Gjorgjev et al., 2008). Fresh cabbage has healing properties when treating stomach ulcer, protects mucous membranes, strengthens the immune system against many diseases, increases hemoglobin in the blood and cures anemia, affect the normalization of blood pressure, cures rheumatism, reduces the risk of colon hose etc (Vlahović, 1999).

During the drying process on appropriate temperature, free water evaporates which preserves the stability of the product from microbiological and chemical aspect. The content of water in the dried vegetables ranges from 8 to 12%. At the same time, it the concentration of sugars and other ingredients is increased (Katalinić, 2006). During the drying process it is important to apply appropriate temperature, otherwise it will adversely affect the texture and the color (Karakashova, 2003).

Hussein (2012), determined the chemical composition of the dry cabbage: water content 7.25%, proteins 14.34%, fat 0.97%, fiber 21.64%, ash 5.82%, carbohydrates 71.62% and phenolic components 81.58 mg/100 g.

Materials and methods

The research in both hybrid cabbage, transam (white cabbage) and maestro (red cabbage), in fresh and dried samples showed: total dry matter content, soluble dry matter, total acids, reducing sugars, ash, calcium, phosphorus, vitamin C, chlorophyll and anthocyanins.

In the analysis were used the following methods: total dry matter with drying chamber at T 105 °C to a constant mass; soluble dry matter by refractometer at 20 °C; vitamin C by Tillmans method with 2,6-dichlorophenolindophenol; total organic acid with 0.1 M solution of NaOH; reducing sugars by Luff-Schoorl method; total mineral matter (ash) in Muffle furnaces on 550 ºC; calcium and phosphorus were studied by the ICC ENISO14082:2003 method. Quantification of chlorophyll and anthocyanins was performed by using a spectrophotometer, for chlorophyll at a wavelength 662 and 644 nm, and for anthocyanins at a wavelength of 700 and 420 nm.

The obtained results were statistically processed, using a special computer program (SPSS for Windows, Sum of squares procedure, Model III).

* bculeva@hotmail.com

Results and discussion

The tested hybrids transam and maestro have Dutch origin, they have high dry matter content and are suitable raw material for the manufacturing industry.

Determination of total dry matter content is an important parameter according to which are determined the quality features of tested hybrids in fresh state, as well as in dried products. It was determined that the average content of the total dry matter content of the hybrid maestro was 7.50% in fresh and 86.85% in the dried samples, while hybrid transam had a value of 8.45% in fresh and 88.4% in the dried samples. The average content of reducing sugars in hybrid transam was 4.40% in fresh and 39.75% in the dried samples and in the fresh head of cabbage of hybrid maestro is3.6%, while in the dried samples is 33.95%.

The total acids in fresh head of cabbage of the hybrid transam are 0.19% and for hybrid maestro are 0.21%, while for the dried samples they are 1.63% for hybrid maestro, to 1.76% for the hybrid transam. The tests for vitamin C in hybrid maestro in fresh head of cabbage determined average content of 80.95 mg/100 g and 31.69 mg/100 g in the dried samples, the hybrid transam in fresh head of cabbage is 19.50 mg/100 g and 17.7 mg/100 g in the dried samples.

The average ash content in both fresh tested hybrids was 0.53% for the maestro and 0.63% for transam, while for the dried samples were determined values of 5.70% for the maestro and for the hybrid transam the value of ash was 6.20%. By analyzing the average calcium Ca content in tested fresh hybrids determined were the following values: 80.95 mg/100 g in hybrid maestro and 45.15 mg/100 g in hybrid transam. Unlike in the fresh, in the dried samples the Ca is more common in the hybrid transam 41.11mg/100 g than in the hybrid maestro 36.26 mg/100 g. The average content of P for both tested hybrids in fresh head of cabbage was 0.53 mg/100 g for maestro and 0.63 mg/100 g for transam. In the dried samples were determined values of P 23.6 mg/100 g in hybrid transam and 21.2 mg/100 g in hybrid maestro.

By analysis of variance and LSD test at the level of 0.05 and 0.01, it has been determined a statistically significant difference in values for all tested parameters between both hybrids for both the fresh and the dried samples.

In the hybrid transam in fresh state it has not been quantified the presence of chlorophyll. In the dried samples of the hybrid transam was found a greater concentration of chlorophyll b (0.24 mg/cm^3) compared to chlorophyll a (0.20 mg/cm^3). The presence of anthocyanins is found only in hybrid maestro. In the fresh head of cabbage was found more anthocyanins content, 7.14% and 1.5% polymeric dyes, compared to the dried cabbage, where the content of anthocyanins was 26.04% and 1.0% polymeric dyes. Their representation in dried cabbage compared with fresh cabbage, point to possible degradation changes of the anthocyanins colored matters which occur in the drying process of red cabbage.

Conclusion

Based on the analysis it can be concluded that the tested hybrids are characterized by good technological and nutritional properties. Better nutrition values are featured in dried samples of the hybrid transam than of the hybrid maestro. In the hybrid maestro, the value of vitamin C is statistically significantly higher than the hybrid transam. Dry samples of the hybrid transam contain a larger amount of chlorophyll b than chlorophyll a, and of the hybrid maestro contain larger amounts of anthocyanins and polymeric dyes compared with fresh red cabbage. Dried cabbage has a high nutritional and biological value, and the obtained dried product is greatly appreciated and interesting for the European and world markets.

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Macedonian pharmaceutical bulletin, **62** (suppl) 331 - 332 (2016)
ISSN 1409 - 8695
UDC: 663.253:547.56]:543.544.5.068.7
Short communication

Determination of some phenolic constituents in extract of local wine species by using a validated HPLC-DAD method

Ebru Türköz Acar*, Mehmet Engin Celep, Mohammad Charehsaz, Gülşah Selin Akyüz, Erdem Yeşilada

Yeditepe University, Faculty of Pharmacy, 34755, Ataşehir, İstanbul, Turkey

Introduction

In Anatolia, wine production has a long way back in history. In fact, Anatolia is believed to be the birthplace of vineyards and winemaking. The earliest records regarding Anatolian wine production dates back to the Neolithic age (Ozdemir, 2015). Currently, Turkey ranks fourth in the world in terms of area devoted to viticulture and fifth regarding the harvested grape tonnage (Peri et al., 2015). Grapes of local varieties such as Kalecik karasi, Bogazkere, Okuzgozu, and Papazkarasi are the popular native Turkish cultivars of *Vitis vinifera L*. and they are used along with those of French origin for wine production in Turkey. The color of its fruits is black with gray bloom (Bozan et al., 2008; Peri et al., 2015). On the other hand, the interest on fruit wines, which are produced from fruits other than grapes, has also been recently increasing. A fruit wine can be considered as a biotechnological product of yeast fermentation of natural sugars present in the fruit juice. Generally, the process for

fruit wine production resembles that of grape wines. Consequently, a non-grape fruit wine is a mixture composed of fruit juice, alcohol, and a wide range of biomolecules that may already be present in the fruit or be biosynthesized during the fermentation process (Amidžić Klarić et al., 2015; Kalkan Yildirim, 2006). Individuals are advised to boost their intake of dietary antioxidants, since they play a major role in increasing the body's antioxidant potential and to provide protection against the harmful effects of oxidative stress (Willcox et al., 2004). Recent epidemiological data have clearly indicated that regular consumption of wine in moderate amounts may have positive effects on health care. This health-promoting effect is generally attributed to their rich phenolic content and antioxidant effect (Naissides et al., 2006). The antioxidant potential of wine is closely related to its phenols content, which may be affected by a number of factors, including grape variety, fermentation processes, vinification techniques, ageing, and geographical and environmental factors (soil type and climate). In this study, selected Anatolian red wines and fruit wines were assayed for their polyphenols contents.

Materials and methods

Standard materials of gallic acid (1), chlorogenic acid (2), epigallocatechin (3), caffeic acid (4), vanillin (5), p-coumaric acid (6), rutin (7) and quercetin (8) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Ortho-phosphoric acid (85%) solution, ethanol (HPLC gradient grade) and methanol (HPLC gradient grade) were from Merck (Darmstadt, Germany).

Ultrapure water for preparation of mobile phase (18.2 MΩ.cm at 25 °C) was obtained by using Millipore Simplicity UV apparatus (Millipore , Molsheim, France).

The eıght analytes stock solutions were prepared by dissolving weighed amount of the standard substance in ethanol at 1mg/mL concentration value. All stock solutions were stored in a refrigerator at 4 °C. Combined working solutions of mixed analytes at the concentrations of 5, 10, 20, 50, 100 µg/ml were obtained by dilution of appropriate volume of stock solutions in volumetric flasks. Calibration curves were plotted, in triplicate, by analysing these standard solutions prepared freshly. Concentration values of the quality control samples (QC) were 7.5, 30 and 80 µg/ml. Chromatographic experiments were performed by using Agilent 1260 HPLC system consisting of a quaternary pump model G1311B, an auto injector model G1329B,

* ebruturkozacar@gmail.com

a thermostated column compartment model G1316A and a diode array detector (DAD) model G4212B. The chromatograms was monitored and integrated by using Agilent ChemStation software. Chromatographic separations of analytes was achieved on an Agilent Zorbax Eclipse XDB-C18 column (4.6 mm x 150 mm, 3.5 µm particle size) and the column was thermostated at 25±1 °C during analysis. DAD signals for every analyte were selected acoording to their spectrums obtained from Agilent ChemStation Software. Appropriate wavelenghts were selected as: 214 nm for gallic acid, chlorogenic acid and quercetin, 306 nm for vanillin, p-coumaric acid and rutin, 333 nm for chlorogenic acid and caffeic acid. Gradient elution system was used to separate all analytes. For this purpose two different mobile phase were used; Mobile phase A was 10mM phosphoric acid solution and mobile phase B was methanol using a flow rate of 1ml/min. The optimised gradient programme was as follows: 0–15 min (0-60% B), 15–20 min (60–80% B), 20.0–22 min (80-100% B), 22–27 min (100–0% B) and 27–32 min (0% B). Samples were injected into the system as 10 µl. Both fruit wines and grape wine of Papazkarasi type cultivar were purchased from local producers in Turkey. After removal of alcohol by using a rotatory evaporator, the residual part of each wine was lyophilized. The lyophilized extracts were dissolved in water at proper concentrations prior the experimentation.

Results and discussion

To achieve the best separation different mobile phases were investigated like buffers, organic solvents and different concentrations and different mixtures of these solutions. 10 mM phosphoric acid solution was used as mobile phase A and methanol was used mobile phase B for further experiments. On the other hand, other chromatographic conditions like flow rate, injection volume and temperature were investigated. At the end of experiments optimum parameters were determined as 1 ml/min for flow rate, 10 µL for injection volume and 25°C for temperature providing the best separation of eight phenolic compounds. In the light of this information system suitability test results were investigated before validation studies. Six replicate analysis of this standard mixture was performed. Different concentration values of each phenolic compounds were investigated to determine dynamic range for the method developed. For this purpose standard solutions of each analyte as a mixture were prepared daily by diluting from stock solution of compounds. Chromatograms obtained for each standard mixture were recorded and investigated to determine calibration parameters of the method. Accuracy studies for the method developed was performed by three repetitive analyzing samples of known concentration at three different level as low, medium and high level in dynamic range. For this purpose standard mixtures of each compund at three different concentration values were prepared by diluting stock solution and concentration values were as 7.5, 30 and 80 µg/mL. Precision of the method was investigated bythe way of RSD values obtained from three repetitive analysis of known amount of standards at three different level. These RSD values for inra-day studies were lower than 1% value that the method very precise in intra-day studies. The specifity of the method was demonstrated by using spiked wine extract samples. For this purpose each standard solution was spiked to same wine extract and analyzed. It was observed that materials being in wine extract samples do not present overlapping peaks with eight phenolic compounds.The method developed and optimized was applied for analysis of eight different phenolic compound in different wine extract samples.

Conclusion

The method developed was sensitive, accurate and sensitive for analysis of these phenolic constituents in different wine samples.

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Pharmaceutical technology and Biotechnology/ Cosmetology/ Biopharmacy

Macedonian pharmaceutical bulletin, **62** (suppl) 335 - 336 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:620.3
Short communication

Cyclodextrin-based nanoparticles for drug encapsulation

Ruxandra Gref

Institut des Sciences Moléculaires d'Orsay, UMR CNRS 8214, Avenue Jean Perrin, Université Paris Saclay, 91400 Orsay, France

Background

The use of nanocarriers for drug delivery represents nowadays a promising approach to overcome a series of pharmacological shortcomings of drugs, like low target specificity, rapid clearance, poor pharmacokinetics, severe side effects, and multidrug resistance phenomena.

Cyclodextrin-based nanoassemblies as drug carriers

Supramolecular cyclodextrin-based nanoassemblies mediated by host-guest interactions have gained increased popularity because of their "green" and simple preparation procedure, as well as their versatility in terms of inclusion of active molecules (Gref et al., 2012). Here we show that original nanoparticlesof around 100 nm are spontaneously prepared in water, by a lock and key mechanism involving formation of inclusion complexes between CDs on one water-soluble polymer and hydrophobic side chains on another one (Anand et al., 2012; Battistini et al., 2008; Daoud-Mahammed et al., 2009; Fraix et al., 2013; Othman et al., 2011). Whatever the mixing conditions, nanoparticles with narrow size distributions arespontaneously formed upon the contact of two polymeric aqueous solutions. Advantageously, the production of the nanoparticles can be scaled-up using a microfluidic device. In situ size measurements helped understanding the mechanisms involved in nanoparticle self-assembly. Individual nanoparticle tracking analysis enabled to establish that despite the non-covalent nature of the nanoassemblies, they were remarkably stable, even upon extreme dilution (few ng/mL). Contrast agents bearing adamantly moieties were incorporated into the nanoassemblies, showing high relaxivities (Battistini et al., 2008). Spectroscopic and photophysical studies helped understanding the interactions involved in the nanoassemblies loaded or not with drugs (Anand et al., 2012). Finally, another set of examples shows the utility of the nanoassemblies for bimodal anticancer phototherapy (Fraix et al., 2013).

Conclusion

This study paves the way towards the "green" scalable formation of supramolecular assemblies with potential uses in cosmetics and drug delivery.

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Macedonian pharmaceutical bulletin, **62** (suppl) 337 - 338 (2016)
ISSN 1409 - 8695
UDC: 615.014.2
Short communication

Geomaterials in the design of new drug delivery systems

César Viseras

Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Granada, Campus of Cartuja, 18071 s/n, Granada, Spain
Andalusian Institute of Earth Sciences, CSIC-University of Granada, Avda. de Las Palmeras 4, 18100, Armilla (Granada), Spain

Introduction

The lecture is intended to provide an overview of the use of geomaterials in medical and health care applications, including their use as active pharmaceutical ingredients and classical excipients together with new applications in drug and gene delivery, tissue engineering, as well as prospective used in future therapeutics and diagnostics trends. Both natural geomaterials and their derivatives are attracting growing attention in a variety of fields and in particular as biomaterials. Pharmaceutical technology is not an exception and geo-source excipients are competing well with the synthetic materials.

Pharmaceutical uses of geomaterials: from conventional to innovative

For thousands of years, humans have been using available substances to achieve specific functions, including their use in contact with living tissues, organisms or micro-organisms. Like most of the materials used by humans, geomaterials are widely mentioned in the literature, and occasionally exploited clinically, as such, as devices or as part of devices to treat trauma and diseases. These classical used of geomaterials in health care applications will be revised.

In recent decades, research in biomaterials and their use in healthcare have increased all over the world. Applications of biomaterials in human healthcare include the development of new medical devices and prostheses, tissue engineering, bone regeneration, implants and surgical tools, diagnostic techniques, bioadhesives, artificial organs and drug delivery (Larsson et al., 2007). Biomaterials for medical applications must be biocompatible, and either bioresorbable or biodurable. Nanotechnologies and inorganic–organic hybrid technologies are currently two of the most active approaches to achieve these requirements. Many organic–inorganic hybrid materials and nanotechnology based targeted drug delivery systems imply the use of geomaterials.

Arrangement, structure and properties of pharmaceutically interesting geomaterials

Some geomaterials, and significantly carbonates (and carbon structures), phosphates and silicates (special clays and zeolites) induce no adverse effect in contact with a living organism (biocompatibility) and their presence may be beneficial (bioactivity) or without any significant health effect (bio-inert) but with biodurable properties. The specific function that a geomaterial have in any formulation depends on its composition, structure and properties, including both physical properties (particle size and shape, specific surface area, texture,…) and chemical features (surface chemistry, charge,…).

Calcium phosphate and calcium carbonate are natural nanosized geomaterials than can be used in drug delivery and tissue engineering. Calcium phosphate nanoparticles can be tailored to be biocompatible and show high adsorption capacities to be used in controlled and targeted drug release.

It must be also remarked all the possibilities in medicine of graphitic forms deriving from graphene as fullerenes, carbon nanotubes and graphite. The possibilities of these carbon materials in health care products are a new frontier of pharmaceutics (Hong et al., 2015; Pan et al., 2012; Sánchez et al., 2012).

* cviseras@ugr.es

Calcium carbonate appears as three different anhydrous crystalline polymorphs: calcite, aragonite, and vaterite. Under standard conditions for temperature and pressure, calcite is the stable phase, while aragonite and vaterite are the metastable forms that readily transform into the stable phase. Vaterite and other polymorphic forms of carbonate are receiving great attention as nanocarriers in drug delivery.

Silicates, including some tectosilicates, as zeolites (Cerri et al., 2016), but mainly phyllosilicates (clay minerals) have also featured in pharmaceutics due to their adsorptive and ion exchange properties (Aguzzi et al., 2007). These minerals appear abundantly at the surface of the Earth as natural nanoscale particles with layered structures and interlayer spaces, which have potential to play a significant role as biomaterials. Structurally, the layers, with thickness around one nanometre, consist in most cases of one or two tetrahedral silicate sheets and one octahedral metal oxide/hydroxide sheet. Many clay minerals form sheet-like particles or platelets, for example, kaolinite (a 1:1-type clay mineral) formed by one tetrahedral sheet and one octahedral sheet, or talc, vermiculite, montmorillonite or saponite and others (2:1-type clay minerals), which are composed of an octahedral sheet sandwiched between two tetrahedral silicate sheets. Chemically, clay minerals are hydrous aluminium or magnesium phyllosilicates, usually with variable amounts of iron, magnesium, alkali metals, alkaline earths, and other cations present either in the interlayer space or in the lattice framework by isomorphous substitution. Uses of clay minerals in healthcare include traditional applications but also, as a result of the nanometre-scale layering, interlayer spacing and strong electrostatic interactions, the possibility of developing functional materials for such advanced technologies as nanotechnology and, in particular, biomaterials. Clay minerals and zeolites have been proposed as carriers of drugs, genes and proteins (López-Galindo and Viseras, 2004). The investigation of geomaterials–drug interaction and release mechanisms is an essential contribution for the formulation of geomaterial-based drug delivery systems. The possibilities of such systems depend on the amount of drug retained by the geomaterial as well as on the release kinetics and the total amount released in regard of the therapeutic regime.

Some polymorphic forms of silicates resulted in structures with particular interest. As for example, natural mineral nanotubes comprise hollow tubular minerals with nanoscale diameters.

Modification, functionalization and polymer conjugation of geomaterials.

Sometimes, properties of natural geomaterials cannot achieve the desired objectives, requiring their modification or functionalization as well as the incorporation into polymeric matrices to obtain nanocomposites with improved properties compared to the individual components (Viseras et al., 2008). All these improvements are notably prompting the availability of optimized and well characterized geomaterial-derivatives, suitable for a wide range of biomedical applications.

Concluding remarks

The therapeutic advantages of geomaterials in the design and development of technologically advanced drug products make this field of investigation a high-priority development area in which the development of new applications will require the close collaboration of pharmaceutical technologist with geologist, and chemists.

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Macedonian pharmaceutical bulletin, **62** (suppl) 339 - 340 (2016)
ISSN 1409 - 8695
UDC: 615.849.2(497.7)
Short communication

University Institute for positron emission tomography in Skopje - unique facility for the new challenges in the regional health care system

Emilija Janevik-Ivanovska[1,4]*, Katerina Kolevska[1,4], Maja Velickovska[2,4], Filip Jolevski[1,4], Marija Atanasova[1,4], Marina Zdraveska-Kocovska[3], Meri Angeleska[3], Maja Chochevska[1,4], Zlatko Filipovski[1,4], Saso Nikolovski[1, 4]

[1]*Faculty of Medical Sciences, GoceDelcev University Stip, Republic of Macedonia*
[2]*Project Unit for Implementation of Positron Emission Tomography - PET Center Skopje in Republic of Macedonia, Ministry of Health of the Republic of Macedonia*
[3]*Institute of Pathophysiology and Nuclear Medicine, Faculty of Medicine, University "Ss Cyril and Methodius", Skopje, Republic of Macedonia*
[4]*University Institute of Positron-Emission Tomography, Skopje, Republic of Macedonia*

Background

The objective of our new facility of the University Institute of Positron-Emission Tomography are the production of PET radiopharmaceuticals for clinical service of in-house patients, commercial distribution of PET radiopharmaceuticals and precursors and development of new PET radiopharmaceuticals for diagnostic and therapeutical purpose. The factors foremost in the planning and design phases were the current regulatory climate for PET radiopharmaceutical production, radiation safety issues, and effective production flow. A medium-energy cyclotron (16.5 MeV) was installed in a bunker with the high-proton energy to offer a higher product radioactivity. This new state-of-the-art Positron Emission Tomography (PET) Institute and with included research capabilities is dedicated to providing the highest quality of nuclear imaging research.

Positron emission tomography in the last decade is one of the most promising methods of detecting oncological, cardiological, neurological diseases and enters slowly in the other fields showing hopeful results. The introduction of the new radiopharmaceutical for diagnosis and therapy in the clinical practice including the clinical trials are possible only with the appropriate production site according all cGMP requirements.

The new University Institute for Positron Emission Tomography is in the final official establishment as a unique facility in the country and in the Balkan Region. The new facility is result of the Government investment and joint project with International Atomic Energy Agency (IAEA).

Methodology

Establishing a PET institution is a large scale process that requires careful planning, inputs from multiple stakeholders, the support and approval of the authorities, secure funding, and a detailed implementation strategy. The need for a carefully planned strategy is even more essential in the conditions prevailing in a developing country, where the introduction of PET may be impeded by a scarcity of financial resources and, in many cases, an inadequate understanding of the potential roles and contributions PET imaging can play in a health care system.

Our new institution integrates Department for Production of Radiopharmaceuticals and Department of Molecular Imaging. They are connected in one unique system of Radiation protection, QA, BMS, Informatics and technical and administrative support of the network from the unified Health Care System in the country (IAEA, No.1, 2009).

* emilija.janevik@ugd.edu.mk

Results

The facility include cyclotron (GE, PET trace 860) for production of ultra-short-lived isotopes (F-18, C-11, N-13 and optional for solid targets that is planned already during the construction). Adjacent to the cyclotron is an area that houses the support equipment and a large dedicated workshop to support machine maintenance and target development. The Radiopharmacy production site contains two clean room with controlled-air environment class 10,000 (M5.5) and access via an interlocking entry change area. One completely dedicated for FDG production and second for the production of other F18, C-11, N-13, Ga-68 radiopharmaceuticals and Cu-64. The third production laboratory is dedicated for small scale production of diagnostic and therapeutical radiopharmaceuticals for clinical trials and investigation (White S, 2016). A fully shielded hot cells (class 100 [M3.5]) is located in all clean rooms. The PET radiopharmaceuticals are delivered via shielded tubing between the synthesizers and hot cells. Inside the hot cells, there is an automated device for dispensing the PET radiopharmaceuticals into either a bulk-activity vial or a unit-dose syringe (IAEA 2008). The dispensed PET radiopharmaceutical then passes through a hatch to a dedicated area where it is packaged for in-house use or commercial distribution. Unit doses for in-house patients are transported via elevator to the PET imaging area away. There is extensive radiation area monitoring throughout the facility that continuously measures radiation levels (IAEA No.1, No.58, 2009).

The integrated parts are two QC laboratories well equipped and one research laboratory for preclinical investigation including toxicological studies of new radiopharmaceuticals and imaging of small animal models.

The Department of Molecular Imaging is located in the second floor with two PET/CT cameras dedicated for clinical investigation and advanced biomedical imaging in human using extensive suite of state-of-the-art internally produced radiopharmaceuticals as imaging tools (IAEA 2010, Calabria and Schillaci, 2016).

This institution will be the full partner to the physicians not only in the country, but also in the region.

PET in that condition may serve as a magnet for recruitment in many areas and promote national and international interdisciplinary cooperation, to provide university educational opportunities for master, doctoral and postdoctoral studies, specialties in Nuclear Medicine, Radiopharmacy and Medical Physics with distinctive strength in education and research and an entrepreneurial dimension.

Conclusion

The University Institute for Positron Emission Tomography in Skopje will move a nationally and internationally recognized unit with true integration of research and service to the high level. The heath care for patients will be improved in many aspects and will help keeping the PET at the forefront of imaging procedures. Our Institute will serve as a model for future and more widespread clinical use of PET radiopharmaceuticals and provide proficiencies how PET technology may facilitate research and development opportunities.

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Macedonian pharmaceutical bulletin, **62** (suppl) 341 - 342 (2016)
ISSN 1409 - 8695
UDC: 615.453.6.014.2
Short communication

Design and evaluation of differently produced glyceride based mini-matrices as extended release systems for highly soluble model drug

Aleksandar Aleksovski[1]*, Chris Vervaet[2], Rok Dreu[1]

[1]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Univeristy of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia.*
[2]*Laboratory of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium*

Introduction

Mini-tablets (d≤3mm or 5 mm) are emerging as promising type of drug delivery platoform suitable in the production of both single- or multiple-unit systems. Mini-tablets proved to be suitable dosage forms for targeted patient groups such as pediatrics, geriatrics and hospitalized patients since they may offer ease in swallowing (alone or mixed with soft food) and flexible dosing regarding age/weight/condition. When formulated as multiple unit systems, mini-tablets offer broad GIT distribution, less significant ''all or nothing'' effect and combining different release kinetics/compounds in one system (Aleksovski et al., 2015).

Hot-melt extrusion (HME) using circular dies and subsequent extrudate cut is emerging as promising technology in the production of mini-matrices aimed to deliver API by modified fashion. HME is solvent free process based on mixing, kneading, melting and transporting powdered materials through a pre-heated barrel equipped with one or two rotating screws up to an end plate die which determines final size and shape of the extrudate (Repka et al., 2012).

The aim of this research was to develop and evaluate mini-matrices based on either glyceryl behenate (GB) or glyceryl palmitostearate (GPS) for extended drug release of highly soluble model drug metoprolol tartrate. Mini-matrices were produced by two different techniques: hot-melt extrusion (mini-extrudates, EX) and compression of mini-tablets from untreated powder mixtures (direct compressed mini-tablets, DCMT) or granules obtained by milling extrudates (granule based mini-tablets, GMT).

Materials and methods

The following materials were used: metoprolol tartrate (MPT, EsteveQuimica) (20-40%), glyceryl behenate(GB, Compritol 888 ATO, Gattefosse) or glyceryl palmitostearate(GPS, Precirol ATO 5, Gattefosse) (80-60%), colloidal Si dioxide (0.5% in extrudates and tablets) and magnesium stearate (1% in tablets). The last two were replacing suitable amount of the glyceride.

Production methods: HME was performedon a co-rotating twin screw extruder with pre-heated barrel segments (entry to die T°C, GB EX -77/75/75/75/72/66 and GPS EX - 57/57/57/55/53/50). Obtained strains (d=3mm) were manually cut into mini-extrudates of 3 or 5 mm height. Mini-tablets (30 mg, d = 4mm) were produced on a single punch tablet press by compressing untreated powder mixtures (DCMT) or milled extrudates (GMT) 3kN compression force. When investigating influence of granule size on drug release, GMT samples were prepared by granules in the range of 0.150 mm ≤ d ≤ 0.250 mm (GMT S) or granules ranging 0.500 mm ≤ d ≤ 0.750 mm (GMT L).

Evaluation methods: Drug release studies (USP I) were carried out in H_2O, 0.1 M HCl, phosphate buffer pH 6.8, FESSIF with addition of pancreatin and $CaCl_2$ and blank FESSIF (without pancreatin, CaCl2, lechitin, and Na taurocholate). Calculation of similarity factor *f2* was performed. Solid state studies were performed using DSC and X-ray diffracttometry. Drug- glyceride interactions were evaluated by ATR-FTIR, while drug distribution by Raman mapping. Matrix porosity was determined by microcomputed tomography (μCT). Stability testing was performed at room and accelerated conditions in vapor protective bags.

* aleksandar.aleksovski@ffa.uni-lj.si

Results and discussion

HME and granule compression proved to be robust and reliable techniques when producing mini-matrices for extended drug release in all drug to glyceride ratios. Direct compression was seen as unsuitable technique due to poor flow properties of powders. Drug release from both freshly produced GB and GPS EX, GMT and DCMT was affected by the MPT loading.Increasing the loading (from 20% up to 40%) led into faster drug delivery, supposedly due to increase in system's hydrophilicity and formation of more open pore structure and thus more rapid MPT leaching in the medium. Formulations produced by melting technology (EX and GMT) provided increased embedment of the drug in the hydrophobic carrier and thus provided slower extended release pattern compared to formulations made by direct compression (DCMT). EX and GMT formulations based on 20% MPT were chosen for further studies due to most suitable prolonged drug release outcome over 24 hours. DCMT was not selected as further platform due to abovementioned problems with poor flow properties and the low extent of drug release retardation. When comparing the delivery of 20% MPT form either GB or GPS units, GBEX and GB GMT matrices gave similar delivery patterns between themselves (square root of time pattern, $f2$=72) while in case of GPS EX and GPS GMT release outcomes were versatile with EX sample showing specific sigmoidal release fashion and GMT square root of time release profile ($f2$=28). The sigmoidal outcome could be related to the appearance of ''wall depletion'' effect, where due to shearing appearance at the inner surface of the extruder MPT is emerging towards the extrudateinterior leaving very thin layer containing mainly glyceride which behaves as a diffusion barrier and thus limits drug release just from the cut sides of the matrix surface. During dissolution GPS EX cracks (after 8 h) which provides faster delivery rate and thus appearance of sigmoidal release pattern. GB EX and GB GMT showed higher porosity and thus faster drug release compared to the same units based on GPS. Porosity of GB EX is probably high enough to overcome the ''wall depletion'' effect. GB EX has more porous and less dense surface appearance compared to GPS EX. Milling the EX breaks the thin glyceride layer and thus disable its action as diffusion barrier and results in faster release profile of GPS GMT sample. Solid state properties (thermal and diffractional) of freshly produced matrices were slightly different than the ones of physical mixtures and also pointed partial solubilisation of MPT in the molten glyceride. Raman mapping and ATR-FTIR measurements indicated uniform drug distribution through either GB or GPS matrices and no significant interactions between the drug and used glycerides, respectively. Increasing the size of the EX from 3mm to 5mm in both GB and GPS samples led into decreased delivery rate due to increased diffusion route and decreased specific surface area of the units. Increasing the size of the granules in GB GMT led to faster release pattern of GB GMT L compared to GB GMT S due to higher porosity and larger pores of first ones compared to last ones. GPS GMT showed in general lower porosity compared to GB GMT and thus probably better compactibility of GPS granulate, which minimized the impact of the particle size on the drug delivery profile. GB units were insignificantly affected by the pH of the dissolution medium and presence of biorelevant compounds while GPS samples demonstrated faster delivery rate in phosphate buffer pH 6.8 compared to 0.1 M HCl and in FESSIF compared to blank FESSIF. Glycerides having higher acid number (GPS ≤ 6mg KOH/g vs GB ≤ 4mg KOH/g) and shorter fatty acid chains (GPS: C16 and C18 vs GB: C22) tend to exhibit increased ionization at phosphate buffer pH (decreasing hydrophobicity) and are more prone to lypolisis respectively, therefore giving slightly faster drug release outcome. Storing of GB units and GPS units at increased temperature for two months provoked changes in glyceride's solid state properties (GB – thermal changes: increased enthalpy and melting point maximum; GPS –thermal changes: increased enthalpy and peak maximum and change in the peak appearance; x-ray pattern changes), which was related to the alterations seen in the unit's drug release outcome after storage. GB units gave slower drug release probably due to blooming effect, while GPS units gave faster release probably due to increase in unit's porosity after storage. Storing units at room conditions affected only GPS samples,leading to faster drug release compared to freshly prepared units.Drug release changes were again connected to changes in the solid state properties of the glyceride. After thermal processing glycerides appear in a layered forms with lower crystallinityand are with time and other factors (increased temperature) transformed to a more stable state. Glycerides composed of longer fatty acid chains such as GB tend to change their crystalline form slower and at more extreme environment conditions, while glycerides based on shorter fatty acid chains such as GPS are prone to faster crystal alterations already at less extreme conditions.

Conclusion

Obtained results outline GB as more reliable matrix former for developing extended release mini-matrices compared to GPS. HME appeared as robust and reproducible process for mini-matrix production. However, understanding of material features and, HME equipment, process optimization and coupling with suitable down-stream processing are prerequisites for successful introduction of this technique as a viable alternative to the compression of mini-tablets.

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Macedonian pharmaceutical bulletin, **62** (suppl) 343 - 344 (2016)
ISSN 1409 - 8695
UDC: 615.222.015.153
Short communication

Formulation of chronotherapeutic delivery systems for delayed release of verapamil hydrochloride using polyethylene oxide polymers

Sanja Bundalo[1,2]*, Jelena Đuriš[1], Svetlana Ibrić[1], Zorica Đurić[1]

[1]*Department of Pharmaceutical Technology and Cosmetology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia*
[2]*University Clinical Centre of Republic of Srpska, Dvanaest beba bb, 78000 Banja Luka, Bosnia and Herzegovina*

Introduction

Chronotherapy refers to a treatment method in which in vivo drug availability is timed to match rhythms of disease in order to optimize therapeutic outcomes and minimize side effects (Sajan et al, 2009). The pulsatile drug delivery system is intended to deliver a rapid drug release after a predetermined lag time (Kalantzi et al., 2009). Simple and inexpensive production methods, such as compression-coating of tablets, might be effective for these formulations to gain widespread use. Compression-coated tablets are composed of an inner core (immediate release tablet) which is embedded in an outer coating shell containing either a hydrophilic, hydrophobic or a mixture of both polymers, that dissolves or disintegrates slowly to produce the lag time. The outer layer surrounds the inner core, therefore selection of the outer layer materials has a significant impact on the performance of the tablet, including the mechanical strength, drug release characteristics, and tablet stability (Lin and Kawashima, 2012).

The possibility of formulating compression-coated tablets of verapamil hydrochloride was examined using polyethylene oxide (PEO) as polymer in the outer layer. The influence of different types and concentrations of polyethylene oxides (PEOs) on the drug release rate was investigated. Verapamil hydrochloride was selected as the model drug since the symptoms of hypertension are more prevalent during the early hours of the morning and development of chronotherapeutic formulation could be useful to fulfill the needs of drug delivery at the required time.

Materials and Methods

Materials

Different grades of polyethylene oxide polymers – Polyox® WSR 1105 (Mr ~ 0.9 x 106) and Polyox® WSR 301 (Mr ~ 7 x 106) were kindly provided by the manufacturer (Dow Chemical Company, USA). Verapamil Hydrochloride (Ph. Eur. 8.0), direct compression excipient based on coprocessed lactose and polyvinylpyrrolidone (Ludipress®, BASF AG, Germany), and anhydrous colloidal silicon dioxide (AEROSIL® 200 Pharma, EVONIK Industries AG, Germany) were used for the tablets preparation.

Methods

Preparation of tablet cores and compression-coated tablets

The inner cores of the compression-coated tablets were prepared by direct compression on an eccentric tableting machine (Korsch EK-0, Germany) using 9 mm concave punches. Tableting blend consisted of verapamil hydrochloride (30% w/w), anhydrous colloidal silicon dioxide (1% w/w) and Ludipress® as a diluent.

Compression-coated tablets consisted of the core tablet and the PEO polymer (Polyox® WSR 1105 or Polyox® WSR 301) as the outer coating agent. F 1 and F 2 batches were prepared using Polyox® WSR 1105 with core: polymer at the following ratios 1:1 and 1:2, respectively. Batch-

* sanja.bund@gmail.com

es P 1 and P 2 were prepared using Polyox® WSR 301 with core: polymer at the same ratios 1:1 and 1:2, respectively. Firstly, half of the amount of the polymer (outer layer) was placed in the die to make a powder bed. Then, the core tablet was carefully placed in the centre of the die and the remaining half of the polymer was filled into the die. The contents were then compressed using the eccentric tableting machine (Korsch EK-0, Germany) to form a flat-faced tablet with a diameter of 12 mm.

In vitro drug release studies

The prepared core and compression coated tablets were subjected to in-vitro drug release studies in suitable dissolution media to assess their ability to provide the desired release. The *in-vitro* drug release study of the core tablets was performed in the rotating paddle apparatus (Erweka DT600, Germany). The dissolution medium was 900 mL of phosphate buffer (pH=6.8) maintained at temperature 37 ± 0.5 °C, and the rotating paddle speed was 50 rpm. The in-vitro drug release study of the compression coated tablets was performed in the reciprocating cylinder apparatus (VanKel's Bio-Dis, USA). The dissolution medium was 250 mL of 0.1 N HCl for the first 2 h followed by 6.8 pH phosphate buffer for the remaining period. Temperature of the medium was maintained at 37 ± 0.5 °C, and the dpm rate was set at 10 dpm.

In all experiments, an aliquot of 5 mL sample was withdrawn at the predetermined time intervals and replaced with an equal volume of drug-free dissolution fluid in order to maintain the sink conditions. The samples were filtered and analyzed spectrophotometrically at 273 nm by using UV/VIS spectrophotometer Evolution 300 (Thermo Fisher Scientific, Cambridge, UK). The amounts released were expressed as a percentage of the drug content.

Results and discussion

The prepared core tablets showed immediate release with more than 80% of the drug released in less than 15 minutes. With compressed-coated tablets, different release patterns were obtained using either different polymer type or polymer concentration. It was observed that the lag time increased as the concentration of the PEO in the outer coat increased. For instance, formulation F 1 had a lag time of 2 hrs before burst release. On the other hand, F 2 had lag time of 4 hrs. For batches P 1 and P 2, the results indicated that that the burst release of the drug occurred at 3 and 6 hrs, respectively. This also confirms that the lag time increased as the concentration of the PEO in the outer coat increased, but this increase were different with different types of polymers. An increased molecular weight leads to increase in polymer chain length and greater degree of chain entanglement, therefore stronger gel layers are formed in contact with water (Colorcon, 2009). Stronger gel layer with the greater viscosity decreases drug diffusion rate and water diffusion within the core tablet which consequently delays the drug release. Lower molecular weight PEOs form weaker gel layer, which is more susceptible to erosion. There is no general rule for selection of the appropriate polymer concentration that should be optimized taking into account the formulation composition, polymer molecular weight and drug solubility.

Conclusion

In the present investigation, compression-coated tablets with Polyox® WSR 1105 and Polyox® WSR 301 in outer layer showed delayed-release, and drug release from formulated tablets showed the dependence on both polymer concentration and its molecular weight. Further optimization of the Polyox® WSR 1105 and Polyox® WSR 301 coated tablets could produce the desired predetermined lag time.

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Macedonian pharmaceutical bulletin, **62** (suppl) 345 - 346 (2016)
ISSN 1409 - 8695
UDC: 615.015.4
Short communication

The role of cocrystallization screening for the assessment of structure-activity relationship in drug development

Aleksandar Cvetkovski*, Bistra Angelovska

Faculty of Medical Sciences, University "GoceDelcev", Krste Misirkov 10-A, P.O 201, 2000 Stip, Republic of Macedonia

Introduction

The selection of the crystalline phases in a form of molecular co-crystals has become scientific challenge at the early stage of drug development of pharmaceutical formulations and in the late stage of synthesis and isolation of active pharmaceutical ingredients (APIs) in desirable defined crystalline forms. Optimal crystal form of API interactively interrelates and impacts its aqueous solubility and dissolution rate that are benchmark for drug delivery and absorption determining the extent of its bioavailability and pharmacokinetics profile. Determining the crystal structure and revealing the crystal packing forces and geometry of the API has impact its physicochemical properties. This approach is the criteria for assessment of the performance of the API. The range of crystal forms in which molecular co-crystals of APIs may exist is advantageous comparing to their polymorphs, salts, solvates and hydrates due to the vast number of potential co-formers which extend the limited counterions for salt formation implying the existence of more complex intermolecular interactions based on different H-bonding patterns with API that lead to conformational changes and flexibility for crystal packing in process of co-crystallization.

Co-crystallization became well known bottom-up approach starting from intermolecular interactions among either selected neutral, ionic or zwitterionic molecules to design and control the properties of the multicomponent crystals (Braga, 2004). In the scope of interest for drug design and formulation, Good et al. (2009) and Cheney et al. (2011) emphasized that the main advantage for designing pharmaceutical co-crystals (PCCs) is, through their modulating properties, to improve the performance of the native APIs such are: biopharmaceutical profile (solubility and dissolution rate), thermodynamical stability (phase transition of polymorphs, solvate/ hydrate formation, decomposition) or bulk powder processability (flowability, compressibility, particle size and shape control). Childs et al. (2007) has pointed out the necessity co-crystals (CCs) semantically to be classify based on accomplishments in research of supramolecular chemistry. This approach enlightens the complex reality of multi-component systems and the wide scope associated between salts and co-crystals, and their differences based on the location of the transferred proton within the salt - co-crystal continuum.

Biguanide drugs are well known and wide used oral antidiabetic drugs for oral therapy of diabetes type-2 that directly improve insulin action. Recent studies in the research work carried out by Vujic et al. (2015) has pointed out that biguanides in combination with targeted inhibitors in order to obtain synergy in reduction cell viability, inhibited tumor growth in the mutated neuroblastoma rat sarcoma oncogene (NRAS) protein from melanoma cells. Hence, it is expected that combination of biguanides which affect activation of the AMP-activated protein kinases (AMPK) and the regulation of energy metabolism with outcome to cell's energy sparing, in combination with other anti-cancer drug-models would influence direct blocking cell's signaling and hinder the resistance.

Materials and methods

Co-crystallization screening reveals protocol was undertaken in order to grown single crystalline phases of PCCs composed of drug model metformin (MET), selected from biguanides class of drugs and coformers that belong to different pharmacotherapy and functional group classes, respectively.

Co-crystallization screening was carried out on applying slow-rate solvent evaporation method for growing sin-

* aleksandar.cvetkovski@ugd.edu.mk

gle crystalline phases at room temperature with quality absolute structure to be determined. An equimolar quantity of MET and co-crystal partner was dissolved in the minimum quantity of ethanol and left for slow evaporation at room temperature. Colorless crystals were observed after a few days.

For four MET PCC models, the methods of preparation reproducible batches were optimized. The quality of batches was controlled by Powder x-ray diffractometer comparing the obtained experimental diffractograms with the same one that was theoretically generated from the single crystal for each of four PCC models.

Single-crystal diffraction data were collected on a Nonius Kappa diffractometer equipped with a CCD detector with graphite-monochromatized MoKα radiation (λ = 0.71069 Å). Intensities were corrected for Lorentz and polarization effects. The structures were solved by direct methods with the SIR97 suite of programs and refinement were performed on F2 by full-matrix least-squares methods with all non-hydrogen atoms anisotropic.

Flow-cytometry was applied for measuring viability of the two PCC models.

Results and discussion

Vujic et al. (2015) has carried out research for both pro-cancer and anti-cancer effects of biguanides on cancer cells, indicating existence of association of the antidiabetic therapy and reduced risk of cancer in diabetic patients. Because biguanide represents the π-conjugated system, MET can exist in three resonance-stabilized forms, i.e. as neutral molecule (MET), monoprotonated (MET+) or diprotonated (MET2+) cation, with dissociation constants in water in range from pKa1 ≈ 12.00 to pKa2 ≈ 2.00.

A search of the biguanide fragments in the structural literature, both in the CCDC (Cambridge Crystallography Database Center) database and in patents, shows that in crystals MET exists as monoprotonated (MET+) or deprotonated (MET2+) but never in its neutral form MET.

We have undertaken a systematic study of the crystal chemistry of MET with the aim of understanding its properties in the solid state and finding relationships with its biopharmaceutical profile. We have determined the structures of the 29 MET PCCs. Four of this MET PCC models are "drug-drug" type of co-crystals. The ligand used for co-crystallyzation was from the following classes: inorganic acids (nitric, phosphoric and carbonic acid); organic NH-type acids (saccharine and acesulfame); organic OH-type acids (squaric and picric acid); monocarboxylic acids (fumaric, acetic, trifluoroacetic, trichloroacetic, dichloroacetic, monochloroacetic, glycolic, salicylic, diclofenac) and dicarboxylic acids (oxalic, malonic, maleic, fumaric, succinic, adipic acid).

Conclusion

In the paper are presented structure analyses for "drug-drug" type of PCCs where both API and CF exhibit pharmacological effect. This approach of designing "drug-drug" type of PCC aligned to the strategy for drug repositioning, the idea for use of a drug for treating diseases other than the drug-specified. This concept was prompted in 2012 through the Discovering New Uses for Existing Molecules program, initiated by US's National Institute of Health (NIH).

The case study underlines the crystal growth and the method of preparation for "drug-drug" type of PCCs wherein two different APIs cocrystallized in single crystal cell, and that represent new paradigm for approaching in development of "fixed-doses" or "combo" pharmaceutical formulations. Preliminary results of the Structure-Activity Relationship study on the co-crystals composed of MET with dichloroacetic acid indicate dual and complementary anti-cancer activities of the two selected drug models for co-crystallization.

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Macedonian pharmaceutical bulletin, **62** (suppl) 347 - 348 (2016)
ISSN 1409 - 8695
UDC: 615.373
Short communication

Recombinant monoclonal antibody rituximab – medical uses and structural characterization

Dashnor Nebija[1]*, Christian Noe[2], Bodo Lachmann[2], Kristina Mladenovska[3], Arlinda Daka[3], Pranvera Breznica[3]

[1]*Faculty of Medicine, University of Prishtina, Bulevardi i Deshmoreve 10000 Prishtina, Kosovo*
[2]*Faculty of Life Sciences, University of Vienna, Althanstrasse, 14, 1090 Vienna, Austria*
[3]*Faculty of Pharmacy, University "Ss Cyril and Methodius", "Mother Theresa" 47, 1000 Skopje, Republic of Macedonia*

Introduction

This paper addresses current topics related to CD-20 depleting agent rituximab, which has been widely used as a targeted therapy in the fields of oncology and rheumatology. Apart from its structure and function, relevant issues regarding the analytical methodologies applied for the assessment of the quality of this complex drug will be highlighted as well.

Rituximab is the first approved targeted biologic therapy for the treatment of haematological malignancies and it took very short time from its first description until the approval and clinical application. In USA it was approved in 1997, whereas in EU in 1998. Approved clinical indications of rituximab include non-Hodgkin's lymphoma, chronic lymphocytic leukaemia, rheumatoid arthritis and granulomatosis with polyangiitis and microscopic polyangiitis (European Medicines Agency-EMA, 2016). Since the patents on MabThera and Rituxan expire in 2016 in USA and have already expired (2013) in EU, there are some biosimilars of rituximab in the late stages of clinical trials developed by companies worldwide, whereas 'non-originator biologicals' of rituximab are approved in some South American and Asia countries (GaBI, 2016). Pharmaceutical formulation (Rituxan or Mabthera) is administered intravenously and supplied at a rituximab concentration of 10 mg/ml in either 10 ml or 50 ml single-use vials.

Structure – activity relationship

Rituximab is genetically engineered chimeric monoclonal antibody (mAb) that is produced in mammalian cell culture using Chinese hamster ovary cells. This IgG1antibody contains murine light and heavy chain variable regions, and human gamma 1 heavy chain and kappa light chain constant regions. During the chimerization process, murine variable domain of heavy and light chain was cloned into the human IgG1 immunoglobulin framework. In the chimerised version only variable domains have murine origin (around 30%). The rest of molecule is human (around 70%). Rituximab binds specifically to the antigen CD20, which is found on the surface of normal and malignant B-lymphocytes (Reff et al., 1994), which in addition play a role in the pathogenesis of rheumatoid arthritis. The Fab domain of rituximab binds to the CD20 antigen on B lymphocytes, and the Fc domain recruits immune effector functions to mediate B-cell lysis through complement-dependent cytotoxicity (CDC), antibody-dependent cell mediated cytotoxicity (ADCC) and apoptosis.

As all biological drugs, rituximab has very complex nature, it contains 1328 amino acids with theoretical Mw of non- glycosylated form 144.54 kDa. It contains two identical heavy and two identical light chains linked via disulfide bridges. Each heavy chain contains 451 amino acids (49.2 kDa) and pI 8.67, whereas each light chain contains 213 amino acids (23.06.kDa) and pI 8.26. The apparent Mw of rituximab is higher than 144.54 kDa, due to the presence of N-linked oligosaccharides attached in the N-glycosilation site at conserved asparagines N-301. Variable regions of both chains comprise complementarity determining regions (CDR1-3), being responsible for antigen recognition and binding. On the other hand, human Fc γ1 (IgG1) fragment is responsible for ADCC and CDC. Heavy chains are linked *via* two interchain disulfide bonds at the flexible hinge region, while heavy and light chains are linked with a disulfide bridge.

* dashnor.nebija@uni-pr.edu

Structural characterization

As all biopharmaceuticals, especially mAbs, rituximab is extremely complex and heterogeneous drug. The main source of the antibody heterogeneity results from differences in protein glycosylation which is one of the most common post-translational modifications of proteins produced in eukaryotic cells although, primary structure variations, deamination, deamidation, lysine truncation, methionine oxidation, sulfation and phosphorilation can create additional IgG variants. The characterization and stability study of rituximab are extensive and require a battery of orthogonal techniques as amino acid analysis, amino-terminal sequence analysis, peptide mapping, and analysis of oligosaccharides, ion-exchange chromatography (IEC), cIEF, SDS-PAGE, and circular dichroism (CD), UV spectroscopy, size exclusion chromatography (SEC), cellular mediated cytotoxicity (CMC). The oligosaccharide structure was investigated by CZE and MALDI-TOF after isolation from HPAEC-PAD (European Medicines Agency-EMA, 2016). Two-dimensional gel electrophoresis (2-DE) complemented with MALDI TOF MS analysis was used for the characterization of identity, purity and structural integrity of rituximab. Experimental data revealed typical migration behavior of mAbs, resulting in poorly resolved spots with different isoelectric points and very small differences in Mw. Heavy chains migrated at about 50 kDa and light chains at about 23 kDa. After tryptic digestion of 2-DE separated proteins, peptide mass fingerprinting analysis was also used for the identification of rituximab heavy and light chains (Nebija et al., 2011). Glycosylation of IgG is critical for effector functions including complement fixation, Fc receptor binding on macrophages and ADCC. In addition clearance of IgG-antigen complexes from circulation is influenced by IgG glycosylation. It was shown that predominantly N-linked oligosaccharides present in rituximab belong to asialo, neutral complex biantennary oligosaccharide type different terminal galactose residues, whereas terminal sialylated oligosaccharides are present only in minor amounts (Kamoda et al., 2004).

Manufacturer of pharmaceutical product MabThera® as primary assay for characterization and lot release of N-linked glycans on glycoprotein drugs employed CE-LIF. A method for direct characterization of glycans using CE-LIF/MS has been reported, as well. Apart from major glycan components this method allowed accurate identification of minor glycans such are asialo- and afucosylated species. Since the fucosilation affects biological activity and sialylation affects the pharmacokinetics of glycoprotein drugs, the identification of these species is of particular significance for the characterization of rmAbs. Charged variants of rmAbs can be studied with different methods such as IEC, IEF, CZE. A simple and rapid method for determination of relative amounts of rituximab glycoforms differing in terminal galactose was reported. Most abundant ions corresponding to the glycoforms found on the rituximab heavy chain were monitored by mass selective detection in the selected-ion monitoring mode (Nebija et al., 2011).

Lot release testing of the pharmaceutical product Rituxan included physicochemical and biological methods, such as SDS-PAGE, peptide mapping, fragment IEC-HPLC, SECHPLC, glycan content, cIEF, CDC, UV-VIS. Validated human CDC assay was used for the determination of potency. Galactose content on the heavy chain oligosaccharide was found to be critical parameter for biological activity, therefore another release test for glycan content was developed (CE-LIF). cIEF was used to positively identify rituximab from other recombinant mAbs made by the manufacturer (US FDA CDER, 2016; Zhang et al., 2016).

Conclusion

As a conclusion, since rituximab belongs to rmAbs, class of drugs obtained by rDNA technology, biological processes are involved in its production. Therefore it demonstrates high degree of inherent heterogeneity and complexity and the combination of different techniques should be used for the extensive characterization of its quality attributes, including identity, structural integrity, purity and stability. This is of primary importance for the safety and efficacy of this class of drugs. In this regard, it was shown that gel electrophoresis and peptide mass fingerprinting analysis may represent an important strategy for the assessment of the quality of rituximab and other biopharmaceuticals.

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Macedonian pharmaceutical bulletin, **62** (suppl) 349 - 350 (2016)
ISSN 1409 - 8695
UDC: 615.262.2(410)
Short communication

Comparison of emollient efficacy - a single centre, randomised, double-blind, bi-lateral comparison of two emollients prescribed in the UK for the management of dry skin conditions such as atopic eczema

Jasmina Gallagher*, Phil Rosher

Dermal Laboratories Ltd, Tatmore Pl/Preston Rd, Hitchin SG4 7QR, United Kingdom

Introduction

In the UK, the National Institute of Clinical Excellence (NICE) clinical guidance on atopic eczema management in children from birth to the age of 12 years establishes emollient therapy as the treatment modality that should underpin all else (NICE quick reference guide, 2007). The guidance recommends continuing emollient therapy even when the skin appears healthy. Healthcare professionals are advised to offer children with atopic eczema a regime of 'complete' emollient therapy involving a choice of non perfumed emollients to use every day, both as leave on moisturisers and as soap substitutes for routine washing and bathing.

Emollient formulations suitable for such use typically contain both oily occlusive substances, such as petrolatum, paraffin or mineral oil, which form a water impermeable film over the skin to decrease evaporation of physiological water from beneath, and humectant substances, such as glycerol and urea, which attract water to the skin (Cork, 2007; Loden, 2003; Rawlings et al., 2004; Watkins, 2008). In order to encourage treatment concordance, however, it is also crucially important that these products are formulated in such a way that their physical characteristics render them appealing for patients to use over large surface areas and for long periods (Cork et al., 2003).

Ideally, emollients should exert their skin softening and moisturising effects within the upper layers of the skin. Thick, greasy ointments undoubtedly exhibit good emollient characteristics, but are not very popular with patients because they are not well absorbed into the skin and leave an oily residue which can feel uncomfortable and has a tendency to soil clothing and bed linen (Cork, 2007; Sidbury and Poorsattar, 2006). More popular emollients are formulated as oil-in-water creams or lotions to make them 'feel' lightweight and to encourage better absorption into the skin (Ersser et al., 2007). However, these dosage forms are less effective than ointments owing to their lower oil content, resulting in reduced occlusive capabilities (Clark, 2004) Moreover, cream and lotion formulations exhibit poor substantivity and have a tendency to be easily rubbed off onto clothing, so they necessitate more frequent re-application than would be practicable for many patients. Emollient gels are alternative pharmaceutical presentations for atopic eczema sufferers and usually contain high concentrations of oily ingredients in semi-solid aqueous systems. To achieve maximum benefit, some of these gels, just like other emollients, need to be applied regularly and frequently.

Performance evaluation methods for emollients and moisturisers are mainly focused on sensory aspects, skin visual appearance, perceived efficacy, and measurements of skin barrier hydration and integrity (Rawlings et al., 2004). However, few studies have involved patients directly comparing the effectiveness of different emollients by using them concurrently or under conditions mimicking normal therapeutic use (Clark, 2004; Simpson, 2006).

Consequently, the aim of this study was therefore to compare the effects on skin hydration of two emollients prescribed in the UK, DELP gel and ZB cream, and using a dosage regimen consistent with most patients' practical circumstances which limits their use of emollients to twice daily only.

* jasmina.gallagher@dermal.co.uk

Materials and methods

This was a single centre, randomised, double blind, concurrent bi-lateral (within-patient) comparison in 18 females with atopic eczema and dry skin of similar severity on their lower legs. Following 7 days' run-in with no use of emollients or moisturisers on the lower legs, DELP gel and ZB cream were each applied to one lower leg twice daily (approximately at 9am and 9pm) for 4 days and on the morning only on day 5. Washing of the lower legs was permitted only during the evening on days 2 and 4. The efficacy of both products was assessed by hydration measurements using a Corneometer CM825 probe (Courage-Khazaka electronic). The measurements were made on days 1 to 5 at approximately 9am immediately prior to the first daily application (the measurement on day 1 being baseline), and around 1pm and 5pm. The primary efficacy variable was the area under the curve (AUC) of the change from baseline corneometer readings over the 5 days.

Results and discussion

The two emollients showed very different effects on skin hydration. The AUC for DELP gel significantly outperformed ZB cream. For DELP gel, the skin hydration effect was substantial, long lasting and cumulative, with the readings each day generally increasing over the treatment period. Even the morning readings on days 3 and 5, following washing the previous evening, were significantly better than baseline. In contrast, for ZB cream, skin hydration was not significantly different from baseline at any time point.

Conclusion

This study, performed by subjects with atopic eczema and dry skin using a dosage regimen simulating normal/practical use, has demonstrated very significant performance differences between two marketed emollients. Whereas DELP gel achieved substantial, long lasting and cumulative skin hydration, ZB cream achieved no measurable improvement compared to before treatment. Healthcare professionals should be aware of this when prescribing these products.

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Macedonian pharmaceutical bulletin, **62** (suppl) 351 - 352
(2016)
ISSN 1409 - 8695
UDC: 616.5-003.829-085.26
Short communication

Implementation of mexametry in periorbital hyperpigmentations studies

Dragomirescu Anca Octavia*, Simu Georgeta-Maria, Dehelean Cristina

*"Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy,
2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania*

Introduction

Usually known as Periorbital Hyperpigmentation (POH), this deficiency seems to have in literature many other denominations, such as: Idiopatic Cutaneous Hyperchromia in the Orbital Region, Infraorbital dark circles, Cutaneous idiopatic hypercromia of the orbital region or Periorbital melanosis. The lack of a unique denomination should not be a serious problem, but behind these inconsistencies are usually some different approaches concerning the ethiopathogeny, as well as the targeted pathogenic treatment by default.

The literature data originating from European authors, as well as some articles of North Americans ones include the periorbital hypercolorations into the chronologic aging process, with implications resulted from vascular and sanguine deficiencies. In other words, it is considered that this deficiency is not necessarily originating from a genetic anomaly. This point of view is due to the fact that most of the subjects from this geographic area are of pale or fair skin phototype (I, II or II phototypes). According to Suppa et al. (2011) the determinants of periorbital pigmentations and of the skin ageing processes are: UV exposure, smoking, vascular problems. Lupo et al. (2011) consider the intrinsic aging role in the periorbital hyperpigmentations pathogeny and suggest a new topical treatment based on human growth factors, cytokines, caffeine and glycyrrhetinic acid.

On the other hand, literature data attributed to Asian or South American authors indicate that the primary cause of periorbital hypercolorations pathogeny should be rather melanic, constitutional, and they refer to as subocular melanosis. This is probably due to the dark skin phototype (IV, V and VI phototypes), specific in these geographic areas. Some authors point-out the post-inflammatory pigmentations' role onto color intensification of this pathogeny, while in other situations it is exclusively assigned to the melanic cause of this process (Kanika and Kassir, 2013). In the same time, in the case of subjects with dark skin phototype, it was stipulated that the main causes of periorbital hyperpigmentation could be melanic, without excluding the aging specific endogeneous processes as aggravating factors.

Corroborating these different approaches, one could consider that the main determinants of periorbital hyperpigmentations, for any skin phototype could be: the genetic melanic pigmentation, the post-inflammatory hyperpigmentation, a tegument periorbital atrophy due to chronoaging, the venous congestion, hemoglobin and oxygenated hemoglobin accumulations, hemosiderine deposit, as well as some other anatomic causes.

Materials and methods

The chemicals used in this study were of high purity grade. Acetyl tetrapeptide-5 (trade name EYESERYL®) was obtained as a gift from Lipotec S.A. (Isaac Peral, 17 Pol. Ind. Camí Ral; E-08850 Gavà Barcelona, SPAIN) as 0.1% aqueous solution. An o/w microemulsion was used as vehicle for the transport of acetyl tetrapeptide-5 through the skin. The main ingredients of the microemulsion were: deionised water, glycerol, liquid paraffin, vaseline, jellifying and lubricant mineral agents, emulsifying agent (Montanov L), preservative agents, and acetyl tetrapeptide-5. The rheological properties of the microemulsion were assessed using the Rheometer RC1 (Rheotec), and two type of probes: CC8 and C25-1.

The study was carried-on on 2 test groups of healthy women volunteers aged of 22 to 64 years. The first group applied on both (right and left) eyes hemispheres the o/w

* ancadrag65@yahoo.com

emulsion containing 0.1% peptidic complex, twice daily, during 60 days. The second group (control) applied the cosmetic emulsion containing 0.1% acetyl tetrapeptide-5 only on the right eye suborbital area, while the left eye was treated with the simple cosmetic formulation. The effects of the cosmetic emulsion were evaluated every 15 days, both by clinical and instrumental methods. The instrumental evaluation was performed by Mexametry, using the MX 18 Mexameter (Courage-Khazaka), which is able to measure two components, mainly responsible for the color of the skin: melanin and haemoglobin (erythema).

The aim of the present work was to study in vivo the efficiency of the acetyl tetrapeptide-5 in the treatment of periorbital hyperpigmentations and eye puffiness, using mexametry as one of the most recent non-invasive method of evaluation of the coloration's intensity of the sub-orbital area.

Results and discussion

In our study, the melanin parameter was excluded due to the fact that acetyl tetrapeptide-5 is not considered as a melanic depigmentant, lacking any tyrosine inhibitor in this composition. Considering that periorbital hyperpigmentations are dependent by individual metabolism, the relevant parameter used as a measure of suborbital coloration was the erythem one.

In the case of the control group, a significant reduction of the erythem values was obtained for the right eye, while for the left eye, the reduction of the erythem corresponding values was quite slight, or (in some cases) none, even at the end of the eight weeks of treatment. In the case of the study group which applied the o/w cosmetic emulsion containg the peptidic complex on both eyes hemispheres, significant reduction of the erythem values was recorded, in some cases even after 15 days of treatment. For this group, 25% of the volunteers showed a slight reduction, 30% a fairly good reduction, and 40% a good one.

Conclusion

In conclusion, mexametry was successfully used as instrumental non-invasive method for the investigation of periorbital hyperpigmentations as consequence of a specific cosmeceutic treatment. The obtained results showed that the cosmetic formulation containing different amounts of acetyl tetrapeptide-5 was well tolerated at the tegument level and visible results were obtained, even after only 15 days of treatment. Moreover, 95 % of the volunteers showed a significant reduction of the periorbital hyperpigmentations and eye puffiness after the treatment with the cosmetic preparation containing acetyl tetrapeptide-5.

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Macedonian pharmaceutical bulletin, **62** (suppl) 353 - 354 (2016)
ISSN 1409 - 8695
UDC: 615.26:577.115
Short communication

A novel natural mixed emulsifier of alkyl polyglucoside type as liposome and skin-friendly cosmetic ingredient

Mila Filipović[1]*, Milica Lukić[2], Sanela Đorđević[2], Gordana Vuleta[2], Snežana Savić[2]

[1]Higher Education School of Professional Health Studies, Cara Dušana 254, 11080 Belgrade, Serbia;
[2]University of Belgrade-Faculty of Pharmacy, Department of Pharmaceutical Technology and Cosmetology, Vojvode Stepe 450, 11221 Belgrade, Serbia

Introduction

Liposomes are phospholipid-based vesicular carriers that still attract great attention of formulators. However, the selection of an appropriate carrier for liposomes remains one of the most important issues. Although, gels are still considered as the most suitable bases for liposomes (Kulkarni, 2005), they are usually not appropriate, neither for the long-term care nor for all skin types. Therefore, emulsion systems are believed to be more convenient carriers. On the other hand, formulation of an emulsion with liposomes entails another problem: selection of suitable emulsifier(s) necessary for system stabilization, considering the fact that emulsifier may interfere with the mechanical stability of vesicles and may enhance propensity for fusion or lead to their solubilization (Kulkarni, 2005). An interesting and highly promising group of novel emulsifiers which could be used for development of emulsion carriers for liposomes are alkyl polyglucosides (APGs). Due to their favorable toxicological and dermatological properties they are considered as environment- and skin-friendly (Lukic et al., 2013).

Therefore, our aim was to investigated whether a novel APG emulsifier, hydroxystearyl alcohol & hydroxystearyl glucoside, could be considered as an appropriate stabilizer for emulsions containing liposomes. To accomplish this, a model emulsion carrier with liposomes was formulated and characterized first. Additionally, the in vivo irritation potential of the developed formulation was assessed, as a certain aspect of safety of the used emulsifier.

Materials and methods

Hydroxystearyl alcohol & hydroxystearyl glucoside was used for samples' preparation with ARSC-liposomes-stem cells of alpine rose leaves in liposomes as an active.

Two oil-in-water (o/w) creams (placebo Fp and active cream F1a containing 0.4% (w/w) of ARSC-liposomes) were developed and characterized by means of: polarization microscopy, rheology, differential scanning calorimetry and thermogravimetric analysis as described (Lukic et al., 2013). Afterwards, an in vivo study with investigated creams and commercially available cream containing the same active was conducted. Skin hydration (EC), transepidermal water loss (TEWL), and erythema index (EI) were measured in accordance with the Declaration of Helsinki and relevant guidelines on 16 healthy volunteers.

Results and discussion

Micrographs taken after 7 days revealed randomly distributed distorted Maltese crosses and birefringence at the oil droplets border ("onion rings") within both samples (Fp and F1a), indicating lyotropic interaction of lamellar type (Lukic et al., 2013) and remained relatively unaltered after 30 days. However, obtained micrographs showed the noticeable difference in the colloidal structure between the placebo Fp and the active cream F1a. Considering that liposomes are also visible under polarization microscopy, it seems that liposome-similar structures could be observed near the lamellar phase gel network i.e. close to the "onion rings" in the continual aqueous phase. Intact vesicles were apparently immobilized within the network of lamellar phase of the system and as such are mechanically stabilized, hence their interaction and fusion are limited.

* millatrbovic@hotmail.com

Continual rheology has shown that both samples exhibited "shear-thinning" (pseudoplastic) flow behavior with pronounced thixotropy. The obtained flow curves and calculated hysteresis area values showed that the addition of the 0.4% (w/w) of ARSC-liposomes has led to a slight increase in viscosity and thickening of the placebo cream, but rheological behavior of both creams stayed rather similar. Oscillation frequency sweep test has shown prevalence of the elastic over the viscous component as the one of the general characteristics of lamellar phase (Lukic et al., 2013) for both samples. Active sample (F1a) had higher elastic-storage modulus (G') and viscous-loss modulus (G'') compared to the placebo (Fp) which is in accordance with the result of continual rheological measurements. Increments in all the assessed rheological parameters upon the addition of ARSC-liposomes, could be due to lamellar phase promotion by the cosmetic active and the change in orientation of the lamellae under shear stress or due to the presence of intact liposomes themselves.

Thermal behavior of the investigated samples (Fp, F1a) was similar in terms of the shape of the obtained profiles with one marked peak. The addition of the liposome-encapsulated active (sample F1a) induces a subtle shift of the curve towards higher peak temperature values. Although such results may imply the promotion of the lamellar phase (Lukic et al., 2013), considering the polarization micrographs and the rheology results, it is more likely that the detected thermal behavior is due to ARSC-liposomes themselves.

Regarding TGA results, upon the addition of the active, amounts of the lost water decreased in the first temperature range (25-50 °C, corresponds to the free water in the system) and increased in the third temperature range (70-110 °C, corresponds to interlamellar water) (Lukic et al., 2013). After the addition of ARSC-liposomes there was no change in the percentage of lost water in the second temperature range (50-70 °C, corresponds to the water bonded within the lipophilic gel phase). Based on all results, we assume that vesicles packed near the lamellar crystalline phase around the oil droplets and their inherent water-binding capacity jointly led to the observed transport of the water within the system.

In order to assess the in vivo irritation potential of samples (Fp, F1a and Fc) and the investigated emulsifier, EI was monitored. Additionally, the potential skin barrier impairment was assessed via TEWL and EC. After the 24-hour occlusion, EI was not significantly changed compared to the baseline values for any tested cream. TEWL was significantly decreased for the creams Fp and F1a compared to the baseline. Considering that TEWL was not significantly changed for the commercial cream Fc (the same active, but different carrier), it could be speculated that these results can be attributed to the carrier itself (system with the liquid crystalline structure) and the used emulsifier. Assumption is in accordance with the reported results concerning skin mildness of this type of emulsifiers (Lukic et al., 2013). Regarding EC, it was significantly increased compared to the baseline values in all the treated sites, apart from the placebo sample Fp where merely a trend of increase was observed. Since there was no significant change in the EC for the untreated control under occlusion, the obtained results cannot be attributed to the occlusion. The absence of erythema and/or any impairment of the skin barrier function in a 24-h occlusion study may preliminarily imply a satisfying safety profile of the creams and the used APG emulsifier.

Conclusion

The study confirmed that a novel alkyl polyglucoside emulsifier, hydroxystearyl alcohol & hydroxystearyl glucoside, could be classified as liposome-friendly. It was shown that in the system stabilized with lamellar phases, liposomes are apparently immobilized within its network and as such are mechanically stabilized. Additionally, due to the lack of skin irritation and skin barrier impairment during the application on healthy skin, it could be said that the investigated emulsifier is skin-friendly and may be safely applied as stabilizer for cosmetic or prospective pharmaceutical emulsion carrier for liposomes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 355 - 356 (2016)
ISSN 1409 - 8695
UDC: 615.262.2
Short communication

Development of an improved method for the *in vitro* determination of the Sun Protection Factor (SPF) for sunscreens

Aleksandra Dimitrovska Cvetkovska[1,4]*, Valeria Dissette[1], Ilenia Magri[1], Laura Nucibella[2], Paola Ziosi[3], Silvia Vertuani[1,3], Stefano Manfredini[1,3]

[1]*School of Pharmacy, Master Course in Cosmetic Science and Technology, Department of Life Scences and Biotechnology, University of Ferrara, via L. Borsari 46, 44121 Ferrara, Italy*
[2]*JUST International AG, Unterdorf 62, 9428 Walzenhausen, Switzerland*
[3]*Ambrosialab srl, via Mortara 171, 44121 Ferrara, Italy*
[4]*General Hospital Remedika, 16ta Makedonska Brigada br.18, 1000 Skopje, R. Macedonia*

Introduction

Sunscreen products are cosmetics according to Council Directive 76/768/EC (CD 76/768/EC, 1976). The efficacy and moreover the safety of sunscreen products, as well as the basis upon which they are claimed are part of extensive research because of the importance over the general human health. For the regulation of the sunscreen products referral to the EC 1223/2009 of the European Parliament (EC1223/2009, 2009) and to the Recommendation EC 2006/647 (2006) on the efficacy and claims of sunscreen products was done.

Based on this legislation it is necessary to validate the photo protection separately in the range of UVA and UVB, with *in vitro* and *in vivo* methods, with preference for the latter. The UVA range (320 - 400 nm) *in vivo* tests have been replaced with the *in vitro* tests, because of the consideration of the good correlation found, harmonized and standardized in the ISO 24443 (2012). For the UVB range (290 - 320 nm) the use of the method *in vivo* is still a necessity, standardized by Standard ISO 24444 (2010), due to problems related to lack of correlation between the *in vitro* and *in vivo* data. The Sun Protection Factor (SPF) continues to be widely used for describing the real performance of the product as well as its safety, and it is sometimes misunderstood, as the unique indicator for the efficacy and safety. Besides the numerical value of the SPF other properties and factors have to be taken into account by the consumers. During the last decade the sunscreen formulations are enriched with many other ingredients such as boosters, antioxidants, immune-modulators. Also into many already marketed products for skin care and make up sunscreen filters are added.

Accordingly, it is more appropriate to describe performance as a whole formula protection factor: Formulation Efficiency Factor (FEF) calculated as SPF/actives % x 100 (O'Lenick and Lott, 2011). FEF as a new parameter in the sunscreen research relates the amount of active sunscreens in a formulation to the overall SPF of the finished product. A formulation chemist can quickly and accurately apply the method to access the efficiency of a certain formulation. Sunscreens can be classified into three major categories based on the formulation active ingredients: organic, inorganic/organic and inorganic. In this complex pattern and to properly address our work toward the development of safe and effective solar products, we investigated the factors that influence the in vitro SPF determination. Having increasing problems in the determination of in vitro SPF values predictive of the in vivo assessment. With this work, we intended to evaluate, at first repeatability of the measure and, secondly, the accuracy of the same.

Test variables of the *in vitro* SPF determination, such as substrate surface temperature, substrate choice and pressure of sunscreen spreading have been examined (Miksa et al., 2013).

The purpose of this study was to investigate the impact of the type of product, way of application and method used to determine *in vitro* SPF.

* dmtlsn@unife.it

Materials and methods

Sun Protection Factor (SPF) measurement and in vivo *correlation*

SPF *in vitro* assessment was carried out on 80 random commercially available products, beside the 3 standard products prepared by us as reference formulating SPF from low and medium to high. Two different protocols for *in vitro* determination were used: the Diffey-Robson method (method A) and the ISO-24443 (method B), using tape and PMMA plates respectively with two different pressured applied (100 and 200 g) for spreadability. *In vivo* tests of selected products were also obtained for comparison using a solar simulator SPF Ultraviolet Solar Simulator 600-150 W/300 W Multiport that provides ultraviolet radiation in the region between 290 and 400 nm from 6 independent outputs. The results were collected on 10 volunteers male and female, belonging to the phototype I, II and III, aged between 20 and 35 years.

Different PMMA's and surgical tape Transpore TM, quantity of product, spectrophotometers (JascoV530PC and Shimadzu UV-2600) and method of application (manual and mechanical) were examined. For the ISO-24443 (method B), the tested product was applied to a new PMMA plate of 5 cm x 5 cm area, and 5 µm of roughness (Schonberg GmbH). The application rate of 0.0128 g/cm^2 ± 0.0003 g was controlled by mass. The application dose was determined by measuring the plates before and after the spreading operation. The application of the product has been realized according to the ISO 24443 (2012) guidelines by spotting, with a pipette, the product on several points all over the plate surface and then distributed by a fingertip, pre-conditioned with the testing sample, for 30 second, with light circular movements; the plate is then positioned on a scale where the spreading phase is carry out performing a pattern of movements in horizontal in vertical direction, checking the pressure applied in all the moments. For all the products the spreading pressure is first of 100 g and in a second analyze of 200 g. Before the measure the sample lies for a minimum of 30 minutes in a dark place. The spectrophotometer used in this method is a Shimadzu UV 2600 provided of integrating sphere ISR 2600 60 mm, and coupled with a SPF determination software: for each sample the transmittance is measured from 290 to 400 nm. Every product has been measured 3 times, i.e. three different product applications on three different plates. Each sample has been tested in six different areas at least; therefore the results presented come from an average of 15 set of data.

Results and discussion

The *in vivo* test is only a recommendation (EC 2006/647, 2006) and in fact many companies, limited by the cost of the in vivo procedure, are often relying on solely the results from the *in vitro* or even on simulations from calculating software, based on the concentration of filters used. In our investigation, the limited reliability of *in vitro* method was confirmed on complex formulation. The calculated SPF data indicate that the spreading method developed in our laboratory is reproducible for all the product test e with Cov% values less than 5%. Most accurate results were obtained with the B method that, in terms of internal repeatability and of acceptance parameters, provided some correspondence with values obtained *in vivo*, in particular using PMMA plates standardized as the roughness, pressure, amount of product and way of application. In conclusion, our method B gave statistically sound results and the better correlation between the *in vitro* and *in vivo* data, these data although preliminary, encourages us to further extend the study to a larger number of samples in order to better understand if any affordable method can be drawn on the basis of these premises.

Conclusion

Our study indicates that the variability of response is largely related with the standardization and thus harmonization of the procedure between different labs and thus we suggest joint cooperative efforts toward a possible ISO definition of an SPF-Vitro protocol.

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Macedonian pharmaceutical bulletin, **62** (suppl) 357 - 358 (2016)
ISSN 1409 - 8695
UDC: 615.454.1:615.262.2
Short communication

Emollient gels: characterisation of physical structure and behaviour in the presence of salts

Samuel Owusu-Ware, Beatriz Sanchon-Lopez and Milan D. Antonijević*

Faculty of Engineering and Science, University of Greenwich at Medway, Chatham Maritime, ME4 4TB, UK

Introduction

Emollients are therapeutic moisturisers that are usually available as leave-on formulations of various types i.e. creams, ointments, lotions and gels (Ersser et al., 2007). These products are used for the treatment of dry skin conditions such as eczema and psoriasis (Voegeli, 2011). Due to the wide range of products available on the market it can be difficult to recommend the most effective and appropriate emollients for the patient. As such, prescribers tend to recommend leave-on topical emollient products based primarily on patient preference and cost (Moncrieff et al., 2013). However, to achieve maximum benefit the emollient product must be both clinically effective as well as cosmetically appealing (Dederen et al., 2013). It is also recognised that emollient preparations are not the same even if claimed to be equivalent to other products, hence prescribers are encouraged to avoid false economy in their prescribing practices (Moncrieff et al., 2013). It is therefore critical for manufacturers to develop innovative products with optimised clinical performance and appealing sensory characteristics (Herman, 2007). To achieve this, application of simple and cost-effective analytical methodologies/approaches that can screen and compare such characteristics would be advantageous (Inoue et al., 2013; Stojiljković et al., 2013).

Emollient gels are considered one of the most cosmetically acceptable leave-on topical formulations, because of their high water content and non-greasy feel (Ersser et al., 2007). These emollient formulations are oil-in-water (O/W) dispersions emulsified using, in general, carbomer gelling agents. The gelling agent(s) act as a physical stabilizer of the dispersed oil droplets, preventing phase separation. The effectiveness of these formulations when applied to the skin, however, relies on the ability of the gel matrix to deconstruct, enabling the separation of the oil phase from the aqueous phase. This has the impact of allowing the oils (emollients) to spread easily and form a uniform occlusive barrier over the skin surface, whilst prolonging emollient retention on the skin by rendering the oily ingredients resistant to re-emulsification (removal) upon subsequent contact with water (Ersser et al., 2007). The breakdown of the carbomer gel matrix is influenced by the shear applied, but driven by the interaction of the formulation with salts on the skin (Noveon, 2002). The interaction of the emollient gel formulations with salts is therefore an important consideration when occlusive performance of the product is of concern.

The purpose of this investigation was to assess the physical structure changes under the influence of salts and compare two marketed emollient gel products, namely Doublebase Gel (DBG) and Zerodouble Gel (ZDG) marketed in the UK.

Materials and methods

Visual appearance

Commercial samples of DBG and ZDG were dispensed into a container measuring 13 mm diameter by 1 mm deep and leveled using a glass slide. The samples were then allowed to stand at room temperature for 48 hours to allow evaporation of water.

Behaviour under the influence of salts

Nearly 10% w/w salt (NaCl) to gel was prepared by sprinkling 2.0 ± 0.1 g of NaCl onto 20 ± 0.4 g of each formulation and gently mixed by folding the sample on itself ten times using a spatula. They were then left to stand for

* M.Antonijevic@gre.ac.uk

30 minutes. Untreated control samples for each formulation were folded in the same manner without added salt solution.

Microscopy

Approximately 20 mg of each treated and control sample was mixed with Nile Red fluorescent dye. The samples were then placed on microscopic slides and pressed with cover slips for 5 seconds. After 1h, the samples were viewed under a Laser microscope (Nikon eclipse 90i) x 60 magnification.

Results and discussion

Considerable differences were observed between the surface characteristics and consistencies of the two formulations prior to salt treatment. DBG was found to have a smoother and a more homogeneous textural appearance when compared to ZDG. These differences were found to be even more evident on drying under ambient conditions. These differences in appearance could result from the manufacturing processes and the composition and quality of the ingredients used.

Carbomers are sensitive to the presence of salts. The addition of small amounts of salt can be used to thicken the emulsion. The thickening of the polymer is brought about by repulsion of like charges on the polymer backbone, causing the polymer to swell. Most carbomer grades have a tolerance limit of 0.1% salt (Noveon, 2002). In the presence of a higher concentration of salt, this repulsion is reduced, resulting in the collapse of the extended polymer chain and reduced viscosity. This behaviour of the polymer, in the presence of salt, is important for the delivery of the emollients during the application of these skincare products i.e. the salts on the skin are expected to break down the polymer, making the formulation spread more easily while releasing the emollient.

The behaviour of the two gel formulations after coming into contact with salt (NaCl) were found to be different. The DBG formulation seems to largely break down into a liquid (decreased viscosity). The ZDG formulation, on the other hand, does not break down and instead appears to curdle and become firmer (increased viscosity). This implies that the polymers used in the manufacturing of the products are not similar. Furthermore, this difference in behaviour demonstrates why the DBG formulation might be expected to spread more easily when applied to the skin.

Microscopic examination also revealed differences between the emulsions, both as original untreated formulations and following salt exposure. For DBG, the structural network stabilizing the oil droplets breaks down completely, releasing the oil droplets from the emulsion. In contrast, for ZDG, microscopic examination suggests that the emulsion structure does not break down to the same extent and manner as DBG.

Conclusion

Presented work has shown that simple visual inspection of the physical structures of two emollient gels before and after interaction with salts can reveal important differences between two products that may have implications for their effectiveness and patient acceptability. The two emollient gel formulations studied have quite different visual appearances and behave very differently in circumstances mimicking contact with salts on the skin. In contrast to ZDG, DBG is a homogeneous gel that completely breaks down in the presence of salts, releasing the oil droplets from the emulsion. This has very important implications in relation to clinical performance and patient preference, where the ease with which the oily ingredients can be spread, irreversibly delivering a uniform and occlusive barrier over the skin, are crucial. These results therefore point to qualitative differences between the formulations and very likely reflect important differences between their respective manufacturing methods and ingredients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 359 - 360 (2016)
ISSN 1409 - 8695
UDC: 615.454.1:615.262.2
Short communication

Emollient gels: Characterisation of textural properties and behaviour in the presence of salts

Samuel Owusu-Ware, Beatriz Sanchon-Lopez and Milan D. Antonijević*

Faculty of Engineering and Science, University of Greenwich, Chatham Maritime, ME4 4TB, UK

Introduction

Topical leave-on emollient gel formulations are one of the most cosmetically acceptable therapeutic moisturisers, because of their non-greasy feel (Ersser et al., 2007). These oil-in-water (O/W) dispersions use carbomer as both the gelling and emulsifying agent. Whilst the carbomer acts to physically stabilise the dispersed oil droplets and thus prevent phase separation, its ability to deconstruct once applied to the skin is an important indicator of the effectiveness of these formulations. Deconstruction/breakdown of the gel matrix, due to contact with salts in the skin, results in phase separation of the oil from the aqueous phase, allowing the emollients (oils) to spread easily and form a uniform occlusive barrier over the skin surface. This also has the impact of prolonging the emollient retention on the skin by rendering the oily ingredients resistant to re-emulsification (Ersser et al., 2007).

When applied, the ability of the gel formulation to spread easily and leave a uniform occlusive layer of emollients on the skin is reliant on the textural properties i.e. firmness/stiffness of the gel matrix. These textural properties also have an important impact on the cosmetic acceptability of the formulation, such as the stickiness of the gel, which may influence the patient's willingness to generously apply the product, and therefore obtain the maximum benefits.

Interaction with salts causes the carbomer gel matrix to break down and it is therefore expected to have an important influence on the textural properties (Noveon, 2002). Characterizing the textural properties before and after interaction with salts could be an important indicator for the performance of the product during application.

The purpose of this investigation was to compare the textural properties of two marketed emollient gel products, namely Doublebase Gel (DBG) and Zerodouble Gel (ZDG) marketed in the UK, before and after interaction with salt (NaCl).

Materials and methods

Firmness/stiffness and Stickiness by Texture Analysis (TA)

50 g of commercial samples of DBG and ZDG were weighed into a beaker and subjected to compression using 35 mm diameter cylindrical probe (Stable Microsystems TA-HD plus) to measure firmness/stiffness and stickiness.

The force was measured as the probe compressed the sample by 15 mm distance after an initial trigger force (0.5 N) at a rate of 0.5 mm/sec.

When 15 mm target distance was reached the probe moved back to its starting position at 10 mm/sec recording the force required to separate the probe from the sample. This force is an indicator of stickiness. Samples were analysed in triplicates.

Spreadability by TA

1.1 ± 0.1 g of samples were compressed between two glass plates using predetermined forces, namely 1, 5, 20, 40 and 50 N. At each force the area of spread was marked out and calculated.

Different samples were used for the measurements of speadability at each force applied.

Behaviour under the influence of salts

Nearly 10% w/w salt (NaCl) to the gel were prepared by sprinkling 2.0 ± 0.1 g of NaCl onto 20 ± 0.4 g of each formulation and gently mixed by folding the sample on it-

* M.Antonijevic@gre.ac.uk

self ten times using a spatula. Firmness/stiffness, stickiness and spreadability of these samples were determined in the same manner as pre-treated samples.

Results and discussion

Firmness and stickiness of moisturisers are important sensory characteristics for consumers (Herman, 2007). Furthermore, firmness of the formulations contributes to the ability of the material to spread. These properties of the products were investigated using texture analysis, which allows for the determination of the force required to compress the formulation (a measure of firmness) and the force required to separate the probe from the sample (a measure of stickiness).

Considerable differences were observed between the two untreated formulations in terms of firmness and stickiness. ZDG appears to form a significantly firmer (3.6 ± 0.1 N) and more sticky (3.7 ± 0.2 N) polymeric structure in comparison to DBG formulation (2.6 and 2.5 N for firmness and stickiness respectively).

Upon treatment with salts, the DBG polymeric structure readily breaks down, resulting in extensive loss of firmness. In contrast, significant firmness of the ZDG structure was maintained after exposure to salts. The stickiness of the products could not be differentiated after salt treatment.

Important differences in spreadability were observed between the two emollient gels under applied force. The DBG formulation spread more easily than ZDG. For example at 20 N of applied force, the untreated DBG formulation spread over an area of 24.6 ± 0.1 mm2, whereas the ZDG formulation spread over an area of 16.2 ± 0.1 mm2. The capability of DBG to spread was greatly increased when exposed to salts and demonstrates a 27 % increase in area of spread. Interestingly, no such effect was observed for ZDG as no substantial difference was observed between the ZDG samples prior to and after being exposed to salts (15.4 mm2 area of spread obtained).

Conclusion

The presented work shows that these two emulsified gel formulations have different textural characteristics, with DBG being a less firm and less sticky gel that spreads more easily in comparison to ZDG. These two gels also behave very differently in circumstances mimicking contact with salts on the skin. In contrast to ZDG, the DBG polymeric structure readily breaks down when exposed to salts, resulting in extensive loss of firmness and increased ability to spread. The study data highlights important qualitative differences between emollient gel products, which may have resulted from differences in their respective manufacturing methods and ingredients. This may in turn, have important implications for clinical performance and patient preference. Furthermore, this type of study points out the need for more comprehensive analysis of products as patient and prescriber perceptions may lead them to select products that sometimes may not be as beneficial as they believe. Development of new methodologies for the assessment of emollient gel products is therefore necessary to fully assess the potential performance of such products.

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Macedonian pharmaceutical bulletin, **62** (suppl) 361 - 362 (2016)
ISSN 1409 - 8695
UDC: 615.33:[618.3:616.379-008.64
615.33:[618.3:616.12-008.331.1
Short communication

Influence of diabetes and hypertension on cefuroxime permeation across placenta in pregnant women

Mladena Lalić-Popović, Svetlana Goločorbin-Kon, Nebojša Pavlović*, Jovana Paunković, Zorica Grujić, Momir Mikov

Faculty of Medicine, University of Novi Sad, Serbia, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

The present study investigated the transcellular and placental permeation of cefuroxime, an antibiotic used in cesarean sections, in pregnant women with diabetes and hypertension. Previous studies have shown that infections after cesarean section could be decreased using preoperatively an antimicrobial agent (Chelmow et al., 2001). However in order to achieve therapeutic concentrations in both maternal and fetal serum and amniotic fluid, antibiotic have to pass placental barrier. It is proven that a single dose of intravenously administered antibiotic is as effective as multiple doses given perioperatively (Lamont et al., 2011). Accordingly, single doses of cefuroxime have been widely used for antimicrobial prophylaxis during cesarean delivery. Previous studies have shown that cefuroxime concentrations in amniotic fluid and in umbilical cord plasma are sufficient to combat most microorganisms. However, diseases such are hypertension and diabetes could alter placental transferto the extent that the prophylactic effect is lacking in both the mother and fetus. Therefore, the aim of this study was to determine the effects of diabetes and hypertension on the transplacental permeation of cefuroxime.

Materials and methods

Fifty-three women scheduled for cesarean section were divided into three groups: healthy women (n = 18), women with arterial hypertension (n = 21), and women with gestational diabetes (n = 14). All women received 1.5 g, intravenously cefuroxime. Study was conducted in accordance with international ethical guidelines (CIOMS) and the study protocol was approved by Ethics Committee of the Gynecology and Obstetric Clinic of Clinical Centre of Vojvodina (no. 100-08/9). Informed consent was obtained from each woman before enrolment in the study. The study was designed as an open-label study. Each intravenous injection of cefuroxime was completed in less than 1 min. Sampling points were chosen in order not to disturb regular cesarean section procedures in the clinic. Blood samples were collected from mothers after administration of cefuroxime before delivery (t_1), at the time of delivery (t_2), and after delivery (t_3). At delivery, umbilical venous and arterial samples were obtained from a section of umbilical cord (cross-clamped at delivery). Estimated gestational age at birth, weight, length and 1and 5 min Apgar scores were recorded. Concentration of cefuroxime was measured using a modification of an HPLC method described previously (Szlagowska et al., 2010). Pharmacokinetic parameters were calculated for each woman using plasma cefuroxime concentration data with WinNonLin version 4.1 (SCI software, Pharsight Corporation, Gary, NC, USA). The effects of diabetes and hypertension on cefuroxime placental-permeation were assessed by the fetomaternal plasma concentration ratios (Cv/Cm) and umbilical cord venous blood (Ca/Cv). Results were compared using ANOVA test ($p \square 0.05$).

Results and discussion

Neonates born to women in the hypertensive group had significantly lower body surface area (BSA), weight, 5-min Apgar score, and gestational age than neonates born to women in the control group. Neonate characteristics in the diabetic group did not differ significantly compared with either the hypertensive or control groups.

* nebojsa.pavlovic@mf.uns.ac.rs

Tthe Ca/Cv ratio in all three groups was near 1, with no significant differences among the control, hypertensive, and diabetic groups (1.01±0.16, 1.06±0.23, and 0.95±0.34, respectively). This correlates with the physicochemical characteristics of cefuroxime, which is a very hydrophilic weak acid (Log Poctanol/water 0.4) and is almost completely dissociated at blood pH (7.4) (Holt et al., 1993). Thus, the slightly acidic pH of neonatal compared with maternal blood would not lead to the accumulation of cefuroxime in neonates. In the present study, neither hypertension nor diabetes had any significant effect on cefuroxime accumulation in neonates. The Cv/Cm ratios were significantly lower in the diabetic compared with the control and hypertensive groups (0.36±0.13 vs 0.71±0.46 and 0.59±0.40, respectively). However, there were no significant differences between the control and hypertension group and because gestational age was significantly lower in the hypertensive than control group, the findings confirms those of a previous study that reported that gestational age had no effect on cefuroxime crossing the placental barrier (Holt et al., 1993). Pharmacokinetic parameters, such as drug plasma concentrations at zero time (C_0), mean resident time (MRT_0–t_3) and areas under the time-concentration curves to infinity (AUCinf) were not significantly different among the three groups. The elimination half-life ($t_{½}$) was significantly shorter in the hypertensive than control and diabetic groups because the constant of elimination (λz) was higher in the former group compared with the latter groups. Apparent volume of distribution and clearance were significantly lower in the diabetic group compared with the control and hypertensive groups. Lower transplacental transfer in the diabetic group compared with the hypertensive and control groups could be also due to lower volume of distribution and clearance.

Conclusion

Hypertension had no significant effect on the permeation of cefuroxime nor on its pharmacokinetics. Diabetes led to decreased placental transfer of cefuroxime, as well as volume of distribution and clearance, but did not affect other pharmacokinetic parameters. Prophylactic concentrations of cefuroxime were reached in all groups, but the dosing time of cefuroxime should not be less than 30 min or greater than 2 h prior to delivery.

Acknowledgment

This work was supported by the Ministry of Education, Science and Technological Development of Serbia (III41012).

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Macedonian pharmaceutical bulletin, **62** (suppl) 363 - 364 (2016)
ISSN 1409 - 8695
UDC: 615.214.24.033.1:[618.3:616.379-008.64
615.214.24.033.1:[618.3:616.12-008.331.1
Short communication

Placental transfer of lipophilic drug diazepam in pregnant women with diabetes and hypertension

Mladena Lalić-Popović, Svetlana Goločorbin-Kon, Nebojša Pavlović*, Jovana Paunković, Zorica Grujić, Momir Mikov

Faculty of Medicine, University of Novi Sad, Serbia, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

Due to the widespread rise in early detection of high-risk pregnant women in need of cesareans, its use is rapidly increasing worldwide (e.g. uncontrolled diabetes, hypertension and pre-eclampsia). Diazepam is lipophilic drug that is weak base and it is used in the treatment of maternal eclampsia and as a premedication in cesarean section deliveries. Diazepam readily crosses the blood–brain barrier and the placenta by passive diffusion. It is also excreted into breast milk and studies showed that diazepam reaches equilibrium in the feto-maternal systemic circulation 10-15 minutes after intravenous administration (Bakke et al., 1992). Also, some diseases such as diabetes and hypertension have been associated to impaired placental composition and functions. Previous studies carried out in our laboratories have demonstrated impaired drug permeation in diabetic animals. The development and progression of diabetes have been associated with disturbed drug absorption due to dysfunctional protein expression and functionality, impaired transcellular transport and intercellular trafficking as well as altered gut physiology (Al-Salami et al., 2009). The aim of this study was to investigate the influence of diabetes and hypertension on the placental permeation of diazepam.

Materials and methods

Pregnant women were recruited from the Gynecology and Obstetric Clinic in Vojvodina, Serbia. Pregnant women scheduled for cesarean section, those who were diagnosed with gestational or arterial hypertension as well as those who were diagnosed gestational diabetes were included in this study. The study protocol was approved by Ethic Committee of the Gynecology and Obstetistric clinic in Vojvodina (N°00-08/9) and informed consents were obtained from each participant before inclusion in the study. A total 75 pregnant women were divided into three groups: group 1 (healthy control, n=31), group 2 (diabetic, n=14) and group 3 (hypertensive, n=30). Two sets of diazepam plasma samples were collected and measured (after the administration single dose of 5 mg/day intramuscularly), before (t_1), during (t_2) and after delivery (t_3). The first set of blood samples was taken from the mother (maternal venous). The second set of samples was taken from the fetus (fetal umbilical veins and arteries). Diazepam concentrations in plasma were measured by modified HPLC method previously described (Rouini et al., 2008). Pharmacokinetic parameters were calculated using non compartmental analyses using with WinNonLin version 4.1 (SCI software, Pharsight Corporation, Gary, NC, USA). Values of AUCs after delivery were taken as a measure of diazepam elimination from blood. In order to assess the effect of diabetes and hypertension on diazepam placental-permeation, the ratios of fetal to maternal blood concentrations were determined. Also umbilical cord arterial to umbilical cord venous concentration ratio was determined as a measure of diazepam uptake, distribution and/or metabolism in neonates. Data were analyzed by ANOVA test and differences were considered statistically significant if $p \leq 0.05$.

Results and discussion

All neonates were similar in length, weight and body surface area values. Also, there were no statistically significant differences neither in height, weight nor body surface

* nebojsa.pavlovic@mf.uns.ac.rs

area of the women between three investigated groups. The diabetic and hypertensive groups have 2-fold increase in the fetal umbilical-venous concentrations, compared to the maternal venous concentrations. Feto: maternal plasma-concentrations ratios were higher in diabetic (2.01±1.01) and hypertensive (2.26±1.23) groups compared with control (1.30±0.48) while, there was no difference in ratios between the diabetic and hypertensive groups. Umbilical-cord arterial: venous ratios (within each group) were similar among all groups (control: 0.97±0.32; hypertensive: 1.08±0.60 and diabetics1.02±0.77) and there were no statistically significant differences. There were statistically significant higher AUCs values before delivery in control and hypertension group compared to diabetes group. Meaning that transfer of diazepam in diabetic group was higher even though exposure was lower probably due to increased permeability of placenta in diabetic women. Values of AUCs after delivery were statistically higher in control group compared to hypertension and diabetes group, but there were no statistical differences between hypertension and diabetes group. These results implies that elimination of diazepam from central compartment is higher in hypertension and diabetes group and that is likely that there were more unbound diazepam in the blood in these groups, since total clearance of diazepam is directly proportional to free diazepam fraction (Riss et al., 2008).

Conclusion

On line with our previous findings which demonstrate disturbed transcellular trafficking of lipophilic drugs in diabetes, this study shows significant increase in diazepam placental-permeation in diabetic and hypertensive pregnant women suggesting poor transcellular control of drug permeation and flux, and bigger exposure of the fetus to drug-placental transport.

Acknowledgment

This work was supported by the Ministry of Education, Science and Technological Development of Serbia (III 41012).

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Macedonian pharmaceutical bulletin, **62** (suppl) 365 - 366 (2016)
ISSN 1409 - 8695
UDC: 615.272.4.015
Short communication

Self-microemulsifying drug delivery systems containing simvastatin: formulation and characterization

Zora Ćetković[1], Marko Krstić[2], Sandra Cvijić[2]*, Dragana Vasiljević[2]

1Emergency Centre, Clinical Centre of Serbia, Pasterova 2, 11000 Belgrade, Serbia
2University of Belgrade - Faculty of Pharmacy, Department of Pharmaceutical Technology and Cosmetology, Vojvode Stepe 450, 11221 Belgrade, Serbia

Introduction

Simvastatin is poorly water-soluble drug which belongs to class II (low solubility, high permeability) according to Biopharmaceutical Classification System (BCS) (Kuentz, 2012). One of the strategies to improve its dissolution rate includes development of self-microemulsifying drug delivery systems (SMEDDS). SMEDDS are isotropic mixtures of oil, surfactant, cosurfactant, and a drug, that under dilution in vivo can spontaneously form microemulsions with droplet size less than 50 nm (Gursoy and Benita, 2004; Hauss, 2007).

The aim of this study was to formulate and characterize self-microemulsifying drug delivery systems of simvastatin with increased dissolution rate of simvastatin.

Materials and methods

Materials

Simvastatin (Ph. Eur. grade) was obtained from Hemofarm a.d. (Serbia). Caprylocaproyl macrogol-8 glycerides (Labrasol®), propylene glycol monocaprylate (Capryol™ PGMC) and oleoyl macrogol-6 glycerides (Labrafil® M1944CS) were obtained from Gattefossé (France). Polysorbate 80 was obtained from Sigma Aldrich Chemie GmbH (Germany).

Methods

Formulation and preparation of SMEDDS

Surfactant phase of Labrasol® as surfactant and Polysorbate 80 as cosurfactant were mixed at fixed weight ratio 3:1. Oil (Capryol™ PGMC or Labrafil® M1944CS) was then added to surfactant phase at varios ratios (from 9:1 to 1:9) and mixed on magnetic stirrer. After preparation, all samples were titrated with highly purified water drop by drop. Two single-phase, transparent systems are considered to be microemulsions and selected for further investigations. Both samples contained 67.5% Labrasol, 22.5% Polisorbat 80 and 10% oil (Capryol™ PGMC-sample F1 or Labrafil® M1944CS-sample F2). Simvastatin (5% w/w) was dissolved in these selected SMEDDS by constant mixing on magnetic stirrer at 50-60 °C until a clear solution was obtained (samples F1s and F2s).

SMEDDS characterization

Droplet size determination

Both, unloaded and simvastatin-loaded SMEDDS were diluted with highly purified water (1:10). The average droplet size (Z-ave) and polydispersity index (PDI) of unloaded and simvastatin-loaded systems were determined immediately after dilution by photon correlation spectroscopy (NanoZS90, Malvern Instruments, UK) at wavelength of 633 nm and a scattering angle of 90 °. The results were the mean and standard deviation (S.D.) of three consecutive measurements for each sample.

In vitro dissolution studies

For *in vitro* dissolution studies liquid SMEDDS with 20 mg of simvastatin were filled into hard gelatin capsules (size 0), and compared to commercially available tablet containing the same dose of simvastatin.

The dissolution test was carried out using rotating paddle apparatus (Erweka DT70, Germany). The dissolution medium consisted of phosphate buffer pH 7.0, the volume

* gsandra@pharmacy.bg.ac.rs

was 900 ml, and the temperature of the dissolution medium was maintained at 37 °C with a rotating speed of 75 rpm. At fixed time intervals (5, 10, 15, 30, 45 and, 60 min), 10 ml samples were withdrawn from the dissolution medium and replaced by 10 ml of fresh phosphate buffer. Sink conditions were maintained at all times. All samples were filtered using membrane filter (0.45 μm MF-Millipore® membrane filter, Millipore Corporation, USA) and simvastatin concentration was determined spectrophotometrically at 239 nm (Evolution 300, Termo Fisher Scientific, England). The dissolution experiments were carried out in triplicate, and data were expressed as mean value ± S.D.

Results and discussion

Droplet size analysis

The average droplet size (nm) of samples F1, F1s, F2 and F2s were 18.58±0.04, 46.41±0.17, 11.68±0.12 and 17.45±0.04, respectively. It could be concluded that upon high water dilution both unloaded and drug-loaded SMEDDS are capable to form microemulsions, because the average droplet size is less than 50 nm (Gursoy and Benita, 2004; Hauss, 2007). Slightly higher droplet size of F1s, compared to unloaded F1, might be due to the interference of the drug with self-emulsification process (Gursoy and Benita, 2004).

Polydispersity index represents the uniformity of droplet size within the formulation and for selected samples F1, F1s, F2 and F2s were 0.155±0.005, 0.286±0.001, 0.137±0.016 and 0.168±0.005, respectively. Lower value of PDI in sample F2s indicated better uniformity of droplet size, in comparison to formulation F1s. Both unloaded and SIM-loaded SMEDDS have shown monomodal droplet size distribution.

In vitro dissolution study

Comparative in vitro dissolution profiles of simvastatin from SMEDDS filled in hard gelatin capsules and commercial (immediate release) tablet showed that simvastatin was completely released from both SMEDDS within first 5 minutes. The release rate of simvastatin from SMEDDS was significantly faster compared with commercially available tablet (approximately 14.57% after 1 hour), which might be due to the surfactants present in formulations. Karim et al. (2015) showed that the droplet size of the microemulsion could determine the rate and extent of simvastatin release, since the in vitro drug release was faster from formulations with smaller droplet size. However, in this study there was no significant difference in simvastatin release between two selected SMEDDS.

Conclusion

Self-microemulsifying drug delivery systems (SMEDDS) containing simvastatin were formulated and evaluated. Upon appropriate water dilution SMEDDS formed microemulsions with droplet size less than 50 nm. Although F2s had smaller droplet size and narrower droplet size distribution, in vitro dissolution study revealed that simvastatin was completely released from both SMEDDS (F1s and F2s) in 5 minutes. These results indicated that development of SMEDDS could effectively enhance in vitro dissolution rate of simvastatin compared to commercial tablet and can be used as possible alternative.

Acknowledgments

This work was supported by the project TR34007 (Ministry of Education, Science and Technological Development, RS).

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Macedonian pharmaceutical bulletin, **62** (suppl) 367 - 368 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:620.3]:543.422
Short communication

A spectroscopic insight into the albumin structure on the nano-bio interface

Nikola Geskovski[1]*, Simona Dimchevska[1], Rozafa Koliqi[1], Gjorgji Petruševski[2], Marina Chacorovska[2], Sonja Ugarkovic[2], Katerina Goracinova[1]

[1]*Institute of Pharmaceutical technology, Faculty of Pharmacy, University of Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*R&D, Alkaloid AD, blvd. Aleksandar Makedonski 12, 1000 Skopje, R. Macedonia*

Introduction

In the last decades, the struggle for efficient targeted drug therapy and diagnostics paved the road to clinical use of nanomedicines. However, the research and understanding of the interactions among the engineered nanomaterials and the biological environment, to date, presents quite a challenge. It is becoming clear that, when placed into a biological environment, nanoparticles initiate a cascade of interactions with the biomacromolecules resulting in the formation of the 'protein corona' (a layer(s) of proteins adsorbed on the nanoparticles surface) (Monopoli et al., 2011). These interactions can alter the secondary structure of the adsorbed proteins promoting instability and/or exposure of new epitopes at the protein surface, thus giving rise to unexpected biological responses (Calzolai et al., 2010). Undoubtedly, the protein corona modifies the nanoparticles interface and thus affects their biological fate and overall performance. Therefore the characterization of the interactions at the nano-bio interface will greatly influence the understanding and capability for prediction of the nanoparticles in vivo behavior. The aim of this work is to investigate the effects of the surface properties of different polymeric nanoparticles upon their interaction with a model protein (bovine serum albumin - BSA) in a binary nanoparticle – BSA system.

Materials and methods

Materials

PLGA-PEO-PLGA (Mw 148KDa and Mw 22KDa) was purchased from Akina Inc (USA). Lutrol F127 - Poly(ethylenoxide)-block-poly(propyleneoxide)-block-poly(ethyleneoxide) was kindly donated by BASF (Germany) and BSA was purchased from Sigma Aldrich (USA). Bradford Protein assay dye reagent was obtained from Bio-Rad (USA). All other reagents and chemicals used were of analytical grade.

Methods

Nanoparticles preparation procedure

Nanoparticle formulations were prepared from PLGA-PEO-PLGA (Mw 70,000:8,000:70,000Da) – NP1 and PLGA-PEO-PLGA (Mw 6,000:10,000:6,000Da) – NP2, using the nanoprecipitation method, as described previously (Dimchevska et al., 2015).

Quantification of bovine serum albumin adsorption

All samples were diluted to concentration of 2mg/ml and subsequently 1ml from each formulation was mixed with 1ml of 2mg/mL BSA solution in phosphate buffer (pH 7.4). The NP dispersions with BSA were incubated for 1h at 37°C in a water bath with horizontal shaking at 100 min^{-1}. After the incubation, the samples were concentrated to 1mL using ultrafiltration tubes with pore size of 1000 kDa, and washed with phosphate buffer pH 7.4. Blank (BSA free) and control sample (without nanoparticles) were also used in the experiment. The amount of adsorbed BSA was indirectly quantified using the Bradford protein assay.

Spectroscopic characterization of nanoparticle-bovine serum albumin interactions

The samples were prepared as described above with additional freeze drying cycle (-40 °C, 0.055 mBar, FreeZ-

* ngeskovski@ff.ukim.edu.mk

one 2.5 L, Labconco, USA) in order to remove the water. FTIR spectra of the samples were carried out using FT-IR spectrometer (660, Varian, USA). To calculate the relative proportions of the different secondary structures of albumin, the spectral region of the Amide I (1700-1600 cm^{-1}) was analyzed with appropriate software for spectral analysis (Grams32, Thermo, USA). Using Gauss-Lorentz transformation, six distinctive peaks of the secondary protein structures were analyzed (β-sheets in spectral regions 1696-1690 cm^{-1}, 1642-1624 cm^{-1} and 1618-1613 cm^{-1}; β-turns in the spectral region 1685-1675 cm^{-1}; α-helix in the region 1658-1654 cm^{-1}; random coils in the region 1648-1640 cm^{-1}). To avoid any influence of the vibration of the polymer chains in the region of Amide I, the spectra of empty nanoparticles were previously subtracted from the spectra of the samples with adsorbed albumin.

Results and Discussion

The results from the quantitative BSA adsorption studies revealed that 24.6±1.9 and 13.1±0.9% of BSA were adsorbed on the surface of NP1 and NP2, respectively.

The FTIR spectra of freeze dried BSA revealed broad band at 3287 cm^{-1} that originates from N-H bending vibrations (Amide A). The C-H bending of aliphatic chains can be noted on 2960 cm^{-1}, 2933 cm^{-1} and 2875 cm^{-1}. Strong C-O stretching vibration (Amide I) that originates from the amide bond in the peptide chain appears at 1645 cm^{-1} while the Amide II band (mainly N-H bending vibrations) is on 1516 cm^{-1}. Several bands that represent mainly combinations of C-N stretching and N-H bending can be noticed in the region from 1400 cm^{-1} to 1200 cm^{-1}. Distinctive blue shift of Amide I (1654 cm^{-1}) in the spectra of NP1-BSA complex can be noticed, relative to the FTIR spectrum of BSA. Such shift of Amide I could be attributed to possible secondary structure changes of BSA that are initiated as a result of the interaction with the surface of the nanoparticles. Also, significant decrease in the C-O antisymmetrical stretching vibrations from the PLGA blocks was noticed, indicating to possible growth of hydrophobicity in the microenvironment of surface exposed carbonyl groups. The FTIR spectrum of NP2-BSA complex reveals blue shift of Amide I and II relative to the bands on BSA spectrum. Additionally, there is a slight decrease of the intensity of the carbonyl C-O vibrations band at 1756cm^{-1} and a red shift of the antisymmetrical C-O stretching vibrations on the PEO chains (1087 and 955cm^{-1}) relative to the respective bands in the spectrum of NP2. The curve fitting on Amide I in the previously mentioned FTIR spectra revealed that the secondary structure of the BSA molecule contains 35% α-helix, 32.5% β-sheets, 12% β-turns (short 4 amino acid segments that form antiparallel loops) and 20% of random coils. Having in mind that the spectrum was taken from freeze-dried sample of BSA, one can assume that the portion of α-helix will be reduced on the expense of the increase of β-sheets and random coils in the secondary structure. The NP1-BSA and NP2-BSA demonstrated presence of 77.63% and 34.39% of α-helix, 11.51% and 31.76% of β-sheets, 10.8% and 17.74% of β-turns, 0.05% and 16.11% of random coils in the BSA molecule, respectively.

Considering the above results one can assume that the behavior of BSA towards NP1 and NP2 is different, mainly because of the differences in the density and PEO chain length of the nanoparticle hydrophilic corona. The PLGA/PEO ratio in NP1 and NP2 is 17.5:1 and 1.2:1, respectively. Having this in mind, it can be presumed that the PEO chain length and surface density can affect the accessibility of the hydrophobic nanoparticle core towards nonspecific interactions with BSA. The hydrophilic corona of NP2 is larger and more rigid and as such will be an effective steric barrier in the interaction of BSA with the hydrophobic PLGA core. The loss of α-helix in the secondary structure of BSA in the native BSA sample and NP2-BSA complex can be attributed to the freeze-drying stress of the BSA molecule that initiates hydrogen bonding redistribution in the secondary structure of the protein. The partially adsorbed BSA molecule won't be able to protect its intramolecular hydrogen bonds during the freeze drying process that will initiate the conversion of α-helix to random coils and β-sheets. On the other hand, the structure of the BSA that is adsorbed on the NP1 surface could be associated with its' native structure. The preservation of the native structure during freeze drying is another indication of the hydrophobic nature of the NP1-BSA interactions resulting in significant contribution towards the maintenance of the BSA original hydrogen bonds.

Conclusion

The results unambiguously point to the effect of the hydrophilic outer nanoparticle layer as a steric barrier for nanoparticle-BSA interactions. The formulations with low hydrophilic coverage expose the hydrophobic core and enable strong hydrophobic binding with the proteins present in the corona. Such strong binding could result in appearance of different new protein epitopes on the nano-bio interface and significantly alter the biological fate of the nanoparticles.

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Macedonian pharmaceutical bulletin, **62** (suppl) 369 - 370 (2016)
ISSN 1409 - 8695
UDC: 665.345.4:615.26
Short communication

Preliminary study concerning *Linum usitatissimum* oil as sebum-reducing agent

Anca Dragomirescu[1]*, Ersilia Alexa[2], Georgeta Pop[2], Felicia Andrei[1], Georgeta Simu[1]

[1]*University of Medicine and Pharmacy "Victor Babes" Timisoara, Faculty of Pharmacy, Eftimie Murgu Square no 2, RO-300041, Timisoara, Romania*
[2]*Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara, 119 Calea Aradului, Timisoara, Romania*

Introduction

There are a lot of records in the literature regarding the utility of linseed oil on the skin. Concerning to this, it is worth to mention here the experimentally confirmed effects, such as increased skin hydration after introducing in the diet linum usitatissimum oil (De Spirt et al., 2009). The linoleic and alpha-linolenic essential fatty acids are involved in restoring the cutaneous barrier, important in skin ceramides synthesis. Other authors have noted the favorable effect of the linum usitatissimum oil on wound healing, on an animal model (De Souza Franco et al., 2012). There are also experimental evidences regarding the benefits on skin excoriations. Additionally, the anti-inflammatory and even antibacterial effect leads some authors to cite flax oil as showing a positive effect on treating ringworm.

On the other hand, theoretically, under the effect of lignans, (secoisolariciresinol diglucoside - SDG - the most abundant flax lignan) with estrogen-mimetic action, the oil of this species should have effects similar to other phytoestrogens. This is the premise of our experimental study.

The study aims to: (1) evaluate fatty acids in Linum usitatissimum oil, in order to be, at least, protective and nutritive for human skin, in topical application, (2) evidencing the sebum-reducing capacity of linum oil, (3) observation of a possible improvement of skin texture (a result of skin hydration), for the cases of seborrhea sicca (dry seborrhea).

Materials and methods

Linseed oil was obtained from the seeds of the Alexin cultivar grown in the Didactic Station Timisoara of Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara in 2014. The cutaneous study benefited from the enrollment for testing of 24 healthy female volunteers, aged between 18 and 46, having oily and seborrheic skin and the sebaceous glands were measured in terms of numbers and dimensions, by using the apparatus Proderm Analyser (NU SKIN, Provo, UT, USA). The evaluation of the cutaneous evolution was performed in the Dermatopharmacy and Cosmetology laboratory (University of Medicine and Pharmacy Timisoara). The criteria for including the volunteers in the study were: (1) Written consent of the volunteers after they understood the test procedure, (2) Diagnostic of seborrhea or normal oily skin, based on clinical criteria, (3) Lack of any pathological complications (eg. acne, seborrheic scaling dermatitis) situations in which the oil is potentially harmful (obstructive, comedogenic, or irritating) (4) Elimination of intolerance, after a preliminary test of the oil at a topical skin application. The assessments with Proderm Analyzer were recorded after 7 days of therapy, at the times: 7, 14, 21 and 28 days respectively. For each volunteer, at each time point, were counted the colored points per skin field explored (number of oversized sebaceous glands per cm2) and the average was calculated for each time unit, separately for seboreea oleossa and for seboreea sicca.

* ancadrag65@yahoo.com

Results and discussion

Linseed oil is characterized by a high content of linoleic acid (0.313 mg / ml) and linolenic acid (0.214 mg / ml). The results are consistent with literature data mentioning a high concentration of linoleic acid in oil from linseed seeds cultivated in Romania but also in other countries. The daily use of linseed oil also produced an improvement in the appearance of the skin, reducing the appearance of scaly skin to complete fade-out. There are no obvious differences between the evolution of the seborrhea oleossa and the sicca during the test for the linum oil treated group, meaning that this oil has efficiency in both types of disorders.

The most significant decrease of sebaceous glands number was registrered starting with the 14th day after the first treatment. The 14 days also represent the turnover of the sebocyte. In other words, after two weeks there are other new sebocytes holding secretory activity under another hormonal stimulation, modified in the meantime. As a matter of fact, the sebocyte is a cell with a prompt response to other pharmaceutical treatments as well, for example after the treatment with topical retinoids the sebaceous secretion decreases dramatically, sometimes after only 7-10 days. The problem with retinoids, however, is their adversity to the epidermis, dryness and the erythema generated.

Conclusion

In conclusion, linseed oil is one of the oldest natural remedies for skin nutrition. Recent studies show its benefits in scarring, in surgical wound healing optimization, in skin hydration and also in the domain of cutaneous aging prevention.

This study brings again linum usitatissimum oil in the foreground, this time for its sebum-reducing effect. This property is a confirmation of the hypothesis launched by the Lucas Meyer dermocosmetic laboratories, a property attributed to the lignans in flax seeds, known for their estrogen-mimetic and implicitly anti-androgenic attributes, which means exactly an intervention on the hormonal mechanism of seborrhea. Our study opens a new prospect to lignans determinations, in different sort of linseeds, for a natural alternative in sebum-regulation field.

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Macedonian pharmaceutical bulletin, **62** (suppl) 371 - 372 (2016)
ISSN 1409 - 8695
UDC: 615.26:613.495
Short communication

Safety profile assessment of cosmetic anti-age creams based on natural ingredients using in vivo bioengineering techniques

Ana Žugić, Nada Ćujić, Jelena Živković, Gordana Zdunić, Katarina Šavikin, Nebojša Menković*, Dubravka Bigović

Institute for Medicinal Plant Research "Dr. Josif Pančić", Tadeuša Kosćuška 1, Belgrade, Serbia

Introduction

In the last years, there is a growing trend of utilization of natural cosmetics, that is related to the frequent occurrence of allergies and skin irritations caused by synthetic ingredients commonly included in the formulation of these products (Glampedaki and Dutschk, 2014). Antioxidants, especially the ones naturally derived from plants, have been recognized as excellent source of bioactive components with beneficial effects on skin, especially to prevent premature skin aging and wrinkling. There is an increasing research interest in polyphenolic compounds which have proven to possess antioxidant activity, and have shown promising effects applied as cosmetic active ingredients (Braunlich et al., 2013).

Materials and methods

Therefore, our group developed an anti-age cosmetic line consisting of day (D) and night (N) cream, both based on natural materials. Namely, in the stated creams (D and N) black chokeberry and hibiscus propylene glycol extracts (produced at Institute for Medicinal Plant Research) and hyaluronic acid (low weight, Amedeo Brasca, Italy), were incorporated as active ingredients in the appropriate hydrophilic (D-V, yielding cream D) and hydrophobic (N-V, yielding cream N) vehicles, in which synthetic excipients were replaced with the ones of natural origin. Chokeberry is one of the richest sources of natural antioxidants among fruits and vegetables, because of its bioactive components such as anthocyanins, flavonols, procyanidins, and phenolic acids (Kulling and Rawel, 2008; Rugina et al., 2012; Sueiro et al., 2006). Beside chokebery extract, D and N creams contained hibiscus extract, abundant in vitamin C that stimulates synthesis of collagen, a protein responsible for skin tone and elasticity (Aburjai and Natsheh, 2003). Hyaluronic acid, natural component of the skin located in the extracellular matrix, recovers lost skin moisture and gives the visual effect of skin lifting (Scott and Banga, 2015). Avocado oil, grape seed oil and shea butter (all from Comcen, Serbia) included in both creams, effectively feed and revitalize the skin due to the complex of active ingredients they contain, especially high content of fatty acids. Creams contain components of natural wetting factors of skin (complex of hexylene glycol, fructose, glucose, sucrose, urea, dextrin, alanine, glutamic acid, aspartic acid, hexyl nicotinate) and elastin (both from Chemisches Laboratorium Dr Kurt Richter GmbH, Germany). Elastin as a protein, is responsible for skin tone, gives it back skin moisturize, elasticity and freshness. Both creams contain a complex of UV filters (ethyl hexyl methoxycinnamate, butyl methoxydibenzoylmethane, ethyl hexyl salycilate, Coning PPI, Serbia) that protect the skin from harmful sun rays.

Safety profile/irritation potential of the developed cosmetic products (active creams-D and N, as well as their matching vehicles-D-V and N-V, respectively), was evaluated using noninvasive measurements of the appropriate biophysical parameters of the skin in a 24-h in vivo study under occlusion.

Thirty one healthy female volunteers (mean age 46.09), which participated in the study, were thoroughly informed about the possible treatment effects and the protocol of the examination prior to signing written consents, in accordance with the Helsinki Declaration. The study was approved by the Ethical Committee of the Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade, Serbia (Decision No 01-9337-13). The following param-

* nmenkovic@mocbilja.rs

eters were evaluated: electrical capacitance (EC) (quantifying the stratum corneum hydration), transepidermal water loss (TEWL), as a measure of skin barrier function and erythema index (EI), as a measure of skin colour, using Multi Probe Adapter MPA®9 (Courage & Khazaka Electronic GmbH, Germany). All measurements were conducted on flexor aspects of forearms at square application sites of 9 cm^2, leaving a site per each arm for untreated control under occlusion (UCO) and without occlusion (UC). The volunteers were instructed not to use dermopharmaceutical and/or cosmetic products on the tested areas as well as to spend at least 30 minutes in a room in which the measurements were conducted in order to adapt to the temperature and relative humidity. After initial measurements, 0.016 g/cm^2 of the investigated samples were applied, covered with silicone film and fixed with hypoallergenic adhesive tapes. Two hours upon removal of the 24-h occlusion, all parameters were reassessed (Jakšić et al., 2012; Tasić-Kostov et al., 2011).

Results and discussion

The investigated samples showed overall satisfying preliminary safety profiles (low in vivo irritation potential). Namely, two hours after occlusion removal, all the investigated samples led to the significant upsurge of EC compared to the baselines and controls, revealing skin hydration potential probably related to appropriate vehicles themselves (D-V and N-V), bearing in mind lack of significant differences after treatment with these samples compared to the matching active creams (D and N, respectively). There was no significant change in EI, which was even decreased for all the tested creams, indicating well-tolerated skin formulations. Also, there was no significant increase of TEWL for the investigated samples compared to the baseline values, nor UCO. Significant growth of this parameter was detected after repeated measurement for the sample N compared to UC, but this increase cannot be attributed to the treatment with this sample itself, while the same difference between test-spots N and UC was also noted in the initial measurement.

Conclusion

In conclusion, preliminary safety profile of the investigated creams based on ingredients of natural origin can be considered satisfactory. Tested samples did not cause the change in the measured biophysical parameters-TEWL and EI, while they even increased EC i.e. stratum corneum hydration, a feature considered preferable in the cosmetic products intended for the treatment of aged skin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 373 - 374 (2016)
ISSN 1409 - 8695
UDC: 615.451.16
Short communication

Small-scale production and evaluation of an acetate-and a lactate -based balanced infusion solution

Elena Najdovska*, Zora Veljanova

Clinical Hospital "Dr.Trifun Panovski" Department for Compaunding steril products, Partizanska b.b. Bitola, Macedonia

Introduction

Intravenous (IV) fluid therapy is a commonly used in hospitalcare practice as one of the essential element in patient care. Over the years IV fluids in different forms have been specially designed and developed according to the physiological demands of various patient's medical conditions. Understanding these IV fluids that are administered to the patient is important because each has a differentimpact on the body and particular indications for use. Therefore, the choice of IV fluid should be based upon the hydration status of the patient and metabolic disorders associated with the patient's condition. .

Ringer's solution for infusion is chemically prepared an isotonic crystalloid that contains sodium chloride, potassium chloride, and calcium chloridein sterile water. The dosage of Ringer's solution is dependent upon the age, weight, clinical conditions of the patient, and concomitant therapy.

This solution is indicated to replace extracellular fluid losses, to restore the sodium, potassium, calcium and chloride balances and, as well as for treatment of isotonic dehydration condition In practice, there are different recipes for Ringer's solution composition depending on its intended use. For example, lactated Ringer's solution is a hypotonic solution that the best approximate extra cellular fluid. It may be infused safely in large quantities in patients with conditions such as hypovolemia with metabolic acidosis, shock syndromes and burns. It is well known that lactate is metabolized in the liver, and to a lesser degree inthe kidney while, acetate is metabolized mainly in the muscles and lesser in tissues such as kidneys and heart, so it's a good alternative in patients with impaired lactate clearance such as in advanced liver disease (Santoro et al., 2007; Zander, 2004). In general, there are three independent acid-base variables that need to be determined when studyingthe acid-base properties of IV fluids such as: the partial CO_2 tension, the total concentration of nonvolatile weak acid (AToT), and the strong ion difference (SID) (Kellum, 2002, 2005). Metabolic acidosis and alkalosis are respectively caused by raising and lowering AToT while holding SID constant. Metabolic acidosis and alkalosis are respectively caused by lowering and raising plasma SID while clamping AToT. Fluid infusion causes acid-base effects by forcing extracellular SID and A ToT toward the SID and A ToT of the administered fluid (Morgan, 2005). The SID of isotonic saline being 0, the infusion of large quantities (as in correction of hypovolaemia, acute normovolaemic haemodilution, and cardiopulmonary bypass) will dilute the normal SID of plasma and decrease pH. Using the Stewart equation, a balanced solution with a physiological SID of 40 mEq/Lwould induce a metabolic alkalosis (Chappel, 2008; Morgan, 2005). In order to avoid this inducti; n, balanced solutions using organic anions such as lactate, acetate etc. increases the SID and also decreases the osmolarity of the solution (Bertrand, 2010; Chappel, 2008). Morgan has calculated that a balanced solution should have a SID of 24 mEq/L (Bertrand, 2010; Morgan, 2005).

In the field of surgery and intensive care, hyperchloremic acidosis is well-known problem in patients receiving large amount of standard electrolytes. A series of studieshas emphasized the disadvantageous effects of hyperchloremic acidosis on various organ systems, for example, hemodynamics, NO-production, renal blood circulation, urinary output or hemostasis.

Having in mind that there is a lack of Ringer's lactate and Ringer's acetate solution on the drug market in our country, the aim of presented work was to formulate these IV solutions, and to evaluate theirquality and stability. Prepared solutions, were used in our Hospital in the Department of Anesthesiology.

* elenanaj@yahoo.com

Material and methods

The minimum conditions required for the small scale production and quality assurance of Ringer's lactate and acetate solutions were established by elaboration of the required Pharmacopoeias monographs (Ph.Eur.8th; USP 39). The IV fluids were prepared in laminar flow cabinet (LFC), using an aseptic technique along with sterile filtration and filled in the sterile containers. Solutionswere sterilized by autoclaving. The final solutionswerethen submitted to quality control,

Results and discussion

Prepared IV fluids had good quality in respect to physical properties, physico-chemical parameters and microbiological quality according to Ph.Eur. 8. It was also confirmed that IV fluids were stable for a year in the conditions of the second (II) climatic zone. Each of these IV solutions had a different profile in terms of impact on acid-base status, electrolyte levels, coagulation, inflammation, renal, and liver function. The choice of the best solution for patient resides in a complete understanding of the expected response of each solution and the patient 's risk factors.

In the preliminary comparison study, Ringer's lactate as well as acetate- based IV solution, proved to be suitable for fluid replacement during surgery. Hemodynamic stability remained unaffected by both of the solutions. Concerning consistency of acid base parameters none of the solutions seemed to be inferior.

Conclusions

It has been revealed that prepared formulation of Ringer's lactate and Ringer's acetate solutions can be successfully used for fluid replacement.

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Macedonian pharmaceutical bulletin, **62** (suppl) 375 - 376 (2016)
ISSN 1409 - 8695
UDC: 615.033.1
Short communication

Distribution coefficient of gliclazide as *in vitro* prediction model of blood brain barrier penetration

Mladena Lalić-Popović, Svetlana Goločorbin-Kon, Maja Đanić*, Nataša Milošević, Velibor Vasović, Boris Milijašević, Momir Mikov

Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

Development of effective *in vitro* model for prediction of blood brain barrier (BBB) penetration of drugs is nowadays widely researched. BBB is selective barrier that eclipsed the brain and isolates it from the circulating blood. It represents a major barrier for drug permeation, especially of those molecules that are highly hydrophilic, ionisable, contain more than 10 hydrophilic moieties, low logP value and molecular mass bigger than 400 Da. Distribution of drug in system octanol/water is in correlation with its lipophilicity and its optimal value ranges between 2.5 and 5 (Pardridge, 1998). Most of newly developed drugs have poor permeability. Thus novel pharmaceutical formulations are developed containing drug permeators. Drug transfer across BBB could be facilitated using permeator enhancers (e.g. bile acids). Value logP in system octanol/water is dependent on drug solvent interaction (i.e. hydrogen bonding), and because of this it is a poor predictor for BBB penetration, since this barrier is highly hydrophobic. Better system is cyclohexane/water since cyclohexane has no possibility for hydrogen bonding. If permeator that is most suitable could be predicted *in vitro* in preformulation investigations, it could decrease formulation development costs. Thus the aim of this study was to investigate gliclazide distribution in systems n-octanol/water and cyclohexane/water as *in vitro* prediction models for *in vivo* BBB penetration. Also the aim was to determine whether mentioned *in vitro* models could predict the influence of permeators such is dexycholic acid (DCA) on gliclazide transfer across BBB.

Materials and methods

Distribution coefficient (logD) was determined using a "flask shake" method. In glass tubes 1 ml of organic solvent (cyclohexane or octanol) was mixed with 5 ml of aqueous phase. Partition profile was determined over physiological pH range (pH 1.2 HCl solution, pH 4.5 acetate buffer, pH 6.8 and 7.4 phosphate buffer and pH 7 distilled water) for 5 combinations of n-octanol or cyclohexane with aqueous gliclazide solution (10 µg/ml) of different pH and with or without the addition of DCA (0.5 mM) into n-octanol or cyclohexane phase. The analyses were done in triplicate for each pair of organic solvent/water. Concentrations of gliclazide were determined using modified high performance liquid chromatography method (Mikov, 2008). Values of logD at pH 7.4 were compared with literature date of gliclazide BBB penetration and influence of DCA on its penetration (Lalic Popovic, 2012).

Results and discussion

Gliclazide is a small lipophilic molecule which is expected to readily cross biological barriers. However it has poor BBB penetration, and since it shows antioxidant properties on brain cells its penetration into central nervous system is of interest (Sandoval, 2009). Though some drugs do cross BBB, there is a great number of drugs like gliclazide for which BBB is impermeable. In this investigation a higher partition into organic layer was found in system n-octanol/water than cyclohexane/water. Profiles of distribution after 1 and 24 h were different which leads to a conclusion that 24 h is needed time for a partition to be finished in system cyclohexane/water. There were no observed differences in logD values after 1 or after 24 h between systems with and without DCA in n-octanol (pH

* majadjanic@gmail.com

6.8; 7 and 7.4), but minor differences were noticed in lower pH values (i.e. pH 1.2 and pH 4.5). Also DCA significantly increased partition of gliclazide in system cyclohexane/water but not in system n-octanol/water. Value of logD at pH 7.4 without DCA in organic layer in system n-octanol/water was 0.34±0.05 and in system cyclohexane/water was -0.74±0.11. According to logD value in cyclohexane/water, gliclazide have poor BBB, which is in correlation with *in vivo* data (logBBB 0.23±0.02 healthy animals and 0.85±0.03 diabetic animals) (Lalic Popovic, 2012). Value of logD at pH 7.4 with DCA in organic layer in system n-octanol/water was 0.54±0.07 and in system cyclohexane/water was 0.18±0.01. Both systems showed increased transfer of gliclazide when DCA was present in organic layer. This is also in correlation with *in vivo* investigation where animals were pretreated with DCA (logBBB 0.96±0.03 healthy animals and 1.35±0.14 diabetic animals) (Lalic Popovic, 2012). Thus partition of gliclazide in system cyclohexane/water better correlates with *in vivo* data where penetration of gliclazide was increased with DCA pretreatement, but in diabetic animals, penetration was increased in group with and without DCA pretreatement and investigated *in vitro* systems could not this predict.

Conclusion

Increased logD value in organic layer when DCA is present indicates existence of physicochemical interactions of DCA and gliclazide. Investigated system cyclohexane/water predicted poor gliclazide BBB penetration and the influence of DCA on gliclazide penetration but system n-octanol/water failed to do so. However investigated systems could not predict differences in penetration between diabetic and healthy animals. Further investigations are necessary to determine value of system cyclohexane/water in prediction of BBB transfer.

Acknowledgment

This work was supported by the Ministry of Science and Technology Development of Serbia (41012 III).

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Macedonian pharmaceutical bulletin, **62** (suppl) 377 - 378 (2016)
ISSN 1409 - 8695
UDC: 615.014.8
Short communication

Choosing the right blister packaging film

Biljana Pavicevic, Maja Lazarova*, Biljana Nanova, Milkica Gligorova

Alkaloid AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

The main function of packaging is to ensure protection of the pharmaceutical product, its safety and proper use, information on the identity, instruction of use and storage at any time point before expiration date of the drug product. The pharmaceutical dosage product and its container closure system act together to serve as an integral unit. The primary packaging comes into direct contact with the product and it is the most significant component in the pharmaceutical product packaging. It serves the two main functions of protecting the product from outside influences that would otherwise render it useless while allowing the manufacturer of the product to package it using practices that typically involve automated form-fill-seal equipment. As innovations continue and new films enter the market, a thorough understanding of how regulatory, package performance and film attributes fit together should help ease the selection process and, hopefully, provide some framework to avoid potential problems throughout the life of the pharmaceutical product.

CGMP requirements for packaging materials for parenteral, inhalation and liquid products are wider than for solid oral dosage forms (FDA, 1999).

Objective

Correct selection of contact packaging has a significant impact on safety and efficiency. The goal is to choose the appropriate contact material for blister packaging. Principles and contribution to simplification and proper choice of packaging material are: out of the vast number of packaging materials to choose a range of materials that can be used for packaging a certain product, materials that meet quality requirements and are tested by the manufacturer, from reliable suppliers of high quality and favorable price, to fully comply with the equipment and resources for packaging at the factory. An analysis of the characteristics of materials for blister packaging was made.

Critical factors in choosing the right blister packaging film

For choosing the right blister packaging film it is necessary to have great knowledge of drug characteristics, physical and chemical properties, microbiological quality, technical considerations of blister films, dimensional stability of the webs, ability for thermoforming and cooling of formed film, variation in thickness, compatibility of forming film and lidding foil.

Films for blister packaging

The base in blisters produced by thermoforming process is a polymer plastic film. The blisters are formed under increased temperature. PVC film used for blister packaging represents a rigid film due to the absence of softeners and plasticizers, which provides structural rigidity and physical protection of the pharmaceutical dosage form. It is transparent, rigid material with a greattermoform ability, easy to color and low cost. The main disadvantage is low barrier properties, high water vapor and gases permeability. PVDC has a significant role in blister packaging as lamination or coating of PVC. PVDC provides excellent barrier to gases and water vapor, unlike other polymers that provide either one or other protection. Permeability to gases does not depend on the relative humidity, so it can be used for packaging in various environmental conditions. The properties of the copolymer depend on the content of the VDC, greater amount of VDC result in better barrier properties, the smaller the amount of VDC the flexibility improves. The amount and type of co-monomer, as

* mstojevska@alkaloid.com.mk

well as additives and manufacturing technology affect the properties of PVDC. The weight of PVDC coatings ranges from 40, 60, 90 and 120 g/m^2. PVDC coatings have been used with duplex (PVDC/PVC) and triplex (PVDC/PE/PVC) structure being the most common ones used (Bauer, 2009). Aclar® has the lowest water vapor permeability of all the films used for blister packaging. It has good barrier properties to gases, but this material is not used often in applications where impermeability to gases is primary demand. Aclar®UltRx (CTFE homopolymer) has the highest moisture barrier of any clear thermoplastic film. It processes within the same range as other thermoforming films on conventional blister packaging equipment. PET has a higher permeability to water vapor compared to PVC. PVDC - coated PET has a similar permeability to water vapor as PVDC. PS has very good thermoforming properties, but has high permeability to water vapor. PP has good barrier properties to water vapor (similar to PVDC). The problem is thermoforming. The temperature required for thermoforming PP and the further cooling process must be precisely controlled. There may be distortion of blisters in secondary packaging due to the thickness of PP film. Another disadvantage is the thermal instability and the possibility of collection of the film after performing the process. The process of blister packaging on a standard blister machine with a PP is difficult to perform and is much slower unlike PVC. COC (Cyclic Olefin Copolymer) in multilayer combinations with PP, PE or PETG has very good barrier properties. Cyclic olefins have good thermoforming properties even for deep blister cavities. COC is used in combination with semicrystal PE or PP polymers to improve thermoform properties. The films are produced with co-extrusion or lamination. COC does not contain halogen in molecular structure comprising only from carbon and hydrogen. COC is used for packaging which requires deep blister cavities (Pilchik, 2000).

Blisters produced by cold forming are formed mechanically without the use of temperature and have high barrier properties. OPA/aluminum/PVC/ALU has excellent barrier properties for oxygen and is also impermeable to water vapor and therefore, it is the first choice of packaging material. Its cost per square meter can withstand comparison with PVDC. However, cold forming takes up more packaging material for the same number of tablets or capsules of the same size, unlike PVDC.

Multilayer films can be laminated, co-extruded, or a combination of the two. In laminated films, all film layers are extruded separately and are bonded together by a thermoset adhesive. In co-extruded films, the film is manufactured in a single-step operation in which the film layers are bonded together by a tacky thermoplastic polymer. These two manufacturing techniques can be combined. Flexible materials are used for the layers of the laminates, such as aluminum foils and films of polymer materials of varying thickness. With the right choice of a layer, a laminate with best suited properties for the packaging of a product at a low price could be obtained. Foil for the inner layer usually has good barrier properties, the lowest permeability to gases and water vapor. The inner layer should not have interaction with the product. The possibility of shaping and closing is provided by inner layer that should be termosealable (PE, PVC, PS, PVDC) (Stagnaro, 2011).

Conclusion

The categorization of the polymer film is made according to their barrier properties: WVTR (water vapor transmission rate) and permeability to gases. PVC has a significantly higher permeability to water vapor from all types of PVC/PVDC and all types Aclar®. PVC/PVDC and Aclar® are closely comparable values for WVTR, depending on the thickness of the impermeable layer in the polymer film. Gas permeability, especially O2 and CO2 is important when the product inside the packaging material is sensitive to oxidation. PVC and all types of Aclar® have significantly higher oxygen permeability of all types PVDC film. OPA/aluminum/PVC/ALU has excellent barrier properties for oxygen and is also impermeable to water vapor.

Identifying the most suitable blister packaging material is complex. It requires very good understanding of the film properties in relation to application/ machine parameters for getting an optimal packaging performance.

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Macedonian pharmaceutical bulletin, **62** (suppl) 379 - 380 (2016)
ISSN 1409 - 8695
UDC: 661.12:614.31
Short communication

Qualification of cleanrooms in pharmaceutical industry

Viktorija Veljanoska*, Silvana Gjosheva, Elena Tomovska, Milkica Gligorova

Alkaloid AD, Aleksandar Makedonski 12, 1000 Skopje, R. Macedonia

Introduction

Clean rooms and associated controlled environments provide control of airborne particulate contamination to levels appropriate for accomplishing contamination-sensitive activities. Production of sterile pharmaceutical products is regulated in a separate Annex 1 in European Guideline for Good Manufacturing Practice (EU GGMP, 2008) and contains instructions for minimizing the risk of contamination of these specific products with particles, microbes and pyrogens.

Presented study represent a critical review of some tests in guidance document for pharmaceutical industry, used in the process of qualification and monitoring of clean rooms for the manufacture of sterile medicinal products.

Qualification of clean rooms

Qualification of clean rooms in pharmaceutical industry is comprised of different tests like air supply, air velocity, air changes, flow pattern, filter integrity, pressure test, particle count, temperature, microbial count, relative humidity, noise level and vibration test.

The purpose of air supply capacity test is to demonstrate that the air system is balanced and capable of delivering sufficient air volumes as per design to maintained required air change in the defined area. The air capacity is demonstrated by following the procedure of measuring air flow in supply and returned duct and air volume to meet the design required.

The purpose of air velocity/uniformity test is to present the capability of the air system for delivering sufficient air volumes to maintain a minimum cross section velocity under HEPA terminal filter modules. The measuring is performed by calibrated anemometer at numerous sites in order to provide one measurement for each 0.37 m^2 filter area. For Laminar Flow sections air flows uniformity has to be 0.45 m/sec ± 20%.

Testing of air flow pattern is performed in order to check the interference due to turbulence eddies in unidirectional airflow area, like sampling and dispensing booth and under laminar airflow in microbiological area (Kitain, 2010) Visualizing the air patterns at numerous points in room is performed by Titanium Tetra chloride sticks. In several cases the test is done by operating the HVAC system of the sterile area and releasing smoke into unidirectional air stream at selected sites. There is no minimum GMP requirement for air changes per hour. Air flow into and out of a space should be based on providing the required cooling, heating, relative humidity, pressurization, particulate control, ventilation. These factors generally result in air change rates between 4 and 20. There is also no numerical requirement for relative pressurization in cleanroom. The velocity and direction of airflow between spaces should be adequate to reduce counter flow of airborne particulates or vapour contaminants for spaces where airborne cross contamination is a concern (EU GGMP, 2008).

All HEPA filters installed in the facility are tested for filter integrity test by using PAO (Poly-alpha-olefin) aerosol into supply duct to the HEPA filter. Sampling of stream challenge is done with photometer and the instrument is set for this challenge. Cross contamination can originate from both the internal and external facility environment. In all air handling systems, the filtration should be evaluated for adequate arrestance of outdoor particulates. Any suitable particle counter instrument can be used for the measurements, with no effect of the measurement principle used. During the operation an air flow rate should be 0.03 m^3/min. Measurement should be done at minimum 10 different representative room locations for one minute at each location at 1 meter height from the floor. It must cover the central location of personal traffic during normal production process (WHO, 2011). Measurements should obtain

* vveljanoska@alkaloid.com.mk

information about concentration of two different ranges of particles: particles with size ≥ 0.5 μm as well as particles with size ≥ 5 μm (5 μm particles for ISO 5 class has been excluded from the limit value table), at each location (DIN EN ISO 14644-1, 2015; DIN EN ISO 14644 - 2, 2015).

Room temperature variation often can be a critical parameter in production processes. Most products, materials and processes can handle a wide range in temperature. The width of this range decreases as the exposure time increases. Existing HVAC system had designed for 24 ± 2 °C to all critical area in manufacturing, warehouse and Quality Control department. Sometimes, for some products, in-processing temperature requirement shall be maintained 18 °C ± 2 °C. Relative humidity may affect exposed product or materials that are sensitive to air moisture. Relative humidity generally has effect on aqueous product. Liquid product can lose moisture to a low humidity room over an extended period. HVAC system in sterile area is designed to maintain the required humidity. Relative humidity is checked by using calibrated humidity meter from different locations (DIN EN ISO 14644-1, 2015; DIN EN ISO 14644 2, 2015).

Microbial monitoring of manufacturing clean rooms should include compressed gases, surfaces, room and enclosure air and any other materials and equipment that may produce a risk of contamination. Monitoring of the air should be performed on meter cubic air (active or passive sampling) by using special instruments or settle plates which are opened for 4 hours and after that incubated. Surface sampling is conducted by using swab or contact plat, collecting the microbiological contamination from approximately 25 cm^2 surface from numerous defined locations. Taking samples for microbiological monitoring of the area should be performed for a period of one year routine production in accordance with the sampling plan. Noise level may be present in the facility due to operation of variety of equipment, during processes etc. Requirements when personnel noise exposure exceed an eight hours time weighed average sound level of 85 dBa. HVAC system has designed not to generate more than 70 dBa noises in critical area during its normal operation (Kitain, 2010).

Conclusion

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation involved in clean rooms and advanced aseptic clean rooms operations vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment might be appropriate.

Situations where some of the parameters are out of range with values defined by guidelines of pharmaceutical products manufacturing undoubtedly are reason for deviation. After researching the presumable causes, the goal is finding solution for solving the problem, its future prevention and reseting the qualified conditions of the cleanroom.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous quality improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from contamination.

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Macedonian pharmaceutical bulletin, **62** (suppl) 381 - 382 (2016)
ISSN 1409 - 8695
UDC: 613.2:635.655
Short communication

Effect of formulation and process variables on probiotic viability after microencapsulation by spray-drying in soy protein-alginate microparticles

Jasmina Hadzieva[1]*, Maja Simonoska Crcarevska[1], Simona Dimceska[1], Nikola Geskovski[1], Marija Glavas Dodov[1], Katerina Goracinova[1], Tanja Petreska Ivanovska[2], Lidija Petrushevska[2], Nadica Vanova[1], Milena Nikolovska[1], Kristina Mladenovska[1]

[1]*Institute of Pharmaceutical Technology, Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Applied Biochemistry, Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

Consumption of probiotic products has been linked to improvement of a variety of health conditions such as hypercholesterolemia, hypertension, inflammation and lactose intolerance and reducing health risks, including allergy and even some forms of cancer. However, for probiotics health benefit to be exerted, it is estimated that they should be administered in a therapeutic minimum of 10^{-6} to 10^{-7} CFU live probiotic cells per g final product (FAO/WHO, 2006). Providing and maintaining at least this number of viable cells is a great challenge due to their sensitivity to the harsh processing and GI conditions prior taking their place of action in the low intestine and delivering the claimed health benefits. For incorporation of probiotics into food or pharmaceutical products, the microencapsulation offers protection to the fine particles produced during freeze- or spray-drying of probiotic concentrates. Among the many techniques for microencapsulation, spray-drying is one of the most challenging because of the low cost, industrial application, stability and throughput in cellular integrity while drying when optimized correctly. However, the spray-drying process parameters might have negative impact on the outcome of viable cells. In addition, the choice of carrier material and its interaction with the bacteria have importance on protection efficacy and affect the probiotic delivery. In search for novel probiotic microencapsulated formulation that will combine favorable properties of alginate (ALG) and polymer that will provide controlled and targeted release of viable probiotic cells in the lower intestine, we have encapsulated the probiotic *L. casei 01* in ALG and soy protein isolate (SPI) matrix by spray-drying. The aim of this study was to evaluate the effect of the critical formulation and process variables on viability of the probiotic after spray-drying.

Materials and methods

FD-DVS/*Lactobacillus casei 01* was supplied from Chr. Hansen (Copenhagen, Denmark), SPI from Sojaprotein AD (Becej (Serbia), whereas ALG (10/60 LS, fG 35%–45%) was kindly donated from Protanal FMC Biopolymers (Ayrshire, UK). $CaCl_2$, de Man, Rogosa, Sharpe agar and broth as well as peptone water were purchased from MerckKGaA (Darmstadt, Germany).

Aqueous mixture of ALG and SPI was inoculated with bacterial suspension (cell load ca. 12.5 $\log_{10}$ CFU/g), activated as previously described (Petreska Ivanovska et al., 2014; Smilkov et al., 2014). The resulting mixture was infused into a spray-dryer nozzle unit of Büchi Mini Spray Dryer B-290 (Büchi Laboratorius-Technik AG, Switzerland) and continuously sprayed at following conditions:

* hadzievaj@yahoo.com

nozzle diameter 0.7 mm, aspirator pressure 90%, atomizer pressure 600 Nl h^{-1}, flow rate 5 mL/min. SPI-ALG microparticles (MPs) with encapsulated *L. casei* that were further subjected to additional cross-linking by $CaCl_2$ were prepared by consequent introduction of the MPs obtained by spray-drying into aqueous solution of $CaCl_2$, followed by continuous stirring at room temperature (1 h). MPs thus obtained were removed from the solution of $CaCl_2$ by centrifugation (1000 rpm, 5 min), washed three times with sterile water, frozen at -20 °C and freeze-dried at 0.070 mbar and -50 °C for 24 h (Freeze-Dryer, Labconco, USA). Critical material attributes (concentration of ALG, SPI and $CaCl_2$) and process parameters (inlet temperature, IT) were previously identified varying one parameter at time, while the influence of critical variables on viability of the probiotic after preparation was evaluated using face centered CC-RSM design (Design-Expert® V8, Stat-Ease, Inc., USA). The total of 30 experiments were designed and carried out, with the following actual levels of studied variables: ALG (A, 1 and 4%w/v), SPI (B, 1 and 4%w/v), $CaCl_2$ (C, 0 and 5%w/v) and IT (D, 90 °C and 150 °C). Viability of the encapsulated *L. casei 01* in SPI-ALG MPs was assessed after dissolution of 1 g MPs in 9 g PBS (1 mol/L, pH 8.0), using the plate-count method as previously described (Petreska Ivanovska et al., 2014; Smilkov et al., 2014).

Results and discussion

Viability of *L. casei 01* in designed formulations after preparation was in range of 8.67-13.09 $\log_{10}$ CFU/g or expressed in % related to initial *L. casei 01* cell count from 68.38 to 99.61%. Influence of examined variables upon this response in terms of coded factors was described by reduced cubic model with the following equation:

Viability(after preparation) (%) = + 86.78 + 0.95 x A - 2.36 x B + 1.78 x C - 3.29 x D + 1.76 x A x C + 3.72 x A^2 - 6.84 x A^2 x C

From the equation one can clearly see that the viability after preparation positively correlated with the factors A, C, AC and A^2 and inversely with B, D, and A^2C. With increase in ALG and $CaCl_2$ concentration, the viability of the probiotic increased, while oppositely was observed with increase in concentration of SPI, suggesting competition between the probiotic and SPI for the same bonding sites in ALG molecules. According to the literature data (Rajam et al., 2012), modification of the compactly folded protein molecules from their native form allows SPI-ALG interactions *via* hydrophobic and electrostatic interactions and hydrogen bonding as well. At the same time, a trend to segregation of SPI and ALG into separated microdomains can occur, which has also been confirmed when whey protein as complexation agent was used (Smilkov et al., 2014). One way ANOVA indicated that variable D is significant model term, meaning that increase in the IT significantly decreases probiotic viability after preparation. The loss of probiotics during thermal processing is the main disadvantage of MPs production by spray drying and it is related to cellular injuries (e.g. denaturation of DNA and RNA, damage of ribosomes, dehydration of cytoplasmic membrane, lipid peroxidation and rupture of cell membrane) due to water removal resulting from the combined effect of heat and mechanical stress (Soukoulis et al., 2013). Thus, protection from the IT of the spray-dryer, as a critical point in this method of microencapsulation, is required. In addition, the probiotic bacteria must be alive at the time of consumption of the product and also capable of reaching the large intestine in quantities that are sufficient to facilitate colonization and proliferation. Therefore, probiotic loaded MPs should be customized in respect to their physicochemical and biological/biopharmaceutical properties as well to make them suitable for incorporation into food or pharmaceutical product as well as for administration in healthy individuals and/or individuals with specific disease.

Conclusion

L. casei 01 loaded SPI-ALG MPs were prepared using spray-drying method, with viability of the probiotic after preparation significantly exceeding the minimal therapeutic value. Process and formulation parameters should be further optimized to obtain probiotic MPs with both, high viability after preparation and in simulated GI conditions.

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Macedonian pharmaceutical bulletin, **62** (suppl) 383 - 384 (2016)
ISSN 1409 - 8695
UDC: 615.322:547.949.4
Short communication

Preparation of curcumin loaded nanoparticles: physicochemical characterization and in vitro evaluation

Elena Drakalska[1,3]*, Denitsa Momekova[1], Stanislav Rangelov[2], Nikolai Lambov[1]

[1]*Faculty of Pharmacy, Medical University–Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria*
[2]*Institute of Polymers, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., block 103-A, BG - 1113 Sofia, Bulgaria*
[3] *Faculty of Medical Sciences, University Goce Delchev–Shtip, Krste Misirkov bb, 2000 Stip, R. Macedonia*

Introduction

Curcumin is the active principle of the spice turmeric, produced by the rhizome of Curcuma longa (Zingiberaceae), which is widely used in traditional eastern medicine as a hepatoprotective, anti-infectious and anti-inflammatory remedy (Shehzad et al., 2010). A compelling body of recent evidence has shown that curcumin is endowed by pleiotropic antineoplastic effects, due to modulation of NFkB and other cell signaling pathways, implicated in cell survival, apoptosis and angiogenesis (Shehzad et al., 2010). Regretfully, the enormous therapeutic potential of curcumin can`t be exploited in clinical practice, due to its extremely unfavorable physicochemical and pharmacokinetic characteristics, and also due to the instability in systemic circulation (Singh and Khar, 2006). The contribution is focused on newly-synthetized octopus-shaped macromolecules, consisting of hydrophobic calix[4]arene core and four arms of hydrophilic poly(ethylene oxide) chains as platform for delivery of curcumin.

Materials and methods

Two methods for preparation of inclusion complexes were used:

Heating method described by (Loftsson et al., 2005) with slight modifications. Briefly, to aqueous solutions of increasing concentrations of CX[4]PEG polyoxyethylatedtertbuthylcalix[4]arene) (2 mg/ml – 12 mg/ml) a constant amount of curcumin (1 mg/ml) that exceeded its aqueous solubility (11 ng/ml) was added. The vials were closed and heated at 50 °C for two hours. After that, the samples were left at room temperature for 24 h. Then the samples were subjected to centrifugation at 5000 rpm for 10 minutes. The clear transparent supernatants containing the inclusion complexes were collected and the amount of the curcumin was analyzed using a validated UV/VIS spectrophotometric method at 427 nm.

Solvent evaporation method: series of samples containing a fixed concentration of curcumin (1 mg/ml) and increasing concentrations of CX[4]PEG (2-12 mg/ml) were prepared in absolute ethanol, and evaporated to dryness using a Buchi rotation-type vacuum evaporator (R-215, Sigma-Aldrich). The concentrations of CX[4]PEG were chosen on the basis of its critical micelar concentration (CMC) of 7.7 mg/ml (or 0.24 μmol/ml) (Momekova et al., 2012). Thereafter the dried CX[4]PEG/curcumin containing films were hydrated with deionizaed water and were left for 2 h at 50 °C and then in dark at room temperature for 24 h. Then the samples were centrifuged for 10 minutes at 5000 rpm. The transparent yellow supernatants containing the curcumin-CX[4]PEG complexes were analyzed for curcumin content using a validated UV/VIS spectrophotometric method. Phase-solubility profiles were obtained by plotting the solubility of drug versus the excipient concentration.

Characterization of the CX[4]PEG-curcumin complexation

UV/VIS spectroscopy

The UV/VIS spectra of curcumin (in absolute ethanol and 10% ethanol solution) and its CX[4]PEG complex (in deionized water) were recorded on JASCO V570 UV-Vis-NIR spectrophotometer equipped with thermostatic cell holder (Huber MPC-K6 thermostat with precision 1 °C).

Fourier transform infrared (FT-IR) spectroscopy analysis

Samples of pure curcumin, pure CX[4]PEG, their physical mixture, and a lyophilized complex were characterized by an IRAffinity-1 Shimadzu FT-IR spectrophotometer. The scanning range was between 4000 and 400 cm-1.

* edrakalska@yahoo.com

Dynamic light scattering (DLS) analysis

The size, size distribution patterns and zeta-potential of curcumin loaded supramolecular CX[4]PEG aggregates were investigated by ZetaSizer NanoZS (Malvern Instruments), equipped with a 633 nm laser. The parameters were evaluated from the measurements at the scattering angle of 175 ° at 25 °C.

In vitro *curcumin release*

The *in vitro* curcumin release from supramolecular BEC-X aggregates was evaluated by regular membrane dialysis at 37 °C against phosphate buffered saline (PBS). 1 ml of tested formulations was placed in dialysis membrane tubing (MWCO 10,000). The dialysis bag was then placed in a temperature controlled vessel, containing 100 ml of PBS (pH 7). At various time intervals aliquots were taken from the released medium and assayed for curcumin by UV–VIS spectroscopy.

Results and Discussion

Phase solubility studies

The phase solubility studies of curcumin with BEC-X were performed using the procedure utilized for the evaluation of cyclodextrin inclusion complexes by Higuchi and Connors (Higuchi and Connors, 1965). Due to their amphiphilic nature, polyoxyethylated calyx(4)arenes (CX[4] PEG) can self-associate in water by forming well-defined spherical nanoparticles. At concentration below the CMC, CX[4]PEG drastically increased curcumin solubility by formation of inclusion complexes with high stability constant (Kc). A significantly higher solubility enhancement of curcumin was observed at concentration exceeding the critical micellar concentration, attributed with additional solubilization of curcumin into the hydrophobic domains of the supramolecular aggregates by non-covalent interactions.

UV/VIS characterization

In order to characterize the spectral behavior of curcumin and its inclusion complex, absorption spectra of pure curcumin in absolute ethanol and 10% ethanol are compared with the absorption spectrum of the inclusion complex in water. The characteristic absorption peak of curcumin at 427 nm is identical in the three media under investigation, which demonstrates that the inclusion complex is formed by non-covalent hydrophobic interactions. An interesting finding is the appearance of a shoulder at 361 nm in the spectrum of pure curcumin dissolved in 10% ethanol which cannot be seen in spectra of curcumin in absolute ethanol and in the inclusion complex of curcumin in water. The shoulder can be attributed to the shifting of the tautomeric equilibrium from keto–enol to diketo-form.

FT-IR analysis

FT-IR spectroscopy is a useful tool for characterization of inclusion complexes. Characteristic combination of a sharp peak at 3508 cm^{-1} and a broad peak at 3293 cm^{-1} in the curcumin spectrum implies the presence of aromatic OH group stretching vibrations (Kolev et al., 2005) and the intensive sharp peaks at 1626 cm^{-1} and 1601 cm^{-1} corresponding to mixed C=O and C=C vibrations and symmetric aromatic ring (C=C) stretching vibrations, respectively, did not interfere with the vibrations in BEC-X spectra and can be used as marks for description of curcumin in inclusion complex.

DLS analysis

Physicochemical characteristics of the nanoparticles (size, size distribution and zeta potential were evaluated by DLS and the results revealed particles of app.180 nm with monomodal distribution (PDI below 0.2) and zeta potential of – 20 mV suitable for systemic application.

In vitro *curcumin release*

The in vitro curcumin release profiles from supramolecular CX[4]PEG aggregates were studied under simulated physiological conditions for different incubation periods from 2, 4, 6, 8, 10 and 24 hours. The results showed initial burst release of curcumin, followed by slower drug release.

Conclusion

Thus on the grounds of the excellent in vitro biocompatibility profile and the favorable physicochemical and drug loading characteristics of the tested liposomal nanoparticles, and their ability to retain the intrinsic pharmacological properties of encapsulated drug they could be considered promising drug delivery platforms for lipophilic curcumin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 385 - 386 (2016)
ISSN 1409 - 8695
UDC: 615.276.015
Short communication

Assessing the risk of alcohol-induced dose dumping: diclofenac sodium case

Marija Lukic, Andjela Lipovac, Ivana Aleksic, Sandra Cvijic*

University of Belgrade, Faculty of Pharmacy, Vojvode Stepe 450, Belgrade, Serbia

Introduction

Coadministration of alcohol beverages with extended-release (ER) oral dosage forms may lead to more rapid drug release and altered systemic exposure. Therefore, recent trends in the development of ER dosage forms highlight the importance of the investigation of potential alcohol-induced dose dumping (ADD) (Anand et al., 2011; Lennernäs, 2009). FDA and EMA guidances include general recommendations regarding the assessment of ADD, stating that suitable *in vitro* dissolution tests should be carried out to identify the risk of ADD, and differentiate between rugged and vulnerable formulations (EMA, 2014; FDA, 2016). In addition, the International Pharmaceutical Excipients Council Europe (IPEC) working group on ADD has summarized relevant scientific and regulatory information to help pharmaceutical companies to better handle ADD issues. It has been recognized that increased solubility of drugs or excipients in the presence of ethanol, and/or formulation factors (e.g. impaired ability to retard drug release), in conjunction with changes in physiological conditions after alcohol intake (e.g. delayed gastric emptying) are the key factors affecting the kinetics of drug release from ER oral formulations. Knowledge about these factors can help to identify and develop ADD-resistant formulations.

The purpose of this study was: (i) to evaluate different dissolution test setups to *in vitro* assess the effect of ethanol on dose dumping from ER tablets, and (ii) to evaluate the potential of the combined *in vitro-in silico* approach for the prediction of drug absorption profiles after concomitant alcohol intake, using commercially available diclofenac sodium ER tablets as model formulations.

Materials and methods

Diclofenac sodium solubility was tested in various media (0.1 M HCl pH 1.1 and USP buffers pH 6.8 and pH 7.4 without/with addition of 40% ethanol). Drug dissolution from the investigated hydroxypropyl methylcellulose (HPMC)-based tablets (ER1 - Diklofen® 100 mg diclofenac sodium ER tablets, Galenika a.d., ER2 - Diklofenak 100 mg diclofenac sodium ER tablets, Hemofarm a.d.) was tested under different experimental conditions: (i) in paddle apparatus at 50 rpm, using single medium pH 7.5 (USP Test 1 for diclofenac sodium ER tablets) without/with addition of 5% or 40% ethanol, and (ii) in basket apparatus at 100 rpm, using media change method (pH 1.1 without/with addition of 5% or 40% ethanol for 2 h, pH 6.8 for 2 h, pH 7.4 for 20 h). The later setup was designed to simulate changes in physiological conditions as the drug travels along the gastrointestinal tract, and to approximate conditions in the stomach, since ethanol is mostly absorbed through the gastric mucosa. The obtained dissolution data, incorporated in drug-specific absorption model (Simcyp® Population-Based Simulator, v. 14.1; Certara™, USA), were used for *in silico* simulations of drug plasma concentration-time profiles. Drug physicochemical and pharmacokinetic properties, used as inputs for absorption modelling, were obtained from available literature sources or *in silico* estimated.

Results and discussion

The solubility study results indicated that solubility-limited drug dissolution from the investigated tablets can be expected only in medium pH 1.1 without ethanol. In other words, due to increased drug solubility in the presence of 40% ethanol, concomitant intake of strong alcohol beverages might induce dose dumping from diclofenac so-

* gsandra@pharmacy.bg.ac.rs

dium ER tablets even at low pH in the stomach.

Dissolution data revealed that 5% ethanol in medium pH 7.5 had no significant effect on drug release rate from the investigated tablets (similar dissolution profiles). In addition, drug release profiles from the two products were similar in medium pH 7.5 with 0% and 5% ethanol. The addition of 40% ethanol in medium pH 7.5 affected drug release rate from ER2 formulation, resulting in approximately 4 times shorter mean dissolution time in comparison to the drug release profile in simple buffer. On the other hand, diclofenac sodium release under "media change" conditions was not affected by the exposure to either 5% or 40% ethanol in acidic medium (less than 10% of drug dissolved in 2 h; similar dissolution profiles). Considering these results, the observed difference in alcohol-resistance of the investigated products in medium 7.5 with 40% ethanol is not expected to have significant effect on drug release profiles in vivo.

The simulated pharmacokinetic parameters, based on the selected input data set, including drug dissolution rate under "media change" conditions without ethanol (Cmax 0.50/0.55 µg/ml and AUC 4.12/3.91 µg/ml h for ER1 and ER2 tablets, respectively), were in agreement with the reported data from clinical studies (Altman et al, 2015). These results indicate that the employed *in vitro* dissolution test conditions for diclofenac sodium ER tablets could be considered biorelevant. As expected based on dissolution data, the presence of ethanol in acidic medium had no effect on drug absorption profiles (the simulated pharmacokinetic parameters were Cmax 0.41-0.58 µg/ml, and AUC 3.97-4.16 µg/ml h). Simulation based on the hypothetical dissolution scenario, illustrating the "worst case", demonstrated that 100% diclofenac sodium release from 100 mg ER tablets in the stomach would alter the rate and extent of drug absorption (Cmax 2.56 µg/ml, tmax 2.16 h, AUC 6.43 µg/ml h), and consequently, the formulation would lose its modified release characteristics. But even this scenario would not pose safety issues for the patients, since the simulated plasma concentration profile is in the therapeutic range (Altman et al, 2015).

Conclusion

The presented case demonstrate that *in vitro* dissolution testing using the proposed "media change" experimental setup could be indicative of drug *in vivo* behaviour in the presence of ethanol. In addition, the combined *in vitro-in silico* approach may provide insight into the effect of ADD on drug clinical performance, and therefore, can serve as an alternative to clinical studies for ADD risk assessment. This approach should be encouraged, and applied to other ER oral drug products.

Acknowledgment

This work was done under the project TR34007, supported by the Ministry of Education, Science and Technological Development, Republic of Serbia

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Macedonian pharmaceutical bulletin, **62** (suppl) 387 - 388 (2016)
ISSN 1409 - 8695
UDC: 615.275.031
615.212.031
Short communication

Small scale production of gel with menthol, benzocaine and procaine HCl

Slavica Maleska Stojadinovik[1]*, Bistra Angelovska[2]

[1]*Public Hospital –Ohrid, Dimitar Vlahov St. 33, 6000 Ohrid, Republic of Macedonia*
[2]*Faculty of Medical Sciences, University "Goce Delcev", 2000 Stip, Stale Popov 92/55 Skopje, Republic of Macedonia*

Introduction

Antirheumatic and analgesics drugs are the most commonly used drugs to treat diseases of the musculoskeletal system and to alleviate all sorts of pain that are caused from different origins. Their systemic use causes long series of side effects, so combination therapies of drugs with systemic and local action or only medicinal products intended for topical use wherever possible, especially in physical therapy, would be treatment of choice.

Various formulations of dosage forms, by composition and consistency, allow different therapeutic approach, suitable for achieving the desired therapeutic effect and for being capable to adapt to the physiotherapist treatment.

According to the regulations of the pharmacopoeia or to other regulations, the galenic drugs are made in small batches in a galenic laboratory, which are intended to be administrated directly to a pharmacy or a health institution.

According to the health requirements, the preparation of pharmaceutical dosage forms in a galenic laboratory, mainly aims to provide a dosage form, which is designed as an opportunity for changes in the composition and consistency of the product. Therefore, it can be provided a product with a modified properties, compared with a finished and fixed composition of the medicinal and an auxiliary substances, or with the already existing traditional products, which have the same composition of active components, satisfying the needs of the healthcare professionals who work with them and also allowing a simultaneous comfort and a therapeutic effect for the patients.

The usage of this drug, immediately after the preparation, reduces the need for adding more funds to stabilize and to ensure longer shelf life.

Obtaining the quality of this drug product, that satisfies the standards and maintains or enhances the therapeutic properties, requires appropriate conditions for a preparation and knowledge of all the components properties, technological process and requirements for the finished product as well.

The usage of the traditional liquid, a pharmaceutical product known as "Russian water", which contains: alcohol, menthol, procaine hydrochloride and benzocaine, is well received and accepted by the patients and the physiotherapists, but it also has some negative sides, such as, short effect, it is easily volatile, it acts surface and cannot be recommended as a massage supplement. On the other hand, gels are a dosage forms for external use that can be applied easily, to tie up to the skin and to enable the drug to heal the affected area,it can realize deep action, such as the ability to quickly penetrate multi-layers on the skin, to be easily rinsed from the site of application and generally does not irritate the skin, which is largely satisfying for our requirements.

The aim of this study was to formulate and prepare gel formulation from the pharmaceutical composition named "Russian water", with the ingredients: menthol, benzocaine, procaine HCl, which is easy and pleasant to apply on the painful area, also effective to reduce the pain and the inflammation. Formulation challenge was slight solubility of some of the components, getting a stable product with a proper consistency, ensuring the release of the active ingredients, their affection on the skin and a prolonged action.

Materials and methods

In the formulation and preparation the following active substances were used: menthol (Alkaloid), benzocaine

* slavica.maleska@yahoo.com

(Sigma Aldrich), procaine HCl (Sigma Aldrich), at the recommended doses and ingredients, carbopol 940 (Sigma Aldrich), glycerol, (Alkaloid), triethylene tetramine (Sigma Aldrich) and aqua distillate.

Gel with menthol, benzocaine and procaine HClwas prepared in the galenic laboratory in Ohrid -General Hospital.Used ingredients and a compounding procedure described in a rational manner are:

menthol 4.0, benzocaine 2.0, procaine HCl 2.0, base gel ad 200.0. Basic gel was made with the following proscription:Carbopol 940 2.0, glicerolum 7.5, triethylene tetramine 2.5, aqua destilata 190.0.

In the step 1, preparation of base gel, Carbopol 940 and aqua distillate 50 mL were left to bulge. Then glycerolumwas gradually added and mixed to achieve good homogenization. After that, triethylene tetraamin was added and mixed again until thick gel was obtained.In the step 2, preparation of healing gel, the menthol was grinded through a sieve, and it was measured in the prescribed amount. It was mixed with the same amount of prepared basic gel to complete uniformity of the mixture. The benzocaine was grinded, sieved and measured at the prescribed amount and mixed with equal quantity of earlier prepared gel with menthol.

The prescribed amount of procaine hydrochloride was measured and dissolved in distilled water 10mL. Then, the solution was gradually added to the gel with menthol and benzocaine, whileconstantly stirred.The remaining amount of distilled water was added, gradually and with constant stirring, to the homogeneous gel to obtain a homogeneous product.

Results and discussion

The obtained gel is a homogeneous product with milk appearance. The high degree of pulverization of the components should improve solubility and ensure uniform distribution of insoluble components in the mixture. The process of mixing equal amounts of the basic mixture and pulverized substance also provides uniform distribution of the components, and the high density of the base gel reduces the possibility of their sedimentation (Allen, 2002; Jovanovic and Sekulovic, 1987; Racev and Lambov, 2005; Simov et al., 2001; Winfeld and Richards, 1998). Menthol is practically insoluble in water and because of its sole distribution in the preparationit was pulverized and a special way for its incorporation into the system was applied.Benzocaine is poorly soluble in water and an appropriate pulverization and mixing improved solubility and its uniform distribution in the product.The procedure of mixing and gradual addition of a small amount concentrated procaine hydrochloride solution reduces the possibility of decomposition of the gel structure under the influence of the potential ionic solution (Jackson and Lowey, 2010; Simov et al., 2001).

The obtained gel is packaged in plastic recipients of 200 mL, closed with a suitable stopper and signed with red signature marked “For external use.”It is used topically by applying to the affected area and/or with good rubbing in physical therapy.The product has a short shelf life and is prepared immediately before use, and to ensure the quality of the product it is recommended to be kept at a room temperature in well-closed recipients.During application it gives pleasant cooling sensation and pain relief and easily rinse out with water. It is not toxic and does not cause skin irritation.

The product can be prepared in galenic laboratory, and easy application and efficiency makes it acceptable to patients (Jackson and Lowey, 2010). It has been retained at the administration site longer than the expected, and has longer actions regarding to the liquid alcohol solution, which is with the same components.

Conclusion

The results of the treatment of patients according the investigation of the staff that operated and the practical patients show that this gel is with a prolonged analgesic activity compared to the liquid preparation, with an easy and safe application, a pleasant cooling sensation and a pain relief, also an easy flushing and with an increased satisfaction of therapists and patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 389 - 390 (2016)
ISSN 1409 - 8695
UDC: 615.37.074
Short communication

Approaches in evaluation of freeze-dried antibody conjugates

Katarina Smilkov[1]*, Darinka Gjorgieva Ackova[1], Petre Makreski[2], Icko Gjorgoski[2], Emilija Janevik- Ivanovska[1]

[1]*Faculty of Medical Sciences, Goce Delčev University, Krste Misirkov No.10-A P.O 201, 2000 Štip, R. Macedonia*
[2]*Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Arhimedova Str. No. 3, PO Box 162, 1000 Skopje, R. Macedonia*

Introduction

Antibodies, proteins and other biotechnological products are often challenging in terms of their in-solution stability. Various physical and chemical changes occur during their in-solution storage, leading to shorter shelf-life. Freeze drying is often proposed as a method of choice, since removal of water has been reported to greatly reduce the rate of degradation, both in physical and chemical terms. Still, during freeze-drying antibodies and other protein pharmaceuticals can experience in-process changes that may reduce their physicochemical biological and/or pharmacological properties. In this context, many attempts have been made to reduce these changes, both in optimization of the freeze-drying process and in optimization of solution formulation, adding various buffers, cryoprotectants, etc.

The presented experience was in freeze-drying of monoclonal antibody – rituximab, conjugated with three types of bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA, and evaluation of possible changes in post-freeze-drying phase. In order to assess possible defragmentation of the antibody, protein integrity test was performed, using SDS-PAGE electrophoresis and the analysis of several structural elements of FT-IR and Raman spectra pre- and post- freeze-drying process, provided an insight in possible changes in the structure.

Materials and methods

Commercially available rituximab (Mabthera®) was conjugated with three bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA (Macrocyclics Inc. USA). The conjugates were synthesized, purified, adjusted to concentration of 1 mg/mL and freeze dried, using Labconco Free Zone Stoppering Tray Dryer (USA), as previously described (Smilkov et al., 2014). The protein integrity was assessed using SDS-PAGE that was performed in about 5 μL of reconstituted samples and 1 mg/mL purified, commercial rituximab (Mabthera®). Samples were mixed with sample buffer and boiled 5 min at 95 °C. Approximately 5 μL of each preparation was applied in 12% bisacrylamide under reducing conditions. Visualization of the bands was enabled using Coomassie Brilliant Blue R-250 (Sigma, USA). For comparison, low molecular weight marker (Amersham GE Healthcare, UK) was used.

For determining protein structure, FT-IR spectroscopy was conducted on PARAGON 1000 (Perkin Elmer, USA) spectrophotometer in the spectral range 2000–500 cm^{-1}. Attenuated Total Reflectance (ATR) spectra were acquired at a resolution of 4 cm–1. The obtained data was processed with Grams_32 software (Thermo Scientific). Raman spectra (2000–400 cm^{-1}) were recorded on a micro-Raman multichannel spectrometer Horiba JobinYvon LabRam 300 Infinity, using He:Ne laser. The spectral resolution was set to 4 cm^{-1}. The acquisition time and the accumulation number were set to 10 s and 10 scans, respectively (Gjorgieva Ackova et al., 2015).

Results and discussion

Using SDS-PAGE electrophoresis it is possible to determine the purity and, therefore possible defragmentation. The electrophoresis in reducing conditions resulted in two distinct Mw species which migrated in two bands in all

* katarina.smilkov@ugd.edu.mk

three rituximab conjugates. The upper band corresponded to ~50 kDa and the lower band to ~25 kDa. This migration pattern is characteristic of IgG antibodies that have two identical subunits, each composed of two polypeptide chains: two heavy and two light chains, linked via four disulfide bonds. Under the action of the reducing agent DTT, the antibody is separated to heavy and light chains, with molecular weight corresponding to the two formed bands (Maleki et al., 2013; Nebija et al., 2011). The lyophilization protocol used did not affect structure properties and caused no post-lyophilization modification, as shown in the reducing SDS-PAGE lane patterns, compared to commercially available rituximab sample.

Spectroscopy studies can reveal information to witness preserved secondary structure upon freeze-drying, a mandatory prerequisite for immunoconjugates. Protein denaturation upon lyophilization is usually monitored by IR spectroscopy (Murphy et al., 2012), although Raman spectroscopy can also be applied (Wen, 2007).

The IR spectra of all three rituximab conjugates revealed higher percentage of β-sheet conformation (antiparallel and parallel) in the structure (strong band in the region between 1612 and 1640 cm^{-1}, followed by a weaker band around 1685 cm^{-1}), followed by α-helices (bands at 1655 or 1656 cm^{-1}), as obtained in the band frequencies for amide I, II and III bands which are used as diagnostic bands. We observed that the freeze-dried rituximab conjugates regain their native conformation upon rehydration (reversible unfolding).

Thermally-induced aggregation processes of the majority of proteins can also be studied by FT-IR and Raman spectroscopy. Strong absorption bands below 1620 cm^{-1} can be correlated with aggregation, usually associated with the formation of new strong beta-sheet structures (Schüle et al., 2007). With the lowest frequency band detected at 1620 cm^{-1} (in all samples analyzed), we concluded no obvious aggregation in all three freeze-dried antibody conjugates.

Conclusion

Among the many techniques available for evaluation of the structure and stability of freeze-dried antibody conjugates, SDS-PAGE electrophoresis, FT-IR and Raman spectroscopy can be employed in assessing structural properties as well as in determination of stability of these potential drug candidates.

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Macedonian pharmaceutical bulletin, **62** (suppl) 391 - 392 (2016)
ISSN 1409 - 8695
UDC: 615.282:577.115
Short communication

An injection method for preparation of liposomes as ketoconazole carriers

Olga Popovska[1]*, Jana Simonovska[1], Elena Trajkoska-Bojadziska[1], Zoran Kavrakovski[2], Vesna Rafajlovska[1]

[1]*Institute of Organic Technology, Faculty of Technology and Metallurgy, University "Ss. Cyril and Methodius", Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Applied Chemistry and Pharmaceutical Analyses, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

The drug delivery with a specified rate in the body for a certain period of time has become an important tool for improving therapeutic index (Patel et al., 2009). Reducing toxicity to the health cells and potential drug degradation is important for achieving an effective treatment (Chanda et al., 2011; Kaur and Kakkar, 2010). Liposomes are considered to be excellent models of cell membranes, as well as for stabilizing the pharmaceutical active substances (Chanda et al., 2011; Kaur and Kakkar, 2010). Phospholipids and cholesterol assembled in one or more lipid bilayers with an aqueous core are the main components in the microscopic spherical liposome vesicles (Chanda et al., 2011). The specific characteristics of the liposomes such as non-toxicity, flexibility, targetability to specific cells or tissues, and biodegradation make them drug carriers, reducing drug toxicity through encapsulation (Kaur and Kakkar, 2010; Patel et al., 2009). The importance of their utilization is due to the ability to encapsulate hydrophobic, hydrophilic, and amphiphilic active pharmaceutical substances (Gómez-Henz and Fernández-Romero, 2006; Sahasrabuddhe et al., 2012). Generally, liposome formulations are classified based on their structural characteristics, substances in their composition, size and lamellarity, method of preparation, and their application (Kaur and Kakkar, 2010; Sahasrabuddhe et al., 2012).

Ketoconazole (cis-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) is a chiral lipophilic imidazole antimycotic drug administered mainly as a racemic mixture (50:50) of enantiomers in the cis configuration. The decomposition of the drug could be easily caused through acidic, chemical, photolytic, and oxidative conditions. Commercially available ketoconazole pharmaceutical dosage forms such as topical cream, antidandruff shampoo, ointments, and tablets possess anti-inflammatory and some antibacterial activities (Patel et al., 2009; Sahasrabuddhe et al., 2012). Potential nephrotoxicity, hepatotoxicity, and decomposition of ketoconazole require preparation of new safety formulations (Patel et al., 2009). In the ketoconazole liposome preparation, a thin-film hydration (Patel et al., 2009) and an injection method using either chloroform (Sahasrabuddhe et al., 2012) or dichloromethane (Patel et al., 2009) were described. The potential adverse effects of alkyl halide solvents (WHO, 2004) emphasize the aim of this work for introducing less toxic solvent such as methanol in ketoconazole liposome preparation through injection method.

Materials and methods

Standard ketoconazole was received as a gift from Pharmaceutical Company Replek Farm DOOEL–Skopje, Republic of Macedonia. Analytical grade methanol, HPLC grade methanol and water were supplied from Merck (Germany). Cholesterol was supplied from Calbiochem (Japan) and L-α-phosphatidylcholine (egg yolk) was from Sigma (Germany). Potassium hydrogen phosphate and potassium dihydrogen phosphate were supplied from Alkaloid AD (Republic of Macedonia). An analytical balance Mettler Toledo (Switzerland) was used for the sample

* olga_popovska@yahoo.com

weighing with 0.1 mg accuracy. A magnetic stirrer (type MM–530 Tehnika Železniki, Slovenia), a rotary evaporator (Devarot Elektromedicina, Slovenia), and a centrifuge (MRC, Pharmachem, Republic of Macedonia) were used in the ketoconazole vesicle preparation. An Agilent Technologies 1200 high-pressure liquid chromatographic (HPLC) system (USA) equipped with a diode array detector (G1315D), a binary pump (G1312A), a degasser (G1379B), a thermostatted column (TCC G1316A), and an autosampler (ALS G1329A) were used for the encapsulation efficiency determination of ketoconazole in liposome formulations. The chromatographic separation was achieved on a column LiChrospher®100 C-18 (150 mm length x 4.6 mm i.d., 5 μm particle size) with a mixture of methanol and water (90:10 v/v) mobile phase adjusted to pH 8.90 with a phosphate buffer. The absorbance was recorded at 296 nm. The morphology of ketoconazole liposomes was determined with an optical microscope Konus, (type M–100–FL, Italy), equipped with a digital camera (Sony, Cyber–shot W, type DSCW830V.CE3) and a lens ZEISS Vario–Tessar 8x (China).

Ketoconazole, L-α-phosphatidylcholine, and cholesterol in the weight ratio of 3.3:1:3.3 w/w/w were transferred with 5 mL methanol in a 100 mL round-bottom flask. Following the homogenization process on the magnetic stirrer at 1000 min^{-1}, methanol was evaporated in the rotary evaporator at 55 °C and 80 min^{-1} (10 min). The lipid mixture was added to a portion of 5 mL distilled water (80 °C). The ketoconazole liposome mixture was left overnight in order to mature at 4 °C. The separation of the liposome and free amount of ketoconazole was done in the centrifuge at 5000 min^{-1} for 10 min.

Results and discussion

In the preparation of ketoconazole liposomes through injection method, the solubility of ketoconazole was considered as a criterion of choosing less toxic methanol instead of chloroform (Sahasrabuddhe et al., 2012). The large unilamellar ketoconazole liposome vesicles were prepared using the injection method with methanol. The determined encapsulation efficiency of the ketoconazole liposome formulations in methanol of 45% was smaller in comparison to the encapsulation efficiency of the ketoconazole in chloroform (71%) obtained by Sahasrabuddhe et al., 2012. The smaller encapsulation efficiency was justified with harmless preparation conditions of ketoconazole liposome formation with methanol. The encapsulation efficiency and morphological appearance were followed with evaluated stability of ketoconazole liposome formulations at 4 °C and 25 °C, during a month. After the first week and one month of the liposome preparation, during stability test at 4 °C, the encapsulation efficiency was 43% and 40%, respectively. At 25 °C, the encapsulation efficiency was decreased to 40% after the first week of the preparation, while after one month, the determined encapsulation efficiency was 45% less than the initial value. The uniformed liposome appearance with a tendency of aggregation at higher temperature (25 °C) during one month stability test was confirmed using microscopic analysis. The drug leakage increases at higher temperature as a result of higher lipid fluidity (Patel et al., 2009).

Conclusion

The injection method using methanol could be applied in the ketoconazole liposome preparation. Process and formulation parameters should be further optimized to obtain vesicles with higher encapsulation efficiency, improving stability after preparation, as well as in simulated conditions, and sterilizing the obtained liposomes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 393 - 394 (2016)
ISSN 1409 - 8695
UDC: 615.272.4.015
Short communication

In vitro model for the analysis of 12-monoketocholate impact on simvastatin physico-chemical behavior in octanol/buffer system

Maja Đanić*, Nebojša Pavlović, Mladena Lalić Popović, Bojan Stanimirov, Svetlana Goločorbin Kon, Karmen Stankov, Momir Mikov

Faculty of Medicine, University of Novi Sad, Serbia, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

The octanol-water distribution coefficient represents a physico-chemical property of a compound, being a valuable parameter in the understanding of its biological behavior. It has been widely used in numerous quantitative structure-activity relationship (QSAR) models for predicting the pharmaceutical properties of molecules (Hughes, 2008). Determination of distribution coefficient is commonly used to predict the drug transport through biological membranes and to test the effect of drug promoters on drug distribution (fanić, 2016). Bile salts are known for their function as modifiers of drug penetration across the biological membranes (Stojančević, 2013). The effect of bile salts on drug penetration appears to be complex and it depends on the physico-chemical properties of the drug and on interactions of the bile salt with the physiological environment. Taking into account that simvastatin (SV) is a highly lipophilic compound with extremely low water-solubility and bioavailability (<5%), many efforts have been made to increase the aqueous solubility of the drug consequently leading to the increase of its bioavailability. Accordingly, the aim of this study was to estimate the influence of semysinthetic bile salt, sodium 12-monoketocholate (MKC), on the distribution coefficient of simvastatin and to suggest molecular mechanisms responsible for this effect.

Material and methods

Distribution coefficients (logD) of SV with or without MKC were determined using a "flask-shake" method at pH 5 and pH 7.4, resembling gastrointestinal environment. The aqueous media used for these pH conditions were 0.1 M sodium acetate and 0.035 M sodium phosphate buffer, respectively, while octanol was used as organic solvent. Experiments were performed according to the procedure described by Serajuddin et al. (1991). Concentrations of SV were determined by high performance liquid chromatography method according to method described by Carlucci et al. (1991). The detection was performed at 238 nm. In order to analyze theoretically complexation of SV with MKC, semi-empirical PM3 method implemented in MOPAC software package in the Chem3D Ultra 7.0.0 program has been applied. It was also used for computation of physico-chemical properties of observed compounds to give a better interpretation of obtained in vitro results. Data were analyzed using OriginPro Software (OriginLab Corporation, MA, USA).

Results and discussion

As a highly lipophilic molecule and weak acid, in more acidic environment, SV is expected to be more in its neutral form that can more easily penetrate biological barriers which explains higher values of distribution coefficient at lower pH (4.70±0.01 at pH5 vs. 4.59±0.06 at pH7.4). The experimental logD values of SV were in good agreement with the calculated logD values reported by Serajuddin et al. (1991). Upon addition of MKC, the distribution coefficient of simvastatin significantly decreased at both selected pH (4.60±0.02 at pH 5, and 4.41±0.05 at pH 7.4). This means that the concentration of SV, i.e. the solubility of simvastatin, in buffer layer is increased in the presence of bile salts. Value logD in system octanol/buffer is dependent on drug solvent interaction. Chemical structure of bile acids is different from ordinary aliphatic surfactants, due to

* majadjanic@gmail.com

the presence of a large, rigid, and planar hydrophobic moiety of a steroid nucleus carrying 2–4 hydroxyl groups and an ionic head of a carboxyl group, which provide the molecule a planar polarity with hydrophilic and hydrophobic domains (Mikov, 2006).The increase in SV solubility correlates with known high hydrophobicity of SV for which strong hydrophobic interactions with the lipophilic steroid nucleus of bile salt are to be expected and are confirmed in analyzed complex SV-MKC. Significantly higher values of Conolly Accesible Area (SAS), Conolly Molecular Area (MS), and Conolly Solvent Excluded Volume (SEV) of SV-MKC complex than SV alone additionally support experimentally obtained results. MKC has similar structure to cholic acid, differing only in a keto group at the position 12 instead of hydroxyl group. It has been shown that replacing of hydroxyl with keto group in MKC resulting in decreased membrane toxicity without compromising ability to enhance membrane permeability (Lalic-Popovic, 2013; Stojančević, 2013), which makes it as a good candidate for the novel drug formulations.

Conclusion

Our data indicate that the addition of MKC into the octanol/buffer system decreases the values of SV distribution coefficient. This may be the result of the formation of hydrophilic complexes increasing the solubility of SV that could consequently lead to the increase of its bioavailability. Results of this study could contribute to the development of new formulations with improved pharmacokinetic properties and enhanced bioavailability of SV.

Acknowledgement

This work is supported by Ministry of Education, Science and Technological Development of Serbia, Project III41012.

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Macedonian pharmaceutical bulletin, **62** (suppl) 395 - 396 (2016)
ISSN 1409 - 8695
UDC: 633.843-157.6:581.192
Short communication

Influence of the particle size at oleoresin extraction from red hot pepper

Jana Simonovska[1]*, Olga Popovska[1], Elena Trajkoska-Bojadziska[1], Željko Knez[2], Zoran Kavrakovski[3], Vesna Rafajlovska[1]

[1]*Institute of Organic Technology, Faculty of Technology and Metallurgy, University "Ss. Cyril and Methodius", Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia*
[2]*Laboratory of Separation Processes and Product Design, Faculty of Chemistry and Chemical Engineering, University of Maribor, Smetanova 17, 2000 Maribor, Slovenia*
[3]*Institute of Applied Chemistry and Pharmaceutical Analyses, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

The pepper (*Capsicum annuum L.*) which belongs to the genus Capsicum is a widely cultivated and distributed vegetable crop (Huq and Arshad, 2010). It is consisted of carbohydrates, proteins, carboxylic acids, minerals, vitamins, and other biologically active compounds. The green, yellow, orange, and red pepper colour is dependent on a carotenoid pigment profile. Capsaicin as the main capsaicinoid compound determined the pungency in the red hot pepper (Kumar et al., 2011). Although the pepper is produced seasonally, it is consumed throughout the year in either various specialties or spices and oleoresin extracts (Huq and Arshad, 2010; Kumar et al., 2011). The pepper is not only popular spice, but it could also be used in plant based insecticide formulations (Huq and Arshad, 2010). The *Capsicum annuum L.* could be differed from shape, pungency level, colour, and content of biochemical compound. The colour could be evaluated from surface and extractable colour aspects (Malacara, 2011). The red colour intensity is considered as the main quality attribute of the red pepper and oleoresins, since it influences on both consumer acceptance and commercial value. The surface colour is dependent on pepper species and conditions during growing, storage, and processing (Belovič et al., 2014). Many factors affect pepper colour loss such as photolytic and thermal conditions, as well as the oxidative degradation of carotenoid pigments (Belovič et al., 2014). Several different methods for the pepper colour evaluation, based on the surface colour measurement and profiling of the carotenoids are reported in the literature (Belovič et al., 2014). Surface colour measuring is the method for describing colour changes closest to sensory visual perception (Malacara, 2011). In this work the influence of the particle size at extraction of pericarp, placenta, seed, and stalk of red hot pepper of Macedonian origin was studied by the determination of the extract yield and surface CIE Lab colour values.

Materials and methods

The red hot pepper (*Capsicum annuum L.,* spp. microcarpum longum conoides, convar. Horgoshka) was grown in the locality Markova Cheshma, Prilep, Republic of Macedonia. Dried fruits of the red hot pepper were cut manually longitudinally with a knife. Pericarp was separated from the seed, placenta, and stalk. Analytical grade petroleum ether (40–60 °C) was supplied from Merck (Germany). An analytical balance Mettler Toledo (Switzerland) was used for the sample weighing with 0.1 mg accuracy. The samples were ground using a Retsch ZM1 mill (Germany). The extraction of the red hot pepper (10 g ± 0.1 mg) was carried out with Soxhlet procedure using petroleum ether (40–60 °C). After 420 min extraction, the solvent was removed at 40 °C, 200 mPa using a rotary evaporator (Devarot Elektromedicina, Slovenia). The steps of

* j_klopcevska@yahoo.com

drying, cooling, and weighing were repeated until the difference between two consecutive weights was smaller than 2 mg. The yield of the extract was calculated based on the dry matter weight of the red hot pepper sample used. The extraction procedure was performed in duplicate under the same operating conditions. Visual colour of the samples investigated was evaluated measuring the CIE Lab coordinates L* (lightness–darkness), a* (red−green), b* (yellow−blue), chroma (C), and Hue (h°) using Dr. LANGE spectra colorimeter (Chelmsford, UK). Colour difference (ΔE) was calculated from a*, b*, and L* parameters, using Hunter-Scotfield's equation (Ergüneş and Tarhan, 2006).

Results and discussion

At extraction with petroleum ether (40–60 °C), 6.42% extract yield was obtained from pericarp with 0.25 mm particle size. Increasing the pericarp particle size from 0.5 to 1 mm, the extract quantity decreased, from 4.60 to 2.68%, respectively. The extract yield from red hot pepper placenta with 0.5 mm particle size was 7.05%. The difference in the extract quantity obtained from the placenta with 0.5 mm and 1.0 mm particle size was 1.49%. At seed extraction, the highest quantity of the extract was obtained in comparison to pericarp, placenta, and stalk. The highest extract yield of 22.32% was determined at seed extraction with 0.5 mm particle size. The obtained stalk extract quantity was the lowest, 3.70, 2.98, and 1.98% with particles size of 0.25, 0.5, and 1.0 mm, respectively. The empirical models tested showed a good agreement between experimental and calculated data for the extract yield ($R^2 > 0.90$). The values of colour parameters varied between the samples of pericarp, placenta, seed, and stalk of red hot pepper. According to the L* values, seed samples were characterized by the brightest (72.28), while pericarp samples by the darkest colour (54.26). The determined values of the colour parameter L* for placenta and seed were close to each other (65.59). High values of redness, a* (32.11) and saturation, C (41.94) were measured for the pericarp samples. The estimated value of a*/b* ratio in seed was the lowest. Hue (h°) values were linear correlated ($R^2 = 0.9793$) with the values for the ratio of red (a*) and yellow colour (b*). The values of the L*, a*, b*, and C decreased with increasing the sample particle size ($p < 0.05$). The sample with 0.25 mm particle size was whiter, vivid, and deep red in colour than samples with particle size of 0.5 mm and 1.0 mm. The highest colour difference before and after extraction with petroleum ether (40–60 °C) was determined for the pericarp with 0.25 mm particle size (ΔE = 27.12). Increasing the particle size from 0.5 to 1.0 mm, ΔE for pericarp was decreased from 22.72 to 14.78. The influence of the particle size during extraction of placenta is insignificant. For placenta with 0.5 mm and 1.0 mm particle size, estimated ΔE values were 19.28 and 18.67, respectively. The lowest ΔE values were determined during stalk extraction. The values of colour parameters for obtained extracts from pericarp, placenta, seed, and stalk at extraction with petroleum ether (40–60 °C) correlated with the determined colour values of the samples before and after extraction. The colour of red pepper is controlled by carotenoids. Capsanthin, capsorubin, and xanthophylls are responsible for the red, while β-carotene and zeaxanthin for the yellow-orange colour. The red colour of different spices of pepper is mainly due to the biosynthesis of keto-carotenoids such as capsanthin and capsorubin (Ergüneş and Tarhan, 2006). The published data for the surface colour were related to the pericarp as an edible part of the pepper fruits. The differences in the pepper varieties, the maturity degree of the pepper, as well as the influence of drying and processing conditions were studied using characteristics of the surface colour (Belovič et al., 2014; Zaki et al., 2013). Results for the visual colour samples of pericarp are in agreement with those reported in the literature (Belovič et al., 2014; Ergüneş and Tarhan, 2006; Zaki et al., 2013).

Conclusion

The extraction of pericarp, placenta, seed, and stalk of red hot pepper was carried out with petroleum ether (40–60 °C). The bigger particle size enabled to obtain less extract yield. The highest quantities were extracted from seed, while the lowest quantities from stalk. The colour differences increased with decreasing the particle size. The highest colour differences were estimated at extraction of pericarp.

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Macedonian pharmaceutical bulletin, **62** (suppl) 397 - 398 (2016)
ISSN 1409 - 8695
UDC: 635.71:581.135.5
Short communication

Development of nanoemulsion formulations of wild oregano essential oil using low energy methods

Elena Trajkoska-Bojadziska[1]*, Jana Simonovska[1], Olga Popovska[1], Željko Knez[2], Zoran Kavrakovski[3], Biljana Bauer[4], Vesna Rafajlovska[1]

[1]*Institute of Organic Technologies, Faculty of Technology and Metallurgy, University "Ss. Cyril and Methodius", Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia*
[2]*Laboratory of Separation Processes and Product Design, Faculty of Chemistry and Chemical Engineering, University of Maribor, Smetanova 17, 2000 Maribor, Slovenia*
[3]*Institute of Applied Chemistry and Pharmaceutical Analyses, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*
[4]*Institute of Pharmacognosy, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

Essential oils are aromatic, volatile, natural oily liquids obtained from plants, mainly leaves and flowers, as well as from fruits, seeds, roots, and stems. The most common known essential oils are those from lemongrass, oregano, peppermint, rosemary, thyme, cinnamon, and clove. A variety of volatile compounds such as terpenoids, phenol-derived aromatic and aliphatic compounds were determined in the essential oil composition. The essential oils are recognized for their medicinal value such as analgesic, sedative, anti-inflammatory, spasmolytic, and locally anaesthetic remedies. As a powerful natural products with bactericidal, virucidal, fungicidal, antiparasitical, and insecticidal properties, the essential oils take a significant place in cosmetic, pharmaceutical and food industry, as well as in agriculture (Hüsnü Can Başer and Buchbauer, 2010). Concerning the complex chemical composition, the essential oils could be easily decomposed. Oxidative and photolytic conditions resulted in a quality loss and reducing of the biological activity (Bilia et al., 2014). In order to protect and to use in drug delivery systems, nanoencapsulation of the essential oils was proposed due to their capability of improving the solubility, stability, and efficacy of essential oil-based formulation. Nanoemulsion is dispersion of two immiscible liquids with droplet size ranging from 20 to 200 nm (Solè and Solans, 2013). Low and high energy methods are used in the development of nanoemulsion formulation. The high energy method required the mechanical energy such as an ultrasonic procedure and a high pressure homogenization. On the other hand, surfactants as emulsifiers either in aqueous phase titration or in spontaneous emulsification low energy method are used (Schuh et al., 2014).

The objective of this study was to develop stable nanoemulsions of wild oregano essential oil using low energy emulsification methods.

Materials and methods

Surfactants: Tween 80 (polyoxyethylene sorbitan monooleate, Sigma–Aldrich, Germany), Tween 20 (polyoxyethylene sorbitan monolaurate, Merck, Germany), and Span 80 (sorbitan monooleate, Fluka, Germany) were used. Propylene glycol (PG) was supplied from Aldrich (Germany). Wild oregano essential oil (Origanum minutiflorum) produced in Turkey was purchased from Inter Connection Group DOOEL (Republic of Macedonia). MilliQ PURElab Classic system (ELGA, USA) supplied ultrapure water.. An analytical balance Mettler Toledo (Switzerland) was used for the sample weighing with 0.1 mg accuracy. A magnetic stirrer (type MM–530 Tehnika Železniki, Slovenia) and a vortex (EV–102 Tehnika Železniki, Slovenia) were used in the preparation of the wild oregano na-

* ekatrajkoska@gmail.com

noemulsion formulations. Pseudo ternary phase diagrams consisting of wild oregano essential oil, mix of surfactans Tween 20, Tween 80 and Span 80 (Smix), and water were constructed using aqueous titration method (Shaaban and Edris, 2015). In glass vials, the wild oregano essential oil was mixed with Smix in weight ratios from 1:9 w/w to 9:1 w/w and was titrated with calculated amount of aqueous phase (water or water and PG in the ratio 1:1 w/w). After 24 h the samples were monitored, visually inspected for transparency and followability and water was added. The phases were identified as transparent/translucent emulsion, transparent gel, turbid gel or turbid/milky emulsion. The visually evaluated mixtures and nanoemulsion phase were marked on the phase diagram. The droplet size, polydispersity index (PDI), and ζ-potential were measured with Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) 24 h after the emulsion preparation.

Spontaneous emulsification in three step process method was employed (Schuh et al., 2014). In the first step organic and aqueous phase were prepared. The homogeneous organic phase contained oregano essential oil, lipophilic surfactant Span 80, and ethanol as water miscible solvent, while aqueous phase was composed of water and hydrophilic surfactants, Tween 20 or Tween 80. In the second step organic phase was injected into the aqueous phase by stirring on the magnetic stirrer for 30 min at 750 min^{-1} to reach phase system equilibrium. In the third step, ethanol was evaporated on a BUCHI rotary evaporator system type R-114 and vacuum controller B-721 (Switzerland) at 40 °C, 250 bar, 1 h. The influence of the surfactant mixture (Tween 80 and Span 80 or Tween 20 and Span 80) and the surfactant to oil ratio on the droplet size, polydispersity index (PDI), ζ-potential, the particle size distribution, and average particle diameter were determined 24 h after the emulsion preparation.

Results and discussion

In the phase diagrams constructed using the oil, Tween 80, and a surfactant mixture of Tween 80 and Span 80 in the ratio of 2:1 w/w and 1:1 w/w, the effective surface of formed nanoemulsion was smaller. The maximum amount of water incorporated in the system was 23%. As a result of the small amount of water, as well as the high nanoemulsion density, the preparation of water based nanoemulsion was disabled. Substituting Tween 80 with Tween 20 and changing water with a mixture of water and PG in the ratio of 1:1 w/w, nanoemulsion surface increased. When the oil and the Smix in the ratio of 1:9 w/w were used, the formed nanoemulsion could be fully diluted with water without the formation of turbid-white emulsion. The average droplet size measured 24 h after preparation was 44 nm, while PDI was 0.349. Transparent emulsion was not formed when mixture of Tween and Span 80 in the ratio 1:2 w/w was used.

The droplet size of the nanoemulsion prepared using the spontaneous method influenced by the surfactant mixture (Tween 80 and Span 80 or Tween 20 and Span 80) and the ratio of the mixed surfactants and oil (the weight ratio of Smix:oil was 0.5, 1 and 1.5 w/w). The droplets with 160 nm particle size, 0.097 PDI, and -26 mV ζ-potential was obtained using a mixture of Tween 20 and Span 80 mixed with the oil in ratio of 0.5. Using the surfactant mixture of Tween 80 and Span 80 in the proportion of 0.5 with the oil, the droplets with 180 nm particle size, 0.134 PDI, and -20 mV ζ-potential were obtained. Increasing the Six to oil ratio at 1 and 1.5, nanoemulsions with bigger droplets were obtained. The average droplet size of Tween 20 or Span 80 mixture oil in the proportion of 1.5 was 361 nm, while PDI was 0.071 and a low ζ-potential value was recorded (-2.69 mV).

Conclusion

The low energy methods, aqueous titration and spontaneous emulsification method can be used in order to produce fine droplet nanoemulsions of wild oregano essential oil. Aqueous titration method requires high concentration of surfactants at low oil concentration. Spontaneous emulsification method can only be applied when low oil concentration, less than 1%, is used. In order to improve the emulsification efficiency of the essential oil with high concentration of carvacrol as a biological active compound and to increase the stability of the nanoemulsions preparation using aqueous titration and spontaneous emulsification method, the process and working parameters should be optimized.

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Macedonian pharmaceutical bulletin, **62** (suppl) 399 - 400 (2016)
ISSN 1409 - 8695
UDC: 615.014.8
Short communication

Risk assessment in blister packaging

Marija Cveevska*, Irena Zdraveska, Biljana Nanova, Milkica Gligorova

Alkaloid AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Every product and every process has an associated risk. Every enterprise should have a methodology for identifying and evaluating the risks it faces and it should have a process for generating intervention plans to reduce the risks to an acceptable level. This process is generally referred to as a Quality Risk Management (QRM). QRM is a systematic process for the assessment, control, communication and review of risks to the quality of the medicinal product across the product lifecycle. Early in development, the purpose of the QRM process may be to acquire sufficient product and process knowledge to assess risks associated with formulation development of the finished pharmaceutical product according to the quality target product profile. As development progresses, in addition to supporting that development, the purpose of the QRM process is to determine and manage risks to bioavailability, safety, efficiency and product quality.

Risk assessment is a systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Packaging represents a critical manufacturing operation requiring strong Good Manufacturing Practices (GMPs) and quality oversight to ensure sustained and robust compliance. Historically, inadequate packaging practices have been a meaningful ongoing contributor to product recall actions industry-wide. A strong understanding of the compliance risks associated with product packaging is a necessary and important component of a good quality system. The goal of risk assessment is to further enhance the quality assurance of existing packaging operations and practices (Guidance for industry "Q9 Quality Risk Managment", 2006) (Quality Risk Management Q9, 2005).

Risk assessment

The aim of risk assessment in blister packaging is to identify, analyse and evaluate risks during blister packaging process validation and the manufacturing processes.

Risk identification is a systemic use of information to identify potential sources of harm referring to the risk or problem. Information can include historical data, theoretical analysis, and the concerns of stakeholders. Risk identification addresses the "What might go wrong?" question and provides the basis for further steps in the quality risk management process (Reddy et al., 2014).

Risk analysis is the estimation of the risk associated with the identified hazards. It is the qualitative or quantitative process of linking the likelihood of occurrence and severity of harms (detectability) is also a factor in the estimation of risk (Reddy et al., 2014).

A team is assembled to collect relevant data about the packaging line. All operations involved with the line function (equipment, procedures, etc.) and all corresponding potential failures are listed. For each potential failure, the team works to understand its potential impact on packaging operations and then respectively to assign a severity category. Following severity classification, the team is reviewing all dominant causes relevant to the defined potential failure and then assigning a frequency category. For each potential failure, all safeguards (e.g. detection capabilities) are reviewed and a detection capability is assigned. For Severity Frequency and Detection determination, all relevant data is taken into consideration, to include maintenance and operation logs, batch records, deviation investigations, customer complaint records, etc. (Reich et al., 2011). The determined parameters and operations are: the environmental conditions during the process of primary packaging, issuing packaging material, issuing bulk product, blister forming, filling tablets into blisters, testing tablet presence in the blister, text printing on the aluminum lidding foil, blister sealing, embossing batch number and expiry

* irenakocevska@gmail.com

date on the blisters, the blister perforation, the blister cutting, insertion of proper number of blisters, leaflets folding, pharma code reading on the boxes, pharma code reading on the leaflet, embossing batch number and expiry date on the boxes, leaflet presence, weight check, determination of yield. For all parameters and operations potential risks are defined, consequences (severity of consequences, probability of occurrence, the possibility of detection). The assessment effort requires that potential product defects from any given packaging operation is defined and graded for severity, frequency and on the ability of the operation and/or an operator to detect the defect (Internal procedure: List of evidence for identification of risk, 2009)

Risk evaluation compares the identified and analyzed risk against given risk criteria. The output of a risk assessment is estimated as quantitative description of a range of risk. A numerical probability is used. Defect detection is categorized and graded on a 1-to-4 scale to reflect a detection capability of "none" (unable to detect) to "always" detect.

For each potential failure, all precautions are reviewed and a detection capability is assigned. For determinations of severity, frequency and detection, all relevant data is taken into consideration, including maintenance and operation logs, batch (Internal procedure: List of evidence for identification of risk, 2009).

Conclusion

The risk of blister packaging process is reduced to an acceptable level. According to the results of the risk assessment on blister packaging on the IMA C80/A81 blister packaging line, we can conclude that the blister packaging process is with low risk and the process is capable to perform effectively and gives reproducible products with quality that matches with the regulatory specifications and quality attributes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 401 - 402 (2016)
ISSN 1409 - 8695
UDC: 615.272:616.892.3
Short communication

Current therapeutic options and trends in drug development for Alzheimer's disease

Maja Simonoska Crcarevska*, Renata Slaveska Raicki, Marija Glavas Dodov

Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril & Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Dementia is characterized by persons` significant cognitive decline and interference with everyday life independency. There are several types of dementia, but Alzheimer's disease (AD) is most common form. AD a neurodegenerative disease is characterized by decline in memory, language, problem-solving and other cognitive skills that affects a person's ability to perform everyday activities (Alzheimer's, 2015).

Currently there are only 5 drugs approved for AD treatment and all are targeted towards metabolic disorders associated with the disease. Four of them, tacrine, donepezil (DON), rivastigmine (RIV) and galantamine (approved in 1993, 1996, 1998 and 2001, respectively) are in group of acetylcholinesterase inhibitors (AChEi) and one, memantine (MEM), approved in 2003, is N-methyl-D-aspartate (NMDA) receptor antagonist (Cummings et al., 2014). Although AD drugs are frequently used for more than 20 years there are evidences about controversies related to their efficacy and clinical significance (Lanctôt et al., 2009).

The aim of the current study was to analyze safety and efficacy of dosage forms of AD most commonly used drugs with focus on meta-analysis as well as to identify trends in AD treatment.

Current therapeutic options

Oral solution, hard capsules and transdermal patches with RIV, for treatment of mild to moderate dementia associated with AD and Parkinson's disease are present on the market. Latest Cohran`s collaboration review is addressing RIV clinical efficacy/safety in treatment longer than 12 weeks (any dose and administration route) in patients with AD dementia compared to placebo as well as comparison of RIV patches and capsules given at the manufacturer's recommended dose (Birks and Grimley Evans, 2015). Results from the study indicated that RIV use is associated with some benefits on some outcomes (cognitive function, activities of daily living and clinician rated global impression) and no differences in behavioural symptoms and quality of life of carers compared to placebo. No difference compared to placebo for withdrawals before the end of the treatment and at least one adverse effect was identified for RIV lower dose (1-4 mg/day), while for higher dose (6-12 mg/day) results favoured placebo. Fewer side effects related to nausea, vomiting, dizziness, effect on decreased appetite and asthenia for 9.5 mg/day patches compared to capsules were identified (Birks and Grimley Evans, 2015).

DON for the treatment of mild to moderate AD is approved in form of film coated and dispersible tablets. Effects of DON (any dose) in mild, moderate or severe dementia in AD treatments longer than 1 day compared to placebo were assessed by Birks and Harvey (2006). Results were similar as for RIV (Birks and Grimley Evans, 2015) with the exception of positive findings for DON effect on behaviour. Dose of 5mg/day DON was found to be more cost-effective as 10 mg/day dose was associated with more adverse effects and only marginally higher effects.

Comparison of safety and efficacy of AChEi for AD was done by Birks (2006). Four studies were identified, but only one (DON vs RIV) met criteria for inclusion in meta-analysis. No differences in efficacy were observed, but DON was associated with less adverse effects, particularly nausea, vomiting, anorexia, weight loss, event of fall and hypertension. However, conducted meta-analysis suggested that their tolerability might be match if RIV is carefully and gradually titrated for more than 3 months (Birks,

* msimonoska@ff.ukim.edu.mk

2006).

MEM is marketed in form of film-coated tablets for treatment of moderate to severe AD dementia. McShane et al. (2006) meta-analysis was related to efficacy and safety of MEM (any dose and any route of administration) for people with AD, vascular and mixed dementia. Clinically significant reduction of deterioration in patients with moderate to severe AD dementia was determined when MEM in dose of 20 mg/day for at least 28 weeks was compared to placebo. In general, MEM was well tolerated with low incidence of adverse effects (McShane et al., 2006).

Latest FDA approved drug for treatment of AD dementia (in 2014) is Namzaric (memantine hydrochloride extended-release + donepezil hydrochloride).

Trends in Alzheimer's disease drug development

Having in mind that we are facing with lack of efficacious therapeutic options for prevention and treatment of dementia and specifically for AD and associated behavioral and physiological symptoms there is extensive scientific research in this field. For ex. search on Scopus database (www.scopus.com.scopeesprx.elsevier.com) performed on 1.11.2015 using key words "Alzheimer's disease" resulted with 105860 items. Annual analysis pointed to the growing scientific interest for AD research as number of published items continuously increased during the years. First 2 published articles related to AD were found to be in 1912 year and the number was relatively low till 1988 (less than 800 per year). Further on, AD research rapidly grew with more than 4000 published scientific papers in 2005, thus reaching its maximum in 2014 with >8000 published scientific items (at the time of search for 2015 there were 6417, and 60 for 2016 year). Also, search on www.clinicaltrials.gov.mk carried out on 27.10.2015 resulted with total of 1769 AD clinical trials allocated to all continents around the globe. At the time of search 541 clinical trials were with status OPEN, mainly in USA, Europe, China and Japan - 231, 175, 67 and 41 respectively. In Europe most of the studies (56.6%) were located in France, followed by United Kingdom (18.3%) and Spain (17.1%). Nearly 30% of active trials were related to drug substances not yet approved for AD. Beside high number of clinical trials we have to be held in reserve as for a period of 10 years (2002-2012) 244 AD drugs were in clinical trials, but only one, MEM in 2003, was approved (0.4% succeed rate) (Alzheimer's, 2015). It is a fact that development of new drugs for treatment of AD is difficult and complicated due to the high costs, relatively long time needed to observe disease progression in AD and blood-brain barrier. Problems associated with blood-brain barrier might be surmounted by innovative therapeutic systems (ITS) for drug targeting and controlled release (liposomes, micelles, nanoparticles etc). Namely, utilizing their unique physico-chemical and biopharmaceutical properties drug targeted and controlled release dosage form with modified/improved pharmacokinetics/dynamics might be developed. Conducted search on Scopus database (accessed on 13.08.2015) retrieved 666 scientific papers (SP) related to micelles, liposomes, solidlipid nanoparticles, nanostructured lipid carriers and nanoparticles as ITS and/or RIV (197 SP), tacrine (124 SP), MEM (189 SP) and DON (156 SP) as drug substances. However in detail analysis of retrieved items revealed that only 15 (2.25%) were original research papers, and others were reviews. A vast majority of the original research papers (86.7%) were related to RIV and only 6.7% were related to DON and MEM, each. Liposomes and micelles as carriers for RIV were studied in 26.7% and 6.7% of the research papers, respectively. On the other hand most of the conducted research (66.7%) was related to nanoparticles, where's 80% were focused on RIV and 10% on DON and MEM, each. All of them were characterized in terms of particle size, drug content/ encapsulation efficiency, zeta potential and in vitro dissolution and majority (66.7%) conducted in vivo animal studies. All obtained results in the reviewed studies pointed to ITS potential use for AD efficacious treatment.

Based on the retrieved data it can be concluded that although ITS for different conditions are already in use for more than 20 years (1989 FDA approved Diprivan® - surfactant-based nanoformulation of anestetic Propofol) exploration of their potentials for AD treatment is still in its begging's.

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Macedonian pharmaceutical bulletin, **62** (suppl) 403 - 404 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:620.3
Short communication

Protein corona evolution on polymer nanoparticles for targeted drug delivery

Simona Dimchevska*, Nikola Geskovski, Rozafa Koliqi and Katerina Goracinova

Institute of Pharmaceutical Technology, Faculty of Pharmacy, University Ss Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

In the recent years, mutual interactions between nanoparticles and various biomolecules have been increasingly studied, in view of the well-known fact that they have major impact on nanoparticle overall performance. After intravenous administration blood is the first biological environment nanoparticles are exposed to, comprising a pool of several thousand proteins in different concentrations (Monopoli et al., 2011). Plasma proteins tend to adsorb on the naoparticle surface forming so-called protein corona, which governs the nano-bio interactions and determines nanoparticles fate following their administration. Modified biological identity of nanoparticles will affect the physiological response including their circulation time, agglomeration, transport, accumulation, internalization and toxicity.

Nanoparticle characteristics (size, shape, surface charge, surface functional groups and hydrophilicity/hydrophobicity), the nature of physiological environment (blood, intestinal fluid, cytoplasm) and the exposure time play a key role in the process of protein corona formation (Masoud et al., 2013). The rate of adsorption/desorption of different proteins, competitive binding, steric stabilization induced by adsorbed polymers and surfactants as well as the protein composition of body fluids lead to dynamic changes of the protein corona.

In the initial phase, usually the most abundant plasma proteins, such as albumin, are adsorbed at the surface of intravenously administered nanoparticles. In time, albumin can be replaced by proteins with higher binding affinity towards NP surface, since the protein corona composition continuously exchange (Vroman effect). It is worth mentioning that there is a possibility for structural and functional changes of proteins that interact with the nanoparticle surface, which could lead to activation of molecular mechanisms resulting in certain pathogenesis (Nel et al., 2009). Therefore, a detailed protein corona characterization is crucial for biocompatibility assessment of the nanoparticulate drug delivery system.

For this purpose, in this study we conducted comparative protein corona composition analysis of three different polymeric nanoparticles at several time intervals.

Materials and methods

Poly(lactic-co-glycolic acid)-b-Poly(ethylene glycol)-b-Poly(lactic-co-glycolic acid) (PLGA-PEG-PLGA) with two different molecular weights (Mw~148000Da and Mw~22000Da) and Poly(DL-lactide-co-caprolactone) (P(DLLACL copolymer, LA:CL 10:90, Mw 77799 Da) were purchased from AkinaInc, USA. Acrylamide-biacrylamide 37.5:1, 30% solution, Electrophoresis running buffer, Laemmli buffer and Comassie brilliant blue were obtained from Bio-Rad, USA. Tris (hydroxymethyl) aminomethane (TRIS) base, Sodium dodecyl sulphate and Amoniumpersulphate were acquired from Sigma Aldrich, USA, while N,N,N',N'-Tetramethylethylenediamine (TEMED) was purchased from MerckMilipore, Germany. All other chemicals were of analytical grade and were used as received.

Nanoparticle preparation

Three types of nanoparticles (NP1, NP2 and NP3) were prepared by nanoprecipitation method as described before (Dimchevska et al., 2015; Koliqi et al., 2016). Following copolymers were used for this purpose: PLGA-PEO-PLGA (Mw~148000Da), PLGA-PEO-PLGA (Mw~22000Da) and P(DLLACL), respectively.

* s.dimcevska@ff.ukim.edu.mk

Protein corona evaluation

Semi-quantitative protein corona evaluation was performed using SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis. 4% stacking gel and 10% resolving gel were used to estimate the protein corona composition of nanoparticles. At first, all NPs (1mg/mL) were incubated with 50% of human plasma at 37 °C for three different time periods (30min, 1h and 4h) in a water bath at stirring rate of 100 oscillations/min (Haake, Denmark). After the incubation, nanoparticles were pelleted by centrifugation (18000xg, 30 min at 4 °C) and the plasma was discarded. The samples were washed twice with 1.5 mL PBS and the supernatant was removed. 25 μL of Laemmli sample buffer containing 5% β-mercaptoethanol was then added to each sample. Samples were vortexed for 30s and heated at 95 °C for 5min in order to provide desorption and denaturation of adsorbed proteins. NPs were allowed to cool down at RT and then 5μL of each sample were loaded into the stacking gel placed on a vertical system (Mini Protean Cell, BioRad, USA). Gels were run for 60-90 min at 110 mV. After the protein separation the gels were removed, immersed in a 0.1% Coomassie blue solution and left to stand for 16h. The next day, each gel was washed with destaining solution (40% methanol, 10% glacial acetic acid and 50% deionized water) in four cycles. Pictures were acquired on white background using VersaDoc, Bio-Rad, USA. Densitometric analysis of proteins was performed using Quantity One 1-D Analysis Software, Bio-Rad, USA.

Results and discussion

Time-resolved protein evaluation confirmed the dynamic character of protein corona for all types of investigated nanoparticles. The results revealed a presence of an intense band around 66 kDa at each time point for all types of NPs, which corresponds to the molecular weight of albumin. Since albumin is the most abundant plasma protein with modular structural organization, high molecular flexibility and variety of binding sites on its surface, mutual non-specific interactions with the NPs were promoted, especially in the early time points of the experiment.

Further, we have noticed that different binding patterns for different NPs over time were displayed, probably due to their specific surface properties. Also, various composition of protein corona for each type of NPs was observed at the later time periods (1h and 4h) which correspond to protein corona dynamic nature and concomitant association/dissociation of diverse protein molecules during time. The results for longer incubation periods have shown that albumin was progressively replaced by other proteins with higher affinity and increased binding specificity to the NP surfaces, regardless of their plasma concentration. Proteins with molecular weights of 43 kDa, 41 kDa, 32 kDa and 25 kDa have gradually replaced the initially adsorbed albumin (66 kDa) as well as proteins with Mw of 55 kDa and 50 kDa at the surface of NP1 and NP2. Additionally, one characteristic band at 8 0kDa was observed at the later time points for NP2 which could not be observed for NP1. Protein corona composition of NP3 formulation was significantly different compared to NP1 and NP2, probably due to the higher hydrophobicity of the NP core. Specific proteins adsorbed onto the NP3 surface were those with Mw of 28 kDa, 36 kDa, 46 kDa, including one high molecular weight protein (Mw 185 kDa).

Our results correspond to the literature data for protein adsorption upon polymer NP surfaces. Namely, in the early time points of the experiment, albumin and fibrinogen were dominant components of the NP protein corona. Later on, significant differences appeared among the adsorbed imunoglobulins upon the surfaces of various NPs and additionally apolipoproteins came to be a significant portion of the total protein quantity.

Conclusion

Displacement of proteins over time was confirmed for all evaluated types of NPs. It was shown that abundant proteins such as albumin and fibrinogen may initially occupy the NP surface and get subsequently replaced by other proteins having higher binding affinity like apolipoproteins.

Different protein binding patterns lead to altered biological identity of NPs that will affect their *in vivo* behavior including their biodistribution, cell internalization, toxicity and efficacy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 405 - 406 (2016)
ISSN 1409 - 8695
UDC: 615.453.6.015.14
Short communication

Formulation development and characterization of modified release matrix tablets with water-soluble drug

Vesna Petrovska Jovanovska[1], Marija Velickovska[1], Aleksandra Petrovska[1], Sonja Ugarkovic[1], Marija Glavas Dodov[2]*

[1] *Research & Development, Alkaloid AD-Skopje, Blv. Aleksandar Makedonski 12, 1000 Skopje, Macedonia*
[2] *Institute of Pharmaceutical Technology, Center for Pharmaceutical Nanotechnology, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Prolonged-release oral dosage forms offer more advantages over a conventional dosage form of the same drug like sustained blood levels and better patients compliance, smaller dosage unit or higher dosage per unit and economical to manufacture (Hanna et al., 1987). The active agent can now be delivered and made bioavailable in a sustained and well-controlled manner up to a desired period of time. The matrix system is commonly used for manufacturing prolonged-release dosage forms. Bio-functional polymers have pharmaceutical interest in recent years and are widely used for formulation of modified release dosage forms (Meidan and Khan, 2007). Cellulose ethers are commonly employed as the hydrophilic, swellable and erodible matrix polymers for orally administered types of prolonged-release systems (Ferrero Rodriguez et al., 2000). As water insoluble excipients, ethylcellulose polymers (Ethocel) can effectively control the release of an active ingredient by modifying the size and length of diffusion path. By varying the type and amount of the insoluble excipient ratio and the particle size, a wide variety of release rate patterns can be achieved.

The aim of the present study was to develop prolonged-release matrix tablets for water-soluble drug using hydroxypropylmethylcellulose (Methocel) and Ethocel in order to reduce dosing frequency, to lesser the side effects and to improve patient compliance.

Materials and methods

Materials

Water-soluble compound (API) was used as a model drug (solubility 10.2 mg/ml, pKa 10.26 and 9.12, Mw 473.49 g/mol, BCS Class III). The other excipients were: Methocel K100M (Colorcon, EU), Ethocel 7cPs FP (Colorcon, EU), Kollidon K90 (BASF, Germany), Avicel PH 112 (FMC Biopolymer, USA), Aerosil 200 (Evonik, Germany) and Magnesium stearate (Mg S) (Carsco GE, Italy). All the other chemicals used were of analytical grade.

Preparation of matrix tablets by wet granulation technique

The tablets were prepared by wet granulation technique. The API and other excipients were accurately weighed and mixed in laboratory mixer granulator (Diosna Dierks & Söhne GmbH, Germany). The mass ratio of API:Methocel K100M:Avicel112:Mg S was 7.56:29.04: 25.72:14.52:1 for sample 1 and API:MethocelK100M:Ethocel:Avicel 112:Mg S = 7.56:14.52: 14.52:29.04:1 for sample 2, respectively.

Kollidon K90 (5% sol. in alcohol 99%) was used as granulation fluid. The wet mass was passed through #6 mesh and granules were oven dried until a moisture content of 1-3% was achieved. Dried granules were further passed through #230 mesh. The granules were lubricated and compressed into round shaped (7.0 mm) tablets using 4-station rotary compression machine (Korsch XL 100, Korsch AG, Germany) with compression forces 3.8 kN and 8.3 kN (sample 1a,1b and 2, accordingly).

Each tablet contained 15 mg of API in 150 mg tablet core weight. The tablets were evaluated for appearance, diameter, thickness and hardness.

Characterization of the final blends

Bulk density was determined by the following formula: *Bulk density =Ws/Vs*; where *Ws* is sample weight and *Vs* is the sample volume (Ph. Eur. 8.7).

Tape density is the indirect measurement of flow, mixing and tableting properties of powder. Tapped density was determined by Ph. Eur. 8.7 method: tablet blend was filled in 100 ml graduated cylinder of tap density tester which was operated for fixed number of taps until the powder bed volume has reached

* magl@ff.ukim.edu.mk

a minimum, thus was calculated by formula: Tapped density = Weight of mixture/volume of mixture after 100 tapings.

Compressibility index of the final blends was determined by *Carr's compressibility (%) = Df - Do/Df*; where *Df* is the bulk density and *Do* is the tapped density (Ph. Eur. 8.7).

Hausner's ratio or index of flowability was calculated by the formula: *Hausner's ratio=V1/V2*; where *V1* is the volume before taping and *V2* is the volume after taping (Ph. Eur. 8.7).

Angle of repose was determined using funnel method. Tablet blend were poured from funnel, that can be raised vertically until a maximum cone height h was obtained diameter heap D, was measured (Ph. Eur. 8.7). The angle of repose was measured using Granulate flow tester (Erweka GmbH, Germany).

Loss on drying is the loss of mass expressed as per cent m/m (Ph. Eur. 8.7). It was measured by drying the substance to constant mass using Moisture Analyzer (Mettler Toledo Excellence HS 153, US).

Physical characterization of the prepared tablets

10 tablets were used to study the weight variation using electronic balance (Sartorius Secura 224-1CU, Sartorius AG,Germany).

The diameter, thickness and tablet hardness was determined for ten tablets using ErwekaTablet Hardness Tester Type TBH 425 TD (Erweka GmbH, Germany).

In vitro *drug release studies*

Dissolution testing was conducted in 900 ml of phosphate buffer pH 6.8 (USP apparatus I, basket, speed 100 rpm) at 37±0.5 °C. A 5 ml of the dissolution medium was withdrawn at regular tome intervals (after 2, 4, 10 and 12 h). The volume of dissolution medium was adjusted by replacing each 5 ml aliquot withdrawn with 5 ml of fresh dissolution medium. Quantity of released API was determined by previously validated HPLC method.

Results and discussion

The bulk density of sample 1 and sample 2 were 0.272 and 0.360 g/ml respectively, while tapped density values were 0.331 and 0.439 g/ml. Compressibility index of sample 1 and sample 2 were 18 and 19%, while Hausner's ratio values were 1.22 and 1.21 correspondingly, which is an indication of fair flow properties.

Angle of repose of the final blends was 41.2° for sample 1 and 40.3° for sample 2. LOD was found to be 2.40% for sample1 and 2.55% for sample2 indicating that both of the final blends had moisture content up to acceptable limit which was good for compression to make uniform matrices.

All tablets were smooth and elegant in appearance. Tablet weight variations for both samples prepared in this study were found to be less than 1%. Sample 1a had diameter of 7.00 mm; thickness 4.23 mm and hardness 5.01 kP (at compression force 3.8 kN). Sample 1b had diameter of 6.97 mm; thickness 3.37 mm and hardness 14.29 kP (at compression force 8.2 kN). Sample 2 had diameter of 6.97 mm; thickness 3.32 mm and hardness 12.12 kP (at compression force 8.2 kN).

Polymers belonging to hydrophilic matrix systems, when exposed to an aqueous medium, do not disintegrate, but immediately after hydration form a highly viscous gelatinous surface barrier which control the liquid penetration into the center of the matrix system as well as drug release from the dosage form (Talukder et al., 1996).

Sample 1a released 57.63, 71.96 and 100.78% after 2, 4 and 10 h, respectively. In the same investigated period, sample 1b released 50.44, 69.88 and 100.55%, indicating that there is no significant difference in the drug release from the Methocel matrices compresses at different compression forces. It can be implied that the porosity and/or tortuosity of the prepared tablets after their hydration were not affected by an increase in tablet hardness from 3.8 kN and 8.2 kN. Obtained results in this work were in accordance with the literature data (Ravi et al., 2008).

Sample 2 released the 30.75, 50.78, 77.49 and 89.13% after 2, 4, 10 and 12 h, indicating that the fine Ethocel particles contributed to retard the drug release from the matrices. The reduction in release rates with addition of Ethocel was perhaps due to slow hydration of the matrix, based upon the hydrophobic character of Ethocel. The insoluble particles of Ethocel were probably acting as barrier to drug release in the gel layer of Methocel.

Conclusion

Based on these results it can be concluded that the combination of Methocel and Ethocel could be a successful solution in modifying the release of water-soluble drug form matrix system tablets.

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Macedonian pharmaceutical bulletin, **62** (suppl) 407 - 408 (2016)
ISSN 1409 - 8695
UDC: 615.225.2.014.8
Short communication

Statistical process control as a tool for process understanding and continuous process verification

Violeta Dinić Milisavljević*, Sonja Georgieva Jovanović, Igor Popović, Aleksandra Zeljković, Viška Miceska, Valentina Ilieva

Alkaloid d.o.o. Beograd, Prahovska 3, 11000 Beograd, Republic of Serbia

Introduction

Statistical process control (SPC) provides a statistical approach for evaluating the process of production and for improving the quality of the process through better understanding. SPC is a process orientated data driven method to improve processes to deliver day after day results that have added value for the customer. In particular this method will help tocontinuously improve processes and reduce variability, describe and control the stability of processes, monitor and prove excellence of processes, alert before any batch is violating specifications, and document process understanding for regulatory agencies (Sven, 2016).

When SPC is effectively implemented within a company, benefits can be derived through a reduced cost of manufacture, improved quality, fewer troubleshooting crises, and improved relationships with customers (Thomas et al., 2006).

In this paper the application of SPC in the process of packaging of tablets in the pharmaceutical industry is shown. As a key process indicator (KPI) for the process of packaging the yield of the final product is observed. As a critical quality attribute an assay of tablets in the final product is observed. For SPC studies, individual values - Control Charts and Moving Ranges-control charts were constructed and Process capability indexes were calculated in order to verify if the process is under control.

Materials and methods

Materials used were as follows: Amlodipin Alkaloid tablets 5 mg (Alkaloid A.D., Skopje, Macedonia), PVC foil 250 μm x 118 mm (Lamp East d.o.o., Serbia), aluminum foil Amlodipin Alkaloid tablets 5 mg (Lamp East d.o.o., Serbia), leaflet Amlodipin tablets 5 mg and 10 mg (Zapisd.o.o., Serbia), Carton boxes Amlodipin Alkaloid tablets 5 mg x30 (Grafolikd.o.o., Serbia).

Equipment used was: blister packaging machine IMA TR100LT (IMA S.p.a. Italy), data printing line Videojet 1210 + Etipack Clear (Videojet Technologies Itc, USA, Etipack, S.p.a., Italy), HPLC Thermo Scientific DIONEX UltiMate 3000 (Thermo Fisher Scientific Inc., USA) STATISTICA 8, StatSoft Inc., USA.

Data for the yield of the blister packaging process were obtained as a ratio of produced number of packages of final product Amlodipin Alkaloid tablets 5 mg x 30 and planned number of packages of each batch, expressed as a percentage.

The content of amlodipine in Amlodipin Alkaloid tablets 5 mg was analyzed according to the Ph. EUR. 8 monograph for Amlodipine besylate with HPLC method (EP 2.2.29).

Control charts were generated by STATISTICA software, after the following steps were performed:

- The data for yield and content of Amlodipine were obtained for all batches of Amlodipin Alkaloid tablets 5 mg x30 that were manufactured in 2015;
- The overall mean and average were calculated;
- Moving ranges were calculated;
- Process standard deviations for the process and moving ranges were calculated;
- Control limits for individual values (UCL and LCL) and moving ranges (UCLMR) were calculated (Bakker et al., 2008).

Process capability indexes can be used as a tool that will show how often the process fits into the specification limits. There are two most common used process capability indexes: Cp and Cpk.

* vdinic@alkaloid.co.rs

The Cp-value is the relation of the range of the specification to the range of the process and it doesn't show if the process is within the specification limits.

The Cpk-value is the relation of the range of the specification to the mean value to the range of the process. If the value is below 1.0 there are values outside specification. If the value is exactly 0 half of the values are outside specification. All process should have Cpk>1.33 to be sure that the process is stable and always in the desired specification limits.

For the purpose of the study Cpk indexes for the yield of the packaging process and for the content of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30 were calculated.

Results and discussion

The new approach of the pharmaceutical industry was developed after the Food and Drugs Administration (FDA) announced initiative in August 2002, Pharmaceutical Current Good Manufacturing Practices (cGMPs) for the 21st Century. The modern approach of the pharmaceutical industry has a basis in good process and product understanding. Quality should be built in each phase of the product lifecycle.

As a result of this approach the use of different tools that will help to gain better knowledge for the process is increased in the last 15 years. These tools can be used in all stages of the lifecycle of the drug product. During the manufacturing of the drug products the most common tools that might help in the understanding of the process are: quality risk management, corrective and preventive action, process capability studies, Six Sigma and control charts.

Individual values control charts and moving ranges control charts should be always evaluated together in order to have a complete picture of the process. After the control charts were generated it was concluded that all of the Western Electric statistical rules were satisfied (Bonnie et al., 1956):

1. One point outside of the control limits (3 σ);
2. Two points out of 3 are more than 2 σ from the centre line;
3. Four points out of 5 are more than 1 σ from the centre line;
4. Eight points on one side of the center line

for both control charts: yield and assay of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30. It was shown on data obtained from 83 produced batches during 2015, that the process of packaging of Amlodipin Alkaloid tablets 30 x 5 mg is under statistical control and there is no special cause variability. Also the Cpk indexes values showed that the process is always capable to give high yield and the active substance content is always within specified limits.

The values for the yield and assay of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30, obtained from the last 30 batches were used to calculate the control limits for continual process verification. The packaging process of all batches that have been produced from 01.2016 onwards will be followed by control charts with the new established control limits.

Conclusion

In the pharmaceutical industry SPC can be used for better understanding of the process, to analyze existing results for Annual Product Review and as a tool for continuous process verification. Different kind of data can be analyzed as per example: in-process control data (tablet's weight, tablet's height), quality control data (dissolution, content of active substance, and content of impurities) and process performance data (yield).

It can be said that individual value-control charts, moving ranges-control charts and process capability indexes are very simple but valuable tools for process understanding and for following the quality of the product continuously.

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Macedonian pharmaceutical bulletin, **62** (suppl) 409 - 410 (2016)
ISSN 1409 - 8695
UDC: 615.356:577.164.2
Short communication

Effects of PSD and wet granulation properties (concentration of granulation aid, temperature and humidity) on physical stability of ascorbic acid 95% granulate

Oja Memed*, Krume Tosev, Natasa Anevska Stojanovska, Gjorgji Petruševski, Marina Chacorovska, Sonja Ugarkovic

Research and Development, Alkaloid AD, AleksandarMakedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

DC (direct compressible) granules nowadays are one of the most popular and most preferable pharmaceutical forms to formulators and as well as to companies because of their easy handling, less time consuming and cost effectiveness aspects.

Granules are manufactured through a complex multistage processes which takes relatively long times under which the starting materials change their physical characteristics a number of times before they take place in final dosage form.

Ascorbic acid is a chemical substance that is best known by its antioxidant activity and free radical scavenging (Heber, 2007). Ascorbic acid is susceptible to oxidative degradation and, therefore, it requires significant physicochemical and stability considerations in its formulations (Odeniyi and Jaiyeoba, 2007).

The purpose of this study was to investigate the variability in the physical stability properties of ascorbic acid 95% granules induced by wet granulation process of ascorbic acid in order to produce ascorbic acid 95% granules used as product which will take place in further processing of conventional tablets.

Materials and methods

Different batches from ascorbic acid 95% granules containing ascorbic acid (95.0% w/w) and hydroxypropyl methylcellulose (HPMC) type 2910 (50 mPas) (5.0% w/w) were prepared by wet granulation technique. Based on before gained experience and literature reference data, concentration of purified water used as granulation aid, process parameters of drying process and sieves/screen size for dry granulate were varied, in order to evaluate the role and check which of this variables is/are most important from the aspect of colour stability of ascorbic acid 95% granules. All manufactured batches from ascorbic acid 95% granulate were stored in stability testing chambers with defined conditions for temperature (40 ˚C) and humidity (75% RH) in open Petri dishes for 15 days and were analysed on zero, fifth and fifteenth day. Physical characterization of granules was conducted using a range of experimental methods.

All excipients used in the present study are approved for use in the pharmaceutical industry. Ascorbic acid 95% granules were produced by using ascorbic acid (DSM), hydroxypropyl methylcellulose type 2910 – HPMC E 5 LV (Methocel, Colorcon, UK).

All methods were performed according to Good Manufacturing Practices (GMP.)

Process parameters: mixing time, mixing speed and chopper speed of the high shear mixer granulator (Diosna 4L, Diosna, Germany) used during wet granulation process were same for all laboratory trials. Mixing of preblend of ascorbic acid (475.0 g) and HPMC E 5 LV (50 g) - mixing time: 3 min, mixing speed: 250 rpm, chopper speed: 1000 rpm. Wet granulation phase was separated in two phases granulation I/II (granulation time: 3 min /6 min, granulation speed: 150 rpm/200 rpm, chopper speed: 1000 rpm/1000 rpm).

Concentration of water purified used as granulation aid during process of wet granulation was varied in different batches as 3.0% w/w, 3.5% w/w and 4.0% w/w.

* omemed@alkaloid.com.mk

Drying process of granules were made in fluid bed drier (Huttling, Germany), by varying of drying parameter in different batches as inlet air temperature 60 °C, 40 °C and 30 °C. As end point determination for drying phase was used loss on drying (LOD) of granules to be maximum 0.5%. In accordance with the varied drying parameters for inlet air temperature, drying time varied from 7 min to 35 min for the drying process. Variations on drying process parameters were made on ascorbic acid 95% granules produced with 4.0% w/w water purified as granulation aid, because based on before gained experience, 4.0% w/w water purified is determined as optimal concentration of granulation aid for obtaining good granulate with acceptable quality parameters.

Prepared dry granules from all batches were separate in two equal parts and were passed through laboratory sieve equipment (QuadroComil, Quadro, Canada) under different sieve size. First part of granules were sieved through sieve with pore size 0.813 mm and the other part from the sieve with pore size 0.610 mm in order to obtained granules with different particle size distribution (PSD).

All manufactured batches of ascorbic acid 95% granules with all variables described above were placed separately in open Petri dish in stability chambers with defined conditions for temperature (40 °C) and humidity (75% RH) and were stored for fifteen days.

Physical and chemical characterization of granules were analysed on zero, fifth and fifteenth day by various experimental methods: Fourier Transform Infrared spectroscopy (FTIR, Varian 660, Australia), Differential Scanning Calorimetry (DSC, Netzsch 204F1 Phoenix, Germany), appearance, colour– organoleptic examination, bulk density and tapped density (Erweka SVM 102, Erweka, Germany), flow characteristics, angle of repose (°) (Erweka granulate flow tester GTB, Erweka, Germany), loss on drying (Mettler Toledo HG 63, Mettler Toledo, Switzerland) and optical microscopy (Morphologi G3S, Malvern, UK).

Results and discussion

From the results obtained in this study it can be easily seen that it is most challenging scientific task to separate just one parameter as responsible for physical (colour) stability issue of ascorbic acid 95% granules. From the produced batches with different variables it is more than obvious that humidity (concentration of granulation aid 4.0% water purified) and time spent under high humidity (concentration of granulation aid 4.0% water purified and dried with 30 °C inlet air temperature which take about 37 min for drying process) are the most responsible factor for changing the colour of ascorbic acid 95% granules and also PSD of the granules plays huge role in colour stability because it was noticed that granules with bigger granules (their fraction with bigger granule) change their colour more drastically than the granules with smaller fraction.

It is important to note that even we have made physical characterization by using range of experimental techniques with none of them except organoleptic and visual examination, changes can't be detected. On the other side by visual examination it can be easily seen that there is big colour difference among the granules.

Conclusion

Results present in this study indicates that production of ascorbic acid 95% granules due to the nature of active substance is very complex process and as most important factors which affect physical stability of granulate, humidity and temperature can be pointed.

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Macedonian pharmaceutical bulletin, **62** (suppl) 411 - 412 (2016)
ISSN 1409 - 8695
UDC: 615.33:616.314.17
616.314.17-085.33
Short communication

Preparation of doxycycline loaded chitosan microparticles for periodontal disease treatment by TPP ionic cross-linking combined with spray drying

Silvana Gjoseva*, Nikola Geskovski, Simona Dimchevska, Katerina Goracinova

Faculty of Pharmacy, University of SS Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Periodontal disease is simply defined as bacterially induced chronic infectious-inflammatory disease that affects the tissues that support and anchor the teeth. It is a disease very difficult to treat, with very little change in the treatment over the last several decades. So, even though with limited efficacy, mechanical plaque biofilm disruption is still at the mainstream of periodontal therapy with the addition of several treatment modalities (systemic antibiotics, topical antimicrobials, laser therapy etc.) but only as adjunct to scaling and root planning. However, optimism for discovery of the treatments with improved efficacy is restored due to continuous research efforts especially during the last two decades, and revised as well as upgraded understanding of the ethyology, pathogenesis and complexity of this disease. Unquestionably, understanding of the complex microbial community, its virulence and the impact upon the host cells response as well as the importance of the host inflammatory-immune response to intraoral plaque for the disease development and progression creates new perspectives in management of periodontitis. Basically, novel research data clarified that the disease outcome depends upon the host response towards the complex dysbiotic oral microbial community (Yucel, 2015). Additionally, it may be modified by genetic and different environmental factors. Consequently, host modulation therapies are being proposed (Deshmukl et al., 2011) in order to tackle excessive host inflammatory reaction and targeting of various aspects of the inflammation pathways (levels of enzymes, cytokines, prostanoids, as well modulation of osteoclast). Host modulating agents include non-steroidal anti-inflammatory drugs (NSAIDS), sub antimicrobial dose doxycycline as well as topical doxycycline, systemic bisphosphonates, host modulating agents antagonizing pro-inflammatory cytokines, selective inhibitors of nitric oxide synthase, inhibitors targeting the signaling pathways like c-Jun N-terminal kinases (JNK) inhibitor; Extracellular-Signal-Regulated Kinases (ERK) inhibitor; NFκB inhibitor; JK3 inhibitor etc.Tetracycline apart from its antimicrobial property, when used in sub-antimicrobial doses works as anti-inflammatory agent and has capability of inhibiting the activities of neutrophils, osteoclasts, MMP 8, thereby inhibiting tissue and bone destruction. However, systemic therapy and conventional polymer implants with Doxycycline hyclate could not supply sufficient concentration of the active substance at the site of action for a prolonged period of time in order to provide efficient therapy. Smart bioadhesive micro and nano drug delivery systems with controlled release of the active substance will greatly contribute to improved therapeutic efficacy, considering their ability to interact with the permeable junctional epithelium as well as elements of inflammation like macrophages, dendritic cells etc, at the same time allocating high concentration of Doxycycline in the vicinity and/or at the site of the inflammation.

Therefore, the main objective of this study is development and evaluation of mucoadhesive controlled release chitosan microspheres for local treatment of periodontal disease, with a size span from 1-10 μm, loaded with Doxycycline hyclate.

* sgjoseva@Alkaloid.com.mk

Materials and methods

Materials

Doxycycline hyclate was obtained form Kunshau Chemical, China, chitosan with degree of deacetylation 75-85%, sodium tripolyphosphate (TPP), sodium citrate, 2-methyl-2-propanol and calcium citrate were purchased from Sigma Aldrich, Germany and tetrabutilammponium hydrogen sulphate was purshased from Merck Millipore, Germany.

Methods

Microparticles were produced by spray drying method (Buchi Mini Spray dryer B-290, Switzerland) with these conditions: inlet temperature 175 ˚C, aspiration 70%, pump flow 2% and air flow of 600 Nl/h. The colloidal solutions were prepared by ionotropic gelation method, with gradual addition of the cross-linking agent solutions (10 ml) to the solutions of polymers (1% chitosan sol.) and active ingredient (50 mg) (magnetic stirrer Variomag, Multipoint HP 15, Germany). Particle size and the swelling index were determined with laser difractometry (Mastersizer 2000, Malvern Instr., Ltd, UK) using cell Hydro 2000S, (Malvern Instr., Ltd, UK). The swelling degree was measured in diluted 1:2 phosphate buffer pH 7.0 (USP) with similar ionic strength of saliva. Drug release rate was followed in a suspension of microparticles in diluted 1:2 phosphate buffer pH 7.0 (USP), placed in closed tubes in horizontal shaker at 37 °C, (Unitronic OR, Selecta, Barcelona, Spain). The concentration of released doxycycline hyclate in different time intervals as well as drug content and efficacy of encapsulation were determined using HPLC method, column Phenomenex® 250-4.6 mm, PolymerX 5µm RP-1 100A (Phenomenex, Torrance, CA, USA), mobile phase of 2-methyl-2-propanol, buffer pH 8.0 R (Ph.Eur.), tetrabutilammponium hydrogen sulphate, and diluted solutions of sodium hydroxide and sodium edetate.

Results and discussion

Doxycycline loaded microparticles were prepared by TPP ionic cross-linking combined with spray drying method. Since the cross-linking of chitosan depends on the availability of the cationic sites and the negatively charged TPP species, the crosslinking process was carried out at pH 4.5, where chitosan as a polycation (pKa 6.3) will present –NH+3 sites and mostly phosphoric ions from TPP will be generated (Desai & Park, 2004). Different concentrations of the TPP cross-linking solutions (formulation A - 0.1% TPP; B - 0.5%; C - 0.75%; D - 1%) were used in order to evaluate their influence upon the physico-chemical properties of the chitosan microparticles like, particle size and particle size distribution, yield, efficacy of incorporation, drug content and dissolution rate.

The results pointed that at higher TPP concentration larger particles were produced with a presence of aggregates after the spray drying process. Mean particle sizes were 5.0 µm; 3.3 µm; 7.5 µm and 50 µm for series A, B, C and D, respectively. Span factor gradually increased with increasing concentrations of TPP solution, confirming the aggregation in series prepared using 0.75% (span = 2.9) and 1% (span = 4.8) TPP cross-linking solution. Yield and efficacy of encapsulation were slightly increased for microparticles prepared using 0.75% and 1% TPP. The dissolution studies showed that although dissolution rate decreased with increasing TPP concentrations, the rate reduction was not significant and it may have resulted from the particle size difference and the differences in the surface area exposed to the dissolution medium (formulation B - Dissolution results at T 1h, 3h, 5h were 40%, 60% and 90 cum.% compared to formulation C Dissolution rate results of 35, 50 and 80 cum.% at T 1h, 3h, 5h, respectively).

Conclusion

The present study investigated the influence of different concentration of the cross-linking agent on the physicochemical properties of the spray dried chitosan microparticles. It was found that the TPP concentration influenced the particle size, particle size distribution, yield, drug loading and efficacy of encapsulation, swelling index as well as the dissolution rate of Doxycyline hyclate. Also, higher concentration of TPP resulted in production of larger particles with very broad particle size distribution and substantial formation of aggregates with increasing concentrations of the cross-linker.

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Macedonian pharmaceutical bulletin, **62** (suppl) 413 - 414 (2016)
ISSN 1409 - 8695
UDC: 615.453.074
Short communication

Preformulation studies as initial phase in development of film-coated tablets with BCS class II active component

Bosilka Stefanova*, Packa Antovska, Sonja Ugarkovic, Gjorgji Petruševski, Marina Chachorovska

Research & Development, Alkaloid AD, blvd. AleksandarMakedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

It may seem obvious to state that a new product should be adequately defined before anyserious product development is undertaken. In many cases, the value of the design phase is often underestimated in the rush to start development and get products to the market quickly.

This can result in much wasted time and valuable resources. Since tablets and capsules account for approximately 70% of pharmaceutical preparations, an investigation into the solid-state properties of candidate drugs is an important task to be undertaken during preformulation (Wells, 1988). Generally speaking, when dealing with high strength solid dosage forms, this formulation will be more susceptible to any drug substance variability. However, other studies are also important since, for example, the same chemical compound can have different crystal structures (polymorphs), external shapes (habits), and hence different flow and compression properties.

Preformulation is the initial phase in the development of pharmaceutical products. Suitable preformulation will inevitably result in obtaining simple and elegant formulation and successful dosage form from a commercial aspect.

The performance of a solid dosage form is dependent on the physicochemical properties of the active ingredient and the excipients.

Preformulation is a critical phase in drug development where the physicochemical profiling of the active pharmaceutical ingredients (APIs) and excipients are determined and prototype formulations are made.

Selection of stable polymorph and solid-state compatibility studies of the compounds proposed for development of new pharmaceutical formulations are essential in the initial stage of formulation in order to identify possible incompatibilities that may affect the stability of the finished product (Gibson, 2004).

A wide variety of methodologies that exist make it possible for the preformulation scientist to effectively study whatever needs.

The aim of this study was to choose the most stabile polymorph as well as to investigate possible solid-state interactions between API of BCS class II and excipients proposed for development of film-coated tablets, based on the changes in the infrared spectra of the both polymorphs and induced changes in the infrared spectra of the stressed binary mixtures compared to the infrared spectra of initial binary mixture, as consequence of possible solid-state chemical reaction. Additionally, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were used to distinguish between the pseudopolymorphs of the studied API.

Materials and methods

Materials used are: anhydrous pseudopolymorph of BCS class II active compound, monohydrate pseudopolymorph of BCS class II active compound, mannitol, dicalcium phosphate, povidone, hydroxypropyl cellulose, low-substituted, sodium stearyl fumarate, cellulose, microcrystalline, partially pre-gelatinazed maize starch and sodium lauryl sulfate.

Fourier Transform Infrared (FTIR) spectroscopy has proved to be suitable technique for these trials. The FT-IR spectra were recorded using ATR method, in the 4000–550 cm^{-1} region, on Varian 660 FT-IR spectrometer (Varian, Australia) (resolution 4 cm^{-1}, 16 scans per spectrum). Attenuated total reflectance (ATR) spectra were obtained by MIRAcleZnSe ATR module (PIKE technologies) with

* bstefanova@alkaloid.com.mk

low pressure micrometer clamp.

FT-IR spectra of the binary mixtures were recorded initially and then the same samples were placed in the stability chambers under various conditions (40 °C/75% RH, 25 °C/60% RH).

Weighed samples of 2-3 mg of the studied API (monohydrate and anhydrous form) were scanned in aluminum pans with a perforated lid at speed of 10 K/min from room temperature to 260 °C. All of the samples were analyzed in dry nitrogen atmosphere using a Netzsch DSC 204F1 Phoenix instrument (Netzsch, Germany). The TGA curves were recorded in the 25–400 °C range, on a Netzsch TG 209 F1 Iris analyzer (Netzsch, Germany) using aluminum oxide pans.

Results and discussion

Results showed spontaneous transition of API from anhydrous to monohydrate form. Significant difference between these pseudopolymorphs was shown in data obtained by Differential Scanning Calorimetry and Thermogravimetric analysis. In the TG curves of monohydrate and anhydrous forms losses of mass of 5.11% and 1.27%, respectively was observed which is in accordance with theoretical value for monohydrate and anhydrous form. The melting endothermic peak at around 253 °C (with decomposition) in the both DSC curves, and continuous evaporation of water in the DSC curve of the monohydrate form was observed. These observations are in good agreement with the studied pseudopolymorphic forms. Stability of the molecule of the monohydratepseudopolymorph was the main reason this polymorph to be chosen as more suitable for the development of film-coated tablet formulation.

Careful inspection of the obtained spectra of the pure API (fresh and stressed) and fresh and stressed binary mixtures, leads to conclusion that no changes in the position and shape of bands in regards to the spectrum of the initial sample are observable. This confirms that the applied stress conditions do not affect the overall appearance of the FT-IR spectra of the API. Therefore, the characteristic vibrational bands from API as well, can be used as relevant spectroscopic markers in order to assess the solid-state stability between APIs in the presence of the studied excipients. Based on the comparison of the FT-IR spectra of the initial and stressed binary mixtures it can be concluded that after exposure on accelerated temperature and humidity levels during the screening period, there is no significant change in the FT-IR spectra of the binary mixtures of API and described excipients in comparison to the corresponding FT-IR spectra of the freshly prepared samples.

Minor spectral changes, in the binary mixture with povidone stored at higher temperature/moisture levels were observed at 1660 cm^{-1} probably due to the higher presence of moisture and hygroscopic nature of the excipients. For this purpose binary mixture with povidone in formulation ratio was prepared and tested by the same procedure as previously described. In this binary mixture there were no significant changes in the FT-IR spectra.

Conclusion

Results showed spontaneous transition of API from anhydrous to monohydrate form. Stability of the molecule of the monohydrate pseudopolymorph was the main reason this polymorph to be chosen as more suitable for the development of film-coated tablet formulation.

The dedicated FT-IR spectroscopy of the binary mixtures between API and the proposed excipients clearly demonstrated the solid-state compatibility of the API and the described excipients. The conclusion was derived based on the absence of significant changes in the FT-IR spectra of the stressed binary mixtures in comparison with the corresponding data obtained from the freshly prepared binary mixtures and the starting materials.

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Macedonian pharmaceutical bulletin, **62** (suppl) 415 - 416 (2016)
ISSN 1409 - 8695
UDC: 615.212.015.11
Short communication

Influence of the formulation factors on the dissolution of highly dose water soluble active pharmaceutical ingredient

Dejan Kuneski*, Packa Antovska, Sonja Dimcevska, Bosilka Stefanova, Blagica Mancevska, Dusica Angelovska, Zoran Zivic, Sonja Ugarkovic

Research and Development, Alkaloid AD, Aleksandar Makedonski 12, 1000, Skopje, Republic of Macedonia

Introduction

Highly dose water soluble active pharmaceutical ingredient (API) with analgesic effect was formulated as an immediate-release film-coated tablet. This formulation is indicated for treatment of mild to moderate pain and to express the analgesic effect faster.

It is important to note that the film is not functional, and the presented data are related to the tablet core.The purpose of our study was to examine the influence of some formulation factors, such as particle size of the API, disintegrant type and concentration (Bolhuiset al., 1994), mass of tablet core and concentration of starch (starch 1500/ partially pre-gelatinized Maize starch) (Cunningham, 1999) on the dissolution profile of the API.

Materials and methods

API, Prosolv HD 90(Silicified microcrystalline cellulose) was supplied from JRS Pharma Rosenberg, Germany, Starch 1500 (partially pre-gelatinized Maize starch) was supplied from Colorcon, Indianapolis USA, Kollidon VA 64 (Copovidone) was supplied from BASF, Ludwigshaften, Germany, Primojel (Sodium starch glycolate) was supplied from DFE Pharma, Goch, Germany, L-Hydroxypropyl cellulose LH-11 was supplied from Shin-Etsu, Tokyo, Japan, Ac-Di-Sol (Croscarmellose sodium) was supplied from FMC Bio Polymer, Wallingstown, Little Island, Cork , Ireland, Aerosil 200 (Silica, colloidal anhydrous) was supplied from Evonik Industries , Rheinfelden, Germany, Kolliphor SLS Fine (Sodium Lauryl Sulphate) was supplied from BASF, Ludwigshaften, Germany, Magnesium stearate was supplied from FACI SpA, Carasco, Italy and Talc from Merck, Darmstadt.

API used in the experimental trials is characterized with high water solubility and has molecular weight 352.48.

Formulations with different disintegrants, partially pre-gelatinized Maize starch (Starch 1500) concentration, different tablet core mass and API's particle size were prepared:

- Formulation I: API is not sieved, has not Starch 1500/ partially pre-gelatinized Maize starch, with mass of tablet core 875 mg and 5% pro tablet (Rowe et al., 2013) of L-hydroxypropyl cellulose.
- Formulation II: API is not sieved, with 5% pro tablet Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation III: API is not sieved, with 10.83 % Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation IV: API is not sieved, has not Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 10% pro tablet of L-hydroxypropyl cellulose.
- Formulation V: API is sieved through 610 µm sieve, with 11.83 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation VI: API is sieved through 610 µm sieve, with 6.33 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 875 mg, 5% pro tablet of L-hydroxypropyl cellulose and 5% pro tablet Ac_Di-Sol/croscarmellose sodium.
- Formulation VII: API is sieved through 610 µm sieve, with 5.67 % pro tablet Starch 1500, mass of

* dkuneski@alkaloid.com.mk

tablet core 860 mg and 4% pro tablet of Primojel/ sodium starch glycolate.

- Formulation VIII: API is sieved through 610 μm sieve, with 10.42 % pro tablet Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of Primojel/sodium starch glycolate.
- Formulation IX: API is sieved through 315 μm sieve, with 5.67 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 860 mg and 4% pro tablet of Primojel/sodium starch glycolate.
- Formulation X: API is sieved through 315 μm sieve, with 10.42 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of Primojel/sodium starch glycolate.
- Formulation XI: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/sodium starch glycolate, mass of tablet core 880 mg and 4% pro tablet of Primojel/sodium starch glycolate.
- Formulation XII: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation XIII: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 880 mg, 3% pro tablet of L-hydroxypropyl cellulose and 3% pro tablet of Primojel/sodium starch glycolate.

Dissolution profile is being conducted on the experimental trials.

The dissolution method conditions were: apparatus II (paddle apparatus) at speed of 75 rpm, dissolution medium (900 ml ± 1% phosphate buffer pH 7.2 ± 0.05), 10 ml sample volume, dissolution medium temperature 37 ± 0.5 ◦C. Samples were collected at six time points: 5, 10, 15, 20, 30 and 45 minutes.

For determination of dissolution rate of active substance UV absorptionspectrophotometric method was used.The content of dissolved API was calculated by measuring the UV absorptions of the test and standard solution at detection of 221 nm.

Results and discussion

Based on the results from the extensive dissolution evaluation on the experimental trials one may conclude that the most influential factor among the following: disintegrant type, tablet average mass and API particle size is the disintegrant type. Namely, the dissolution profile is most highly affected when pre-gelatinized starch is incorporated into the formulation in comparison to sodium starch glycolate and crosscarmelose sodium. This results point to conclusion that dissolution of the highly dosage highly water-soluble API is rather fastened by the partitioning of the dissolution media between the pre-gelatinized starch and the API than with the use of a classical super-disintegrants. The high percent of the API in the formulation support this assumption having in mind that the mechanism of the super-disintegrants is hindered by this high percent of the API (85w/w% of the API in tablet formulation).

As per the second factor, increase of the average mass of the tablet with other excipients (fillers) did not cause any increase on the dissolution of the API due to the fact that this slight percent of mass increase was not enough to modify and support the mechanism of disintegration related to super-desintegrants.

The third factor, particle size of the API (achieved by sizing with different sieve size) was also found to be not influential as the disintegrant type probably due to the API is characterized with high water solubility and penetration of the dissolution media is not hindered by different particle size of the API.

Conclusion

The presented study shows that optimized concentration of Starch 1500/ partially pre-geletanized Maize starch and the combination of two selected disintegrants (L-HPC and Primojel/sodium starch glycolate), as well as sizing step of the active pharmaceutical ingredient have influence on the dissolution rate of the highly dose water soluble API.

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Macedonian pharmaceutical bulletin, **62** (suppl) 417 - 418 (2016)
ISSN 1409 - 8695
UDC: 615.277:615.373
Short communication

Trastuzumab and its radioimmunoconjugates in treatment of cancer

Marija Sterjova[1]*, Paulina Apostolova[1], Predrag Dzodic[2], Katarina Smilkov[1], Darinka Gjorgjieva-Ackova[1], Emilija Janevik-Ivanovska[1]

[1]University "GoceDelcev", Faculty of Medical Sciences,
Str. "Krste Misirkov" No. 10-A, 2000 Stip, Republic of Macedonia
[2]University in Nis, Medical Faculty, Str. "Zoran Dindic" No. 81, 18000 Nis, Serbia

Introduction

Monoclonal antibodies are new type of targeted anticancer therapy, which achieve specificity, selectivity and localization in tumor cells. There are many naked antibodies and immunoconjugates commercially approved for different types of cancer (Mehren et al., 2003). In order to improve specificity and selectivity of cytotoxic drugs and toxins, monoclonal antibodies are used for formulation of immunoconjugates. Many efforts are done to develop stable immunoconjugates of trastuzumab with various drugs, toxins and radioisotopes to improve the general conditions of the patients (Sharkey and Goldenberg, 2006). The aim of this paper is to focus on current achievements in the formulation of radioimmunoconjugates of HER2-targeting trastuzumab.

Trastuzumab is a humanized IgG1 monoclonal antibody active against HER2 positive breast cancer. It originates from murine antibody 4D5 that is potent inhibitor of HER2 positive cancer cells. Subsequently, it was chosen for further clinical development in order to reduce the probability of generation of HAMA (human anti-murine antibody) (Harries and Smith, 2002). Carter et al. (1992) cloned hypervariable regions from 4D5 in plasmids which encode formation of constant regions from human IgG1 antibody and generated a vector that encode formation of chimeric antibody which is additionally humanized. The new humanized 4D5 has higher affinity for the HER2/neu antigen and reduced immunogenicity. Trastuzumab is acting by binding to the IV subdomain of the HER2 receptor and Fc region of the antibody support ADCC (antibody-dependent cellular cytotoxicity) (Gennari et al., 2004).

* marija.sterjova@ugd.edu.mk

Radioimmunoconjugates for imaging and therapy

Because of the easy detection, radioimmunoconjugates can be used for body imaging at a molecular level using sensitive imager like γ camera, computed tomography and positron emission tomography (PET) (Goldenberg, 1997). Significant radiopharmaceuticals based on peptide and antibody for diagnostic and therapeutic purpose use different radioisotopes (99mTc/188Re, 67Ga, 177Lu, 90Y, 131I) (Kassis, 2008). In order to obtain successful labeling, previously conjugaton with a bifuntional chelators (DOTA - 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA - diethylenetriaminepentaacetic acid; TCMC - (1,4,7,10-tetra-(2-carbamoyl methyl)-cyclododecane; HYNIC - succinimidyl-6-hydrazino-nicotinamide; 1B4M-DTPA - 2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triaminepentaacetic acid) is required. These chelators allow binding to the antibody on the one side, and coordinative binding of radioisotopes on the other side (Kang et al., 2012).

Immunoconjugates of trastuzumab for PET imaging

In recent years there have been significant achievements in development of stable immunoconjugates of trastuzumab for PET imaging of HER2 positive lesions (Hooge et al., 2004). Chen et al. (2008) used 99mTc in order to create a stable conjugate, 99mTc-NYCIN-trastuzumab, useful for identification of HER2 positive metastasis. Tamura et al. (2010) have shown the possibility of identification of HER2 positive lesions in patients with pri-

mary metastatic breast cancer with 64Ga-DOTA-trastuzumab. Three years later, Alitezapour et al. (2013) were able to formulate similar conjugate with another gamma emitter 67Ga-DOTA-trastuzumab for the same purpose. Investigations of Palm et al. (2003) for pharmacokinetics of trastuzumab labeled with pure β emitters 86Y and 90Y in mice with ovarian cancershown a selective uptake of the conjugate by the tumor cells and minimal localization in healthy organs. In vitro and in vivo investigations in mice with breast tumor show that 177Lu-DOTA-trastuzumab can be new promising drug in treatment of human breast cancer (Rasaneh et al., 2012). Tan et al. (2012) have shown that 212Pb-TCMC-trastuzumab has a significant therapeutic effect in HER2/neu positive prostate cancer. Borchardt et al. (2003) have tested therapeutic effects of alpha emitters 227Th-DOTA-p-benzyl-trastuzumab and 225Ac-trastuzumab in mice with HER2 positive breast and ovarian cancer. Studies have shown rapid internalization and cytotoxicity in cancer cells which leads to a extend survival and low toxicity.

Our examinations will be focused on synthesis and evaluation of the immunoconjugates of trastuzumab with bifunctional chelators (DOTA, DTPA and 1B4M-DTPA) with already used method for freeze dried kit formulation of rituximab-conjugates. The most stable immunoconjugate will be labeled with gamma emitter Ga-68 for further in vitro characterization and in vivo biodistribution. The simplicity of Ga-68 labeling will increase the access of radioimmunoconjugates in hospitals for PET imaging of HER2 positive metastasis.

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Macedonian pharmaceutical bulletin, **62** (suppl) 419 - 420 (2016)
ISSN 1409 - 8695
UDC: 615.849.2(6-11)
Short communication

Trends in radiopharmacy in developing african countries

Aschalew Alemu[2,3]*, David Mwanza Wanjeh[1,3], Joel Munene Muchira[1,3], Emilija Janevik[3]

[1]*Ministry of Health, Kenya*
[2]*Faculty of Medicine, Addis Ababa; Ethiopia*
[3]*Faculty of Medical Sciences, University Goce Delčev – Stip, R. Macedonia*

Background

This article describes trends in Radiopharmacy in the developing countries and the current status of Radiopharmacy Practice in Eastern Africa.

The main goal of our presentation is to show and to stress the importance of existing problems related to the missing of the exact information on the number, status and size of Radiopharmacy units in African countries as the regional status as well as for the human resources, education, suitable training and local demand for the Radiopharmacy and Nuclear Medicine services is not documented (Dondi, 2006).

The Radiopharmacy Practice by the definition requires well-defined and controlled conditions to avoid any risk contamination with microbes, pyrogens and particulate matter as well as cross contamination with other radiopharmaceuticals, together with established radiation protection.

Implementation of the Good Radiopharmaceutical Practices in all levels in the Radiopharmacy should be planned, introduced by the planned priority and strictly monitored and reordered in the production, preparation, testing and in the packaging areas for all final product ready for use.

The practice of nuclear medicine using established radiopharmaceuticals, mostly from the first generation has clinical applications in virtually all systems of the body, for example, the skeletal, cardiac, endocrine, oncologic, gastrointestinal and renal systems. The commoner nuclear medicine procedures in African developing countries are the bone scan, thyroid scan and the renal scan respectively.Almost all radiopharmaceuticals are parenterally administered and requires techniques and procedures that guarantee sterility of the product done according to the clearly defined protocol and and established and controlled conditions.

The critical moment for the realization of all these necessities is to have suitably staff, enough educated and trained to provide the implementation and development in the right direction.

Radiopharmacy professionals should have an adequate training in all aspects of the sterile production, quality control, GMP, GLP, radiation safety and radiochemistry to ensure the competent handling of the radioactive materials (IAEA NUMDAB, 2009).

Methodology

The practice of radiopharmacy combines the expertise of pharmaceutical preparation and the skills needed to handle radioactive substances. Diagnostic radiopharmaceuticals do not normally have any pharmacologicale ffect and their administration is not associated with major clinical side effects. Their clinical use, however, is associated with a risk deriving from radiation exposure and possible contamination during radiopharmaceutical formulation by chemical, biological and microbiological impurities.

Accordingly, principles of Good Practices should be planned in all levels, by priority and strictly observed in the production, preparation, testing and the packaging of the final product ready for use. The main powerful key for implementing Good Radiopharmaceutical Practice is qualified and trained personnel. Trained and competent staffs are essential for achieving high standards and growth in Radiopharmacy. In African Countries there is an acute shortage and in some countries an absence of nationally registered pharmacists with radiopharmacy experience. Most nuclear medicine facilities operate their radiopharmacies with the support of technologists not trained in radiopharmacy practices. For that reason the basic quali-

* aschuradio@yahoo.com

ty systems in the Radiopharmacy laboratory could be improved significantly with the better training.

This competency-based training is the first step to provide the essentials of a trainingprogramme for all radiopharmacy practitioners that addresses the following requirements:

- Standardization of training for staff members that operate in hospital radiopharmacy practice;
- To improve performance and management of the radiopharmacy service;
- To encourage good radiopharmacy practices for the preparation and quality assurance;
- To establish a quality management system which encourages continuous update of core competencies in hot laboratory staff.
- Encourages continuous update of core competencies in hot laboratory staff.

Results

One of the key bottlenecks for Nuclear Medicine is a human resources shortfall, especially radiopharmacists. There is an acute shortage and in many countries absences of pharmacists with radiopharmacy experience. Most of Nuclear Medicine facilities operate at IAEA operational level 1 and 2' mainly with support of technologists. There is a global need for effective implementation of the 'operational guidance on hospital radiopharmacy-a safe and effective approach' under which there is a strong recommendation to strengthen skills, competencies and professional qualifications of all staff involved in clinical radiopharmacy practice. They should be empowered to address the poor state of Radiopharmaceutical laboratories in many countries and be more aware of cost of radiopharmaceuticals.

For safety of patient they should be aware of proper registration of radiopharmaceuticals and quality assessment required locally. At the time when there are difficulties with supply and relative high cost of routine radiopharmaceuticals, trained staffin Radiopharmaceutical laboratories could make the difference to Nuclear Medicine (IAEA NUMDAB, 2009).

The trained radiopharmacist should have a:

1. working knowledge of the radiopharmaceutical terms, abbreviations, and symbols commonly used in prescribing, compounding and dispensing radiopharmaceuticals
2. working knowledge of the procedures and techniques relating to aseptic compounding and parenteral admixture operations.
3. working knowledge of the procedures and operations relating to the reconstitution, packaging and labeling of radiopharmaceuticals
4. the ability to perform the usual functions associated with a specific radiopharmacy.
5. the ability to perform the manipulative and record keeping functions associated with the compounding and dispensing of radiopharmaceuticals
6. manipulative and record keeping function sassociated with quality control testing of radiopharmaceuticals
7. working knowledge of drug dosage by imagingprocedure, routes of administration, dosage forms, and be able to distinguish
8. the ability to perform the essential functions relating to drugpurchasing and inventory control
9. appropriate knowledge and understanding of the specific nuclearpharmacy site with emphasis on the technician duties and responsibilities, including standards of ethics governing pharmacy practice therapeutic from diagnostic radiopharmaceutical utilization
10. ability to perform them a thematical calculations required for the usual dosage determinations and solution preparations in the compounding and dispensing of radiopharmaceuticals.
11. Demonstrate appropriate working knowledge of any additionaltraining or safety requirements mandated by the pharmacy or by any local, state, or federal agency by successful completion of any required program (DAT on-line, 2011).

Conclusion

This paper present our idea how to create suitable training and network of all professionals and state authorities for establishing and develop education for Good Radiopharmacy Practice, qualified personnel and appropriate regulation according to the local and international parameters that will be step forward to have advanced health care system and confidence of the patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 423 - 424 (2016)
ISSN 1409 - 8695
UDC: 615.074:543.422.3-74
Short communication

Solid-state compatibility screening of $CaCO_3$ and $MgCO_3$ with selection of excipients suitable for development of solid-dosage formulation

Marina Chachorovska*, Sonja Dimchevska, Sonja Ugarkovic, Gjorgji Petrushevski

Research and Development, Alkaloid AD, AleksandarMakedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Fourier transform infrared spectroscopy (FT-IR) was applied as suitable screening analytical method to assess the compatibility screening of $CaCO_3$ and $MgCO_3$ with several excipients aimed for development of solid-dosage formulation.

The solid-state compatibility testing of the active pharmaceutical ingredients (APIs) $CaCO_3$ and $MgCO_3$ and the selected excipients was performed by Fourier Transform Infrared (FT-IR) spectroscopy. The compatibility was studied by comparison of the FT-IR spectra of the pure samples (APIs and excipients) and freshly prepared binary mixture with the FT-IR spectra of the corresponding stressed binary mixtures. The appearance of new bands in the FT-IR spectrum, non-typical for the APIs or excipients, can be considered as most significant sign of possible solid-state interaction and formation of new molecular species. Changes due the water/moisture intake, resulting in band broadening or changes in the band resolution and intensities in the FT-IR spectra, can be considered normal, because the samples were exposed directly to the external influence, without any protection or packaging.

The aim of this experiment was to investigate possible solid state interaction between APIs and the described excipients, based on the induced changes in the infrared spectra of the binary mixtures exposed at different external conditions, compared to the infrared spectra of the pure compounds and the freshly prepared mixtures. The obtained results afforded deeper insight into the solid-state stability of the studied binary mixture.

Materials and methods

During development of the $CaCO_3$ and $MgCO_3$ tablets several excipients were evaluated for compatibility with the active ingredients as a screening for potential choice for tablet formulation. APIs: Calcium carbonate ($CaCO_3$, purchased from Solvay Osterreich GmbH, Austria) and Magnesium carbonate heavy ($MgCO_3$, purchased from Dr.PaulLohmann, Germany); Excipients: Silica, colloidal anhydrous (purchased from Evonik Resource Efficiency GmbH, Germany); partially pre-gelatinized maize starch (purchased from Colorcon, USA); copovidone (purchased from BASF (BTC Europe GmbH), Germany); xylitol (purchased from Roquette, France); low-substituted hydroxypropylcellulose (purchased from ShinEtsu, Japan); spearmint flavor SD (purchased from Symrise AG, Germany); menthol L flavourspraydried (purchased from Symrise AG, Germany); talc (purchased from Merck, Germany) and Mg stearate (purchased from FACI SpA, Italy).

Binary mixtures were prepared by dry mixing of equal amounts of APIs and each excipient in 1:1 ratio (*w*/*w*). This ratio is different than the ratio used in the formulation, but according to the literature (Cunha-Filho et al., 2007), the equal masses afford bigger possibility for solid-state interaction among the constituents of the mixture. Pure APIs, excipients, and corresponding binary mixtures were stressed by 30 days exposure in stability testing chambers, at open Petri dishes, at temperatures of 40 ºC and 25 ºC, with a relative humidity (RH) of 75% and 60%, respectively.

FT-IR spectra of pure APIs and excipients, freshly prepared binary mixture and stressed binary mixtures after 30 days were recorded and evaluated.

The FT-IR spectra were recorded using ATR method, in the 4000–550 cm^{-1} region, on Varian 660 FT-IR spec-

* mkajdzanoska@alkaloid.com.mk

trometer (Varian, Australia) (resolution 4 cm^{-1}, 16 scans per spectrum). Attenuated total reflectance (ATR) spectra were obtained by MIRAcleZnSe ATR module (PIKE technologies) with low pressure micrometer clamp.

Results and discussion

Based on the comparison of the FT-IR spectra of pure $CaCO_3$ and pure $MgCO_3$, with fresh prepared binary mixtures and corresponding excipients, it can be concluded that in all cases the most prominent vibrational bands of the APIs can be identified in the initial prepared binary mixtures. Although, generally being obscured and overlapped by strong bands of the APIs, few stronger bands originating from the present excipient can be identified in some of the binary mixture. No additional bands, of unknown origin, were observed.

In regards of the FT-IR spectra of the starting binary mixture and the FT-IR spectra of the same mixture after exposure for 30 days at 25 ºC/60% RH and 40 ºC/75% RH, it can be concluded that no significant spectral changes were observed, except in binary mixtures between studied APIs with xylitol and copovidone. In the FT-IR spectra of these binary mixture exposed at stressed conditions some spectral changes were observed according to higher moisture. Having in mind that both of excipients are hygroscopic at high moisture (Raymond et al., 2009), pure xylitol and copovidone were exposed 30 days at 40 ºC/75% RH. Based on the obtained FT-IR spectra, it can be concluded that spectral changes observed in the FT-IR spectra of the stressed binary mixtures are results from the absorbed moisture from xylitol and copovidone, which can be confirmed by their FT-IR spectra.

Conclusion

The closer inspection of the FT-IR spectra of the obtained binary mixtures of $CaCO_3$ and $MgCO_3$ with the proposed excipients, clearly demonstrated that there are no significant spectral changes induced in the FT-IR spectra during the screening period at all testing conditions. The preformulation studies have shown that in the FT-IR spectra in the binary mixtures between: $CaCO_3$ with xylitol and $CaCO_3$ with copovidone; and $MgCO_3$ with xylitol and $MgCO_3$ with copovidone; exposed at stressed conditions, some spectral changes were observed due to moisture uptake by the excipient with hygroscopic properties.

These findings were confirmed by comparison of the spectral changes observed in the FT-IR spectra of the stressed binary mixtures and the individually stressed xylitol and copovidone.

The compatibility studies as a part of preformulation screening of the excipients aimed for development of $CaCO_3$ and $MgCO_3$ solid dosage form were beneficial and give useful directions for development of a stable and effective formulation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 421 - 422 (2016)
ISSN 1409 - 8695
UDC: 615.453.6.015
Short communication

Formulation development of immediate release tablets with water insoluble drug using fluid-bed granulation

Sanja Simeonovska Gushic[1]*, Dejan Kostovski[1], Aleksandra Petrovska[1], Marija Velickovska[1] Sonja Ugarkovic[1], Marija Glavas Dodov[2]

[1]*Research & Development, Alkaloid AD-Skopje, Blv. AleksandarMakedonski 12, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Pharmaceutical Technology, Center for Pharmaceutical Nanotechnology, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Tablets as solid preparations can range from relatively simple immediate release formulations to complex modified release drug dosage forms. The desired drug release properties could be adjusted by proper selection of excipients used in the formulation as well as by technological process and process conditions selected.

Numerous unit processes are involved in making tablets, including blending, granulating, drying, compaction and coating. Various factors deriving from these processes can affect content uniformity, drug release rate and/or stability of tablets.

The fluid-bed method of wet granulation is well known in the pharmaceutical and other industries as a one-step, enclosed operation. Because several ingredients can be mixed, granulated, and dried in the same vessel, the technique reduces material handling and shortens process times compared with other wet granulation techniques. In addition to granulation for tableting, the fluid-bed top-spray method produces highly dispersible granules with a characteristic porous structure that enhances wettability and improves many of the powder properties for tablet compression. Granules of high quality can be obtained by understanding and controlling the critical process parameters by timely measurements (Parikh, 1997).

The aim of this study was to develop immediate release tablets with water insoluble drug (API), using fluid-bed granulation technique.

Materials and methods

Materials

API from BCS Class II/IV (particle size D_{50} less than 20μm, freely soluble in dichloromethane, sparingly soluble in toluene, insoluble in methanol and water) as model drug substance was used. Other excipients used in the formulation were: lactose monohydrate, microcrystalline cellulose, starch maize, hydroxypropylcellulose, croscarmellose sodium, magnesium stearate and iron oxide. All chemical used in this present study are approved for use in pharmaceutical industry.

Preparation of the granules

Three different formulations of granules were prepared using fluid-bed granulation process (Hüttlin GmbH, Hohe-Flum-Strasse 42, Schopfheim, top spray configuration, Germany). Water solution of hydroxypropylcellulose was used as granulation vehicle. Fluid-bed granulation was performed under the following working conditions: air volume 15 m^3/h; inlet temperature 66 °C; atomizing pressure 0.5-1 bar; mycroclimate 0.3-0.8 bar; filter pressure 0.8-1 bar; filter cleaning period 2 sec.; filter blow time 0.2 sec. After finishing the atomization of the binder solution, the granules were dried for a variable period of time in the same apparatus at 66 °C inlet temperature and air volume 15 m^3/h.

For preparation of sample 1, API was granulated together with lactose monohydrate, while starch maize, microcrystalline cellulose and iron oxide were added extragranularly. For sample 2, API was granulated together with all excipients used in the formulation (lactose monohy-

* ssimeonovska@alkaloid.com.mk

drate, starch maize, microcrystalline cellulose and iron oxide). For sample 3, API was granulated together with lactose, part of croscarmelose sodium and starch maize, while microcrystalline cellulose, iron oxide and the other part of croscarmelose sodium were added extragranulary.

All prepared samples were finally mixed with magnesium stearate (5 min.; Drum blender, Erweka PM5, Germany) and compressed by a rotary tablet press (KorschXL 100 Pro, Korsch AG, Germany), using round shaped 6 mm punches.

Determination of granules and tablets characteristics

Final blends were evaluated for loss on drying and flow properties. Loss on drying (LOD) was analyzed in Sartorius Infrared dryer MA 35 (Germany). Granulate in quantity of 1 g was dried to constant mass. The loss of mass was presented as percent m/m (Ph. Eur 8.7).

Flowability of granulates was determined according to Ph. Eur 8.7 with Granulate flow tester (Erweka type GTB, Erweka, Germany). Nearly 50 g of granulate was poured from funnel and the time required to empty the funnel was measured.

Determination of disintegration time and dissolution test of the compressed tablets was performed according to the official methods from the European Pharmacopoeia. For that purpose 6 tablets were placed in baskets of apparatus for disintegration (Erweka type ZT302, Erweka, Germany). Time required for disintegration was measured automatically.

Dissolution testing was conducted in 900 ml of dissolution medium hydrochloric acid buffer pH 1.2 (USP 38 NF 33) at 37 ± 0.5 °C using Apparatus II paddle (Varian VK, USA). Apparatus was adjusted to a speed of 60 rpm. Aliquots were taken at regular time intervals (after 5, 10, 15, 20, 30 and 45 min) and replaced with an equal volume of pre-warmed hydrochloric acid buffer. Withdrawn aliquots were analysed for drug content using previously validated HPLC method (Hitachi HPLC system, Japan), column 150 mm x 4.6 mm, 5µm, at 25 °C and flow rate of 1.5 ml/min. Quantification of API was detected by UV at 250 nm.

Results and discussion

LOD as a parameter is extremely important for the process of fluid-bed granulation and drying. Obtained results indicated that sample 2 has the lowest loss of mass (3.1%) in comparison with samples 1 and 3 (3.25 and 3.74%, respectively) probably due to the presence of microcrystalline cellulose into the granule matrix. Even though microcrystalline cellulose allows rapid addition of the granulating fluid, the water does not become bound inside, but rather it is easily given up during drying process. This property aids in preventing case hardening and uniform moisture content in granules (FMC Pharma, 2000).

Results obtained for the flow properties of the final blends indicated that the flowability was excellent for all prepared samples (10, 8.7 and 10.8 sec for sample 1, 2 and 3, respectively).

The macroscopic appearance of the tablets was satisfying. All prepared tablets were smooth and elegant and no mottling was observed. Uniform appearance of the tablets was probably due to presence of microcrystalline cellulose, which enables equal migration of added dyes (FMC Pharma, 2000).

The results of disintegration time show that all prepared samples have disintegration time less than 15 minutes (1.38, 2.35 and 1.21 min for sample 1, 2 and 3, respectively).

Sample 1 did not meet acceptance criteria for immediate release tablet dosage forms (80.22% were dissolved for 30 minutes). On the other hand, sample 2 and 3 were characterized by > 85% dissolved API at the time interval of 30 min (90.06% and 91.82%, respectively). Obtained results pointed to the rationality of microcrystalline cellulose intragranular incorporation (sample 2) as well as addition of croscarmellose sodium (sample 3) equally between the intra and extra granulation.

Conclusion

In the present work efforts have been made to develop immediate release tablets with water insoluble API using fluid-bed granulation as a promising approach to enhance the drug release profile. The results showed that the release of the drug was depended on type of excipient used in the formulation. Formulation containing 2% croscarmellose sodium showed minimum disintegration time and better drug release profile as compare to other formulations.

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Macedonian pharmaceutical bulletin, **62** (suppl) 425 - 426 (2016)
ISSN 1409 - 8695
UDC: 615.276:615.454
Short communication

Evaluation of physical properties on nonsteroidal anti–inflammatory gel formulation with different polymers

Milka Mijalkova Dokova*, Ljiljana Krsteska, Dejan Kostovski, Sonja Ugarkovic

Research & Development, Alkaloid AD, Blvd.AleksandarMakedonski 12, 1000 Skopje, R. Macedonia

Introduction

Pharmaceutical semisolid preparations may be defined as topical products intended for application on the skinor accessible mucous membranes to provide localized and sometimes systemic effects at the site of application. However, most of the semisolid preparations are applied to the skin for topical relief in dermatologic conditions (Swarbrick et al., 1990). Several categories of semisolid preparations for cutaneous application may bedistinguished: ointments, creams, gels and pastes. These topical formulations are composed of drug in asuitable semisolid base which is either hydrophobic or hydrophilic in character (Allen et al., 2011).

Depending on the physicochemical properties, desired site of action and formulation strategies for the drug delivery incorporation into semisolids can show their activity on the surface layers of tissues or via penetration into deeper layers to reach the site of action or through systemic delivery. Nonetheless if the drug is to act locally or systemically, it must first penetrate the stratum corneum (Raw et al., 2013).

To treat a number of painful conditions affecting the joints and muscles, such as backache, rheumatic and muscular pain, sprains, strains and sports injuries, an active ingredient that belongs to a group of non-steroid anti-inflammatory drug (NSAID) was used. During formulation development, in order to obtain a stable, transparent, homogeneous hydro-alcoholic gel for topical use with satisfactory rheological properties, several gelling agents were tested such as hydroxyethyl cellulose, sodium carboxymethylcellulose, poloxamer and carbomers.

The aim of this research work wasto show the influence of different types of polymers as gelling agents on physical properties of gel, like grittiness, viscosity and spreadability which are important for achieving therapeutic efficiency.

Materials and methods

Materials

NSAID (BCS class II), Poloxamertype 407, Carbomer, grade 940,C10-30 alkyl acrylate crosspolymer,ethanol 96%, diisopropanolamine, sodium hydroxide, propylene glycol, levomenthol.

Equipment

Laboratory mixer homogenizer (IMA Stephan UMC 5,Germany), magnetic stirrer (IKA Ret control/t, Germany), viscometer (Brookfield model DV2T with T- bar spindle, USA), pH meter (Seven Compact Mettler Toledo, Germany),Morphologi-G3S, (Malvern instruments, UK).

Preparation of gels

Method of preparation of samples iskept to be same. Processing steps include hydration of the polymer with mixing until complete hydration is obtained. Next is the gel forming step,where with appropriate gel forming agent clear gelwasachieved. Separately, process of dissolving of active ingredient, levomentholand propylene glycol into ethanol 96% is performed.This solution is added to gel base and homogenized.

Sample S1 contains cross-linked polyacrylate polymer as a gelling agent and diisopropanolamineas a pH balancer. Organic amines are commonly used to neutralize polymers as agents for gel formation. In sample S2, C10-30 alkyl acrylate crosspolymer, polymer of the same group, but with different physical characteristics was used. The sample S3 was prepared with Poloxamer as a gelling agent. So-

* mdokova@alkaloid.com.mk

dium hydroxide solution as a pH balancer is used in the optimal amount to obtain a transparent gel.

Propylene glycol and ethanol (96 %) were included in formulation as co-solvent and solvent for the active ingredient. As a medium for hydration and swelling of polymer,water purified was used. Levomenthol in the formulation was chosen as a cooling agent and skin penetration enhancer.

Physical properties of the prepared formulation

The produced gels were evaluated for grittiness, viscosity and spreadability as physical tests.

Grittiness (texture)

The samples were evaluated microscopically for the presence of any appreciable particulate matter, seen under light microscope. Proper quantity from the samples was applied directly to the glass for viewing under a microscope with a thickness that is appropriate for the lens.

The gel should fulfil the requirement of absence of particular matter and from grittiness as desired for any topical preparation.

This test was performed on three selected samples with differentpolymers on Morphologi-G3S Malvern instruments, UK.

Viscosity

Rheological properties such as viscosity of semisolid dosage forms can influence their drug delivery. The viscosity of the formulations was performed using Brookfield viscometer DV2T model with T – Bar spindle, Brookfield. The test was developed according to European Pharmacopoeia test 2.2.10 (Ph. Eur. current version).

For the measurement approximately 50 g of gel were filled in a 100 ml beaker and the T-bar spindlewas lowered perpendicular in the centre taking care that the spindle does not touch bottom of the beaker. The viscosity was read as a single point measurement after 60 s, rotating with 5 rpm at room temperature.

Spreadability test

The spreadability is a test of the gel easiness of application. The spreadability of the samples was determined according to in-house test and is referenced according to Rao et al.(2010) by measuring 1 g gel between horizontal plates (20 x 20 cm^2), after 1 minute. The standardized weight tied on the upper glass was 125 g. The results were calculated according to a formula.

The spreadability (S) can be calculated using formula:

$$S = m \times \frac{l}{t}$$

Where:

S –spreadability(g.cm/sec)
m - weighttied to upper glass slide (g)
l - lengthmove on the glass slide (cm)
t –timetaken (sec)

Results and discussion

For this purpose three different hydro-alcoholic gelswere prepared for testing certainphysical properties.

Grittiness (texture)

All samples have a homogeneous appearance without particulate matter.That is an expected result for this kind of topical formulations.

Viscosity

The results from measurements are 54 000, 41 600 and 700 000 cP for the samples S1, S2 and S3 respectively. Viscosity results were influenced by the gelling properties andmolecular weight of thepolymers.

Spreadability test

Average result from ten measurementsof the samples S1, S2 and S3 were 10.04 g.cm/sec, 10.96 g.cm/secand 5.67 g.cm/sec, respectively. Results are in accordance with the viscosity values, similar between S1 and S2, S3 being least spreadable.

Conclusion

All examined samples for the physical parameter grittiness gave satisfactory results.From the presented results it can be concluded that gel sample with Poloxamer is the most viscous and it has weaker spreadability properties compared to the samples with cross-linked polyacrylatepolymer and C10-30 alkyl acrylate crosspolymer. Samples S1 and S2 have optimal viscosity values, provide good spreadability properties and are easy to apply on the affected skin area.

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Macedonian pharmaceutical bulletin, **62** (suppl) 427 - 428 (2016)
ISSN 1409 - 8695
UDC: 615.276.032.311:615.451.2
Short communication

Taste masking approach in oral suspension with nonsteroidal anti - inflammatory drug

Roza Markovska Dameska*, Liljana Krsteska, Milka Mijalkova Dokova, Dejan Kostovski, Sonja Ugarkovic

Alkaloid AD, Blvd. AleksandarMakedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Oral liquid pharmaceutical dosage forms are designed to provide maximum therapeutic response in a targeted population, especially for children and people with difficulty in swallowing tablets and capsules, and to produce rapid therapeutic effects. Suspensions as dispersed systems are composed of two or more phases, where the solid phase, usually the drug substance, is distributed throughout the polymeric matrix (Cox, 2008).

Oral suspension described in this study is composed of active substance which belongs to BCS class II, and is nonsteroidal anti - inflammatory agent with very intensive burning taste, as is literally described as "burning mouth effect". Taste masking effect is very difficult to achieve, but is inevitable step which must be satisfied during development of successful formulation of such oral suspension. Different approaches are reported in the literature to achieve successful taste masking of bitter or unpleasant taste of drug, as follows: addition of flavoring and sweetening agents, taste suppressants and enhancers, viscosity enhancer, pH modifier, microencapsulation, coating with inert material, ion-exchange, inclusion complexation, granulation, adsorption, prodrug approach, by using liposomes, by effervescent agent etc. (Baig et al., 2014; Bhalerao et al., 2013; Suthar et al., 2010).

During formulation development several combination of polymers and ion exchange resins in different ratio are being used. Polymers used for achieving the desired viscosity range were xanthan gum, carboxymethylcellulose sodium, microcrystalline cellulose and carboxymethylcellulose sodium complex, maize starch, lambda carrageenan gum, and cross linked polyacrylic polymer.

Materials and methods

For preparation of the oral suspension, the following materials were used: xanthan gum, carboxymethylcellulose sodium, microcrystalline cellulose/ carboxymethylcellulose sodium , maize starch, lambda carrageenan gum, polacrillin potassium, cross linked polyacrylic polymer, surfactants, flavor, pH regulatory agents, sweeteners and purified water.

All the experimental trials were prepared in several continued steps including polymers hydration, swelling and incorporation of the active substance, using appropriate equipment: table balance (Sartorius CP 4202 S, Mettler PM 200, Germany), stirrer (IKA UltraTurrax T-50 basic, Germany), magnetic stirrer (Heidolph MR Hei-Tec, Germany), viscometer (Brookfield model RV with standard spindle set, Germany), pH meter (Seven Compact Mettler Toledo, electrode Inlab® Solids Pro, Germany).

Seven different formulations were prepared. Combinations of different percentage of xanthan gum, and the same percentage of carboxymethylcellulose sodium and microcrystalline cellulose/ carboxymethylcellulose sodium, were included into 3 experimental trials (F1, F2 and F3). In the fourth formulation (F4), maize starch was incorporated instead of carboxymethylcellulose sodium, and the fifth formula (F5) included lambda carrageenan gum instead of carboxymethylcellulose sodium.

Method of preparation of the above mentioned trials consists of several processes which include preparation of polymer medium with hydration and swelling process under continuously stirring, and after that incorporation of the active substance into polymeric matrix. Additional inactive ingredients which enhance the organoleptic properties and consistency of the suspension were added, such as sweetening agents, flavors, pH modifiers and surfactants.

* rmarkovska@alkaloid.com.mk

Into the last two formulations (F6 and F7) ion-exchange resins were included. They were prepared with complexation process between the ion-exchange resins and the active substance.

Viscosity measurements were done of all of the prepared experimental trials using laboratory viscometer (Brookfield DV2T RV with standard spindle set, Germany).

The experimental conditions (600 ml beaker, filled with 350 ml suspension, viscosity measured after 60 seconds at 50 rpm of spindle rotation) are kept the same for all of the performed trials which means that the obtained results are comparable with each other.

The palatability study for the prepared experimental trials was performed by panel method. The study protocol was explained and written. Also, the consent was obtained from the volunteers. For this purpose, 10 human volunteers were selected. About 5 ml of the suspension was placed on tongue and taste evaluated after 15 seconds, using a numerical scale. The numerical scale consists of values as 0 = excellent, 1 = good, 2 = slightly burning mouth effect, 3 = burning mouth effect, 4 = intensive burning mouth effect, which were determined by the formulator.

Also, measurement of the sedimentation volume of all of the experimental trials in the study was done. Sedimentation volume (F) is a ratio of the final or ultimate volume of sediment (Vu) to the original volume of sediment (Vo) before settling. It can be calculated by following equation:

$$F = V u / Vo$$

where,

Vu = final or ultimate volume of sediment

Vo = original volume of suspension before settling

Results and discussion

In the formulations with ion – exchange resins, interaction between the reactive functional group of the polyacrylic polymer and ionisable active substance molecule occurs, until active substance - polymer complex is formed. The reaction is performed under determined pH and temperature values. The complex is insoluble in water and it has no taste, so the bitter taste of the active substance entrapped into the complex is masked.

When such complex enters the gastro intestinal fluid, the bond between the active substance and polymer diffuses and the molecule of the active substance is released throughout a decomplexation process. The process of releasing the active substance is performed very quickly and completely into gastro intestinal fluid so it does not affect the absorption and bioavailability of the active substance. (Bilandi et al., 2014; Sampath Kumar et al., 2012).

Different values for the viscosity measurement of all of the experimental trials were obtained, which logically were in correlation with the used percentage of the selected polymer. Values from viscosity measurement for the formulations F1, F2 and F3 were 1046 cP, 1234 cP and 1748 cP, as follows. For the trials F4, F5, F6 and F7 were obtained the following values 1040 cP, 1730 cP, 738 cP and 1916 cP, respectively.

The sedimentation volume can have values ranging from less than 1 to equal or rarely, more than 1. The ultimate height of the solid phase after settling depends on the concentration of solid and the particle size. To obtain an acceptable suspension the value of F should be at least 0.9. In the presented study there is no sedimentation after 14 days for all of the seven trials.

Conclusion

From the performed experimental trials it can be concluded that increasing the viscosity of the prepared oral suspension with combination of different percentage of xanthan gum, and the same percentage of carboxymethylcellulose sodium and microcrystalline cellulose/carboxymethylcellulose sodium, results into satisfactory taste masking effect which is more intensively achieved in comparison with the rest of the experimental trials. The complexation process does not give a satisfactory taste masking effect, although these formulations were optimistic and promising.

The combination of the three polymeric matrices in the optimized concentration, which results into satisfactory viscosity of the suspension containing nonsteroidal anti - inflammatory active substance, was chosen as a favorite formulation and a candidate to work with in the future trials.

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Macedonian pharmaceutical bulletin, **62** (suppl) 429 - 430 (2016)
ISSN 1409 - 8695
UDC: 615.015
Short communication

Influence of formulation variables on encapsulation efficiency of microsponges

Maja Simonoska Crcarevska, Tanja Kjurkchieva Olumcheva*, Renata Slaveska Raicki, Kristina Mladenovska, Marija Glavas Dodov

Institute of Pharmaceutical Technology & Center of Pharmaceutical Nanotechnology, Faculty of Pharmacy, Ss. Cyril & Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Pharmaceutical/cosmetic industry trends are focused toward development of innovative formulations characterized by controlled release of active ingredient (AI) in order to enhance effectiveness and to reduce AI related side/adverse effects.

Microsponges (MSPs) incorporated into well known dosage forms represent new generation of highly effective formulations. MSPs efficacy is related to particle size and size distribution, porosity, pore size, surface area, AI encapsulation efficiency (EE) and release rate. MSPs` characteristics (physico-chemical, biopharmaceutical) influencing their efficacy might be tailored using certain preparation technique, as well as formulation and process parameters. Effect of formulation variables upon MSPs` EE is going to be presented in this review. Quasi-emulsion solvent diffusion technique was most commonly used method for MSPs preparation, while drug to polymer ratio and composition of internal and outer (external) phase were identified as significant formulation variables that influenced EE.

Influence of drug to polymer ratio

EE of Ethyl cellulose (EC) or Eudragit RS100 (ERS100) based MSPs loaded with fluconazole (Abdelmalak and El-Menshave, 2012), was in a range of ~15 to 90% depending from the variables studied. Results from their study pointed that increased polymer quantity resulted with higher EE (~45% *vs* 55% for 1:1 and 1:2 drug to polymer ratio (DPr), respectively). According to authors the findings might be related to the fact that higher polymer amount would result with increased viscosity of internal organic phase (IOP), thus reducing drug molecule diffusion into the external phase (EP) or simply higher amount of polymer encapsulated more drug molecules.

Contrary, studies related to ERS100 MSPs loaded with diclofenac diethylamine (DPr 1:1 to 1:6) (Osmani et al., 2015a) or domperidone (DPr 1:1 to 1:5) (Osmani et al., 2015b) showed opposite trend. Determined diclofenac diethylamine EE values ranged ~10% for DPr 1:6 to 48% for DPr 1:1 (Osmani et al., 2015a), while domperidone EE were ~73% (DPr 1:5) to 92% (DPr 1:1). Similar were the findings for benzoil peroxide EC MSPs (Jelvehgari et al., 2006) prepared with DPr of 1:1 to 13:1 characterized by EE of 70-98%, whereas probably higher drug quantity would result with increased EE as more drug molecules per polymer unit are available.

Studies of Çomoğlu at al. (2003), Orlu et al. (2006) and (D'souza and More, 2008) related to EE of ERS100 MSPs loaded with ketoprofen (DPr 1:1 to 11:1, EE ~92-96%), flurbiprofen (DPr 3:1 to 5:1, EE ~96-97%) and fluocinolone acetonide (DPr 1:1 to 13:1, EE ~87-94%) accordingly, indicated that EE was not much affected by DPr.

Studies of Arya and Pathak (2014) and Srivastava et al. (2012) associated to ERS100/EC and ERS100 MSPs loaded with curcumin and meloxicam, respectively, were conducted using experimental design studies. EE were ~80-93% for curcumin ERS100/EC MSPs and ~71-99% for meloxicam ERS100 MSPs. Derived correlations between EE and studied variables - EC amount (300-900mg) in the IOP and Polyvinil alcochol (PVA) (0.5-1.5% w/v) in the EP (Arya and Pathak, 2014) and volume of organic media - dichloromethane (DCM)(5-7 ml) and ERS100 content (400-

* tanjakjurkchieva@yahoo.com

1200 mg) in DCM (Srivastava et al., 2012) pointed to the positive influence of polymer quantity, although later notified that wasn`t statisticaly significant as it was expected. However, influence of interactions` factors upon EE was also identified. Similar findings were observed in the study of Gupta et al. (2015) where 5-fluorouracil ERS100 MSPs were prepared using different polymer quantity (400-1200 mg) and IOP volumes (6-8 ml) consisted of ethanol:DCM mixture (7:3) (EE ~36-61%) as well as in the study of Pawar at al. (2015) related to oxybenzone EC MSPs prepared with DPr 1:1 to 1:3 and DCM - 3-5 ml (EE ~90-99%).

Influence of organic phase

Influence of solvent type (ethanol, methylen chloride) upon MSPs` EE was investigated by Abdelmalak and El-Menshave (2012) where determined EE indicated that ethanol was solvent of choice most likely due to the higher boiling point and hence lower evaporation rate thus decreasing the diffusion rate into the EP. Orlu et al. (2006) used 3, 5 and 10 ml ethanol for MSPs preparation, but however MSPs could be prepared only with 3 ml ethanol as IOP. When volume of DCM was increased from 5 to 15 ml in the study of Jelvehgari et al. (2006) a decrease of EE was observed (~87 to 67%), most likely due to the lowering of drug concentration.

Higher EE values in increase of IOP volume were determined by Gupta et al. (2015) and Pawar et al. (2015) probably due to the better drug solubility, but however inverse correlation for interaction term of polymer amount and IOP volume was observed. Alike were the results of Srivastava et al. (2012) where additionally quadratic functions of investigated variables` influence upon EE were determined. Inverse dependency of EE to $(IOP)^2$ might be linked to the formation of more porous MSPs thus facilitating drug partitioning into the EP.

Influence of surfactant concentration in the outer water phase

Curcumin EE of ERS100/EC MSPs (Arya and Pathak, 2014) showed positive correlation with the interaction of EC and PVA, while negative correlation was established with the influence of quadratic terms (EC^2*PVA, EC^2*PVA^2). Influence of PVA on the EE was also determined in the study of Jelvehgari et al. (2006) where obtained results pointed that benzoil peroxide EE was higher at lower PVA concentration (EE of 93.26% at 0.047% PVA and 77.87% at 0.187% PVA). Similar results were reported by Abdelmalak and El-Menshave (2012) where fluconazole EE was inversely dependent from PVA concentration (EE ~40% at 0.75% PVA to > 70% at 0.5% PVA). These observations were explained by probable formation of alternative hydrophobic regions dissolving some drug portions thus resulting with decreased EE.

Opposite findings were determined when PVA solution in a concentration of 0.03 to 0.07% was used as EP for preparation of domperidone ERS100 MPSs (Osmani et al., 2015b) and hence EE was ~76 to 90%, respectively. The difference with the previous studies might be related to the low PVA concentration used. Similar were the findings for diclofenac diethylamine ERS100 MPSs (Osmani et al., 2015a) prepared with sodium alginate solution (0.03 to 0.07%) as EP which were characterized by slight increase of EE (~85 to 93%), consequently.

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Macedonian pharmaceutical bulletin, **62** (suppl) 431 - 432 (2016)
ISSN 1409 - 8695
UDC: 615.451.2.032.211-053.2
Short communication

Optimization of viscosity building agent in oral paediatric suspension

Eleonora Trajanovska*, Suzan M. Sejfulah, Sanja Simeonovska Gushic, Vesna Petrovska Jovanovska, Ana Georgieva, Gjorgji Petrusevski, Sonja Ugarkovic

Research & Development, Alkaloid AD-Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Macedonia

Introduction

Suspensions are two-phase systems having solid, substantially water insoluble active agent particles dispersed throughout liquid medium. They represent thermodynamically unstable disperse system, so it is necessary to add suspending agent which reduces the rate of settling and permits easy redispersion of any settled particulate matter, both by protective colloidal action and by increasing the consistency of the suspending medium. Gums and other polymers are frequently employed in pharmaceutical suspensions as viscosity building, thickening, stabilizing and suspending agents. They are used to delay or prevent sedimentation by affecting the rheological characteristics of suspensions. Viscosity building agents increase the viscosity of an aqueous mixture without substantially modifying its other properties, such as taste. They provide body and improve stability of added ingredients (Moreton, 2010).

The aim of the study is optimization of viscosity building agent, xanthan gum in combination with hydroxyethyl cellulose in order to achieve stable oral suspension. In order to evaluate the optimal concentration of the viscosity building agent, formulations with different concentration of xanthan gum were made. Appearance and homogeneity of suspension, viscosity, sedimentation volume and dissolution were evaluated.

Materials and methods

Materials

Active pharmaceutical ingredient, sparingly soluble in water, para-aminophenol derivative that exhibits analgesic and antipyretic activity with bitter taste was used. Solvents, co-solvent, stabilising agent, sweetening agent, buffering agent and preservative were used. Xanthan gum (Jungbunzlauer, Austria) and hydroxyethyl cellulose (Ashland, France) were used as viscosity building agents. Flavour and taste masking agent were used to mask the bitter taste of the active ingredient. All excipients are approved for use in pharmaceutical industry.

Methods

Suspensions were produced by a common process for preparation of oral suspension by method of mixing without heating, dispersing of active ingredient and homogenization. Each of the laboratory trials was prepared under the same condition and with the same method. Suspensions were prepared with different concentration of xanthan gum 0.15%; 0.17%; 0.20%; 0.25% and 0.35%. The concentration of hydroxyethyl cellulose was kept constant in all five laboratory trials. Visual inspection for appearance and homogeneity of suspension, viscosity, sedimentation volume and dissolution was evaluated as critical parameters.

Homogeneity of the prepared suspensions was evaluated using optical microscopy (Morphologi-G3S, Malvern instruments, UK). Viscosity of the suspensions was measured with rotating viscometer (Brookfield, USA, with RV spindle No. 3; temperature 20ºC). Sedimentation volume is defined as the ratio of the final, equilibrium volume of the sediment to the total volume before settling (F = Vu /V0 x100; Vu – volume of sediment in ml; V0 – total volume of suspension in ml). The values for F range from 0 to 1. Values of F = 1 indicate that no sediment is apparent and that the suspension is stable and flocculated (Mohammad et al., 2010). Sedimentation volume was observed and calculated for a period of 30 days, in the following intervals: 60 minutes; 180 minutes; 24 hours; 7 days and 30 days. Content of dissolved active component was determinate with

* etrajanovska@alkaloid.com.mk

HPLC method. Dissolution testing was conducted in 900 ml + 1% phosphate buffer pH 6.8+ 0.05 using USP apparatus II (paddle), set at rotation speed of 50 rpm and temperature of the medium of 37 ± 0.5ºC.

Results and discussion

Homogeneity of the prepared suspensions was evaluated microscopically and differences due to different concentration of viscosity building agent were not observed. Suspensions with concentration of xanthan gum in range from 0.15%; 0.17%; 0.20; 0.25% to 0.35% were measured and results from 562 cP; 687cP; 752 cP; 1005 cP to 1233 cP, respectively for each concentration, were obtained. The increase of the concentration of xanthan gum resulted with an increase of viscosity of the suspensions, which was easily visually noticed and after that confirmed by measurement with a rotating viscometer. The suspension with viscosity above 1000 cP showed bad pourability which can directly influences the proper dosing of the oral suspension (Zatzx and Knapp, 1984). The suspensions were observed during a period of 30 days and sedimentation effect was not observed. Sedimentation volumes for all evaluated concentrations were 1, indicating that xanthan gum used in a range from 0.15% to 0.35% is capable of forming and maintaining a stable suspension for this period. The increase in viscosity avoids the particle aggregation and helps particles to remain in a flocculated state (Tempio and Zatz, 1980). Dissolution testing was performed on the prepared suspension and the following results were obtained 98.22%, 95.15%, 92.22%, 89.42% and 67.95% for each concentration respectively, for the time point of 30 minutes and 99.52%, 98.21%, 97.36%, 91.50% and 81.65%, for the time point of 45 minutes. The percent of dissolved active component decrease by increasing the concentration of xanthan gum, or viscosity of the suspensions. Obtained results from dissolution of the suspensions were higher for the time point of 45 minutes in comparison with the results for 30 minutes. From the results can be noticed that due to its polysaccharide nature the viscosity building agent has the ability to decrease the dissolution rate of the active component when added in higher concentration (Verhoeven et al., 2006). As the viscosity of the dispersion medium increases, the terminal settling velocity decreases thus the dispersed phase settle at a slower rate and remain dispersed for longer time yielding higher stability of the suspension. On the other hand as the viscosity of the suspension increases, it's pourability decreases and the inconvenience to the patients for dosing increases. Also the increase of the viscosity resulted in decrease of the dissolution rate of the active component, directly influencing the bioavailability of the drug.

Conclusion

With concentration optimization of viscosity building agent xanthan gum, viscosity of the suspension can be maintained within optimum range to yield a homogenous, stable suspension appropriate for oral delivery of the drug.

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Macedonian pharmaceutical bulletin, **62** (suppl) 433 - 434 (2016)
ISSN 1409 - 8695
UDC: 615.014.4
Short communication

Risk assessment of excipients in medicinal drug products: a short review

Borche Stamatoski*, Elisaveta Adamova Abraseva, Miroslav Popovski, Suzan Memed Sejfulah, Sonja Ugarkovic, Miroslava Ilievska

ALKALOID AD - SKOPJE, Blvd. AleksandarMakedonski No. 12, 1000 Skopje, Republic of Macedonia

Introduction

Pharmaceutical excipients are substances other than the active pharmaceutical ingredient(API), which are intentionally included in a drug delivery system. They are inert substances by definition but in many cases represent high proportion of the finished dosage form and their quality significantly influences the effectiveness or safety of the drug. Excipients support the processing of the finished dosage form during its manufacture; they protect, support or enhance stability, bioavailability or patient acceptability; help in product identification, or enhance other attribute of the general product quality attributes.

Despite their critical role in the pharmaceutical formulation, there are no specific GMP regulations obligatory for their manufacturers. On the other hand, there are a huge number of applications of these materials which makes the development of appropriate guidelines very demanding. Currently, in the pharmaceutical industry there is a general expectation that excipients are manufactured to recognised GMP principles (The Joint IPEC – PQG Good Manufacturing Practices Guide for Pharmaceutical Excipients, 2006).

The major problem for Marketing Authorization Holders(MAHs) is that the excipient manufacturers produce ingredients that are not only intended for use in pharmaceuticals but also in food, cosmetics, or as chemicals. The quantitative requirement for excipients in pharmaceutical products is often insignificant compared to their use in other applications; however, the quality of the ingredients for use in medicinal products could be essential to the safety, quality and efficacy of the finished drug product (IPEC Federation Position Paper on EU Risk Assessment Guidelines for Excipients 2015C/ 95/02).

Most important global organizations which instruct and facilitate implementation of excipient quality standards are EDQM (European Directorate for Quality of Medicines and Healthcare) and the IPEC (International Pharmaceutical Excipients Council).

EDQM position

At the moment it is the Eudralex Vol. 4, Part 2 guideline, which refers to the quality system of API manufacturers and is sufficient and even higher standard for the excipient manufacturers.

According to this guideline excipients and excipient manufacturers should be controlled based on the results of a formalised quality risk assessment (EudraLex, Volume 4 Good manufacturing practice (GMP) Guidelines, Part 1, Chapter 5: Production). The MAHs are required to have a documented risk assessment/management system for appropriate GMPs for excipients, available on site for review by EU GMP inspectors.

According to The Guideline of 19 March 2015 on the formalised risk assessment for ascertaining the appropriate good manufacturing practice for excipients of medicinal products for human use, the risk assessment principle consists of three parts:

1. Determine the appropriate GMP level that the excipient manufacturer needs to achieve;
2. Determine the current GMP level that the excipient manufacturer has;
3. GAP analysis between these two GMP levels and proposing actions.

The following specific areas of potential risks should be considered, understood and assessed when reviewing the excipient manufacturers' GMP level: risk aspects related to excipient quality and safety, and risk aspects related to excipient function in the drug product.

* bstamatoski@alkaloid.com.mk

Once the appropriate GMP for the excipient and the risk profile of the excipient manufacturer have been defined, the EU guidelines specify that ongoing risk review should be performed.

IPEC's position

The newest and customized guideline for excipient manufacturers that covers topics from the EU GMP guidelines and ISO 9001 chapters is The Joint IPEC – PQG Good Manufacturing Practices Guide for Pharmaceutical Excipients, 2006, which represents good base for implementation of the highest GMP for the excipient manufacturers.

The major concern is that the risk assessment process by different MAHs could eventually classify one manufacturer into different categories of risk (high, medium, low) depending on the different approaches and intended use. This could lead to different quality requirements by different companies for the same excipient. It would be impossible to comply with these different requirements in one quality system unless the highest level of GMP was to be implemented. IPEC is concerned that there is not enough time to complete risk assessments for all excipients by the March 21, 2016 deadline, and that incomplete assessments may jeopardize the availability of high–quality excipients that have been in use for many years.

IPEC will share their views with the EDQM on the challenges to comply with the current timeline and request more realistic goals and timelines (IPEC Federation Position Paper on EU Risk Assessment Guidelines for Excipients 2015/C 95/02).

IPEC views third−party auditing and certification schemes, such as EXCiPACT, and national standards, such as NSF/IPEC/ANSI 363−2014, as playing an essential role to achieve compliance with new requirements for the qualification of excipients and their suppliers. Without additional information about GMP and GDP compliance of the excipient manufacturers through independent third−party audits, it will be nearly impossible for MAHs on their own to gather all the necessary data required (Quality Risk Assessment for Excipients: An Industry Perspective, 2015).

Regulatory authority's position

Even though the groundwork for standardization is established, there is a concern from the regulatory authorities: Ewan Norton of the UK Medicines and Healthcare products Regulatory Agency (MHRA) gave an inspector's view of the new EU requirements on risk−assessment of excipients, expressing concern that despite a 12−month introduction period some pharma companies may not be ready to meet the deadline of 21 March, 2016.

A survey carried out at an MHRA conference last year revealed that a considerable number of pharma industry had either not started the risk assessment process or were unaware of the upcoming requirements. Norton suggested that inspectors would be inclined to serve companies with a deficiency in their report if they were not compliant with the requirements (Excipients insight January/February 2016, IPEC e−newsletter, 2016).

Pharmaceutical industry position

EFPIA (European Federation of Pharmaceutical Industries and Associations) supports the new legislation but is concerned that its implementation may lead to an abundance of regulatory guidance. EFPIA recommends that instead of formalized risk assessment the process should be integrated into existing supplier qualification management programmes. They recommend that it is not necessary to develop additional GMP guidelines for excipients since the risk management principle is already embedded in Part III EU GMP: Quality Risk Management (ICH Q9).

They propose more effective legislation without unnecessary regulatory burden on manufacturers and suppliers to ensure continuous supply of quality excipients and finished drug products (EFPIA TDOC Position Paper).

Conclusion

In the literature, depending on the standpoint there are an abundant number of guidelines, papers and articles that explain the general recommendations for risk assessment principle, but lack those describing hands−on application. There are a limited number of case studies of the pharmaceutical companies applying those guidelines and showing significance of those guidelines and practices.

It is recommended that the literature invests more in the area of application and significance of guidelines and practices. New case studies should be done to prove the success of such practices in risk assessment of excipients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 435 - 436 (2016)
ISSN 1409 - 8695
UDC: 615.014.4
Short communication

Hold-time stability study - a "must-do" for pharmaceutical industry

Ognjenka Rahić , Edina Vranić*, Jasmina Hadžiabdić , Alisa Elezović, Marija Glavas Dodov

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina
Institute of Pharmaceutical Technology, Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Terza 47, 1000 Skopje, Macedonia

Introduction

Three crucial characteristics of medicinal product are its safety, effectiveness and quality required for its intended use. In order to obtain and retain those characteristics, special consideration must be paid to proper storage of medicinal product. Good Manufacturing Practice (GMP) requires that raw materials, packaging materials, intermediates, bulk and finished product are stored under appropriate conditions. This also means that a maximum holding period for intermediates and bulk products before their further processing should be established. In order to avoid any misunderstanding, it is important to define terms "intermediates" and "bulk products" The term "Intermediate" is partly processed product that must undergo further manufacturing steps before it becomes a bulk product. "Bulk product" refers to any pharmaceutical product that has completed all processing stages up to, but not including, final packaging (Eudralex, 2010).

Hold-time studies guidance

Until 2015, relevant guidelines (CPMP/QWP/122/02, 2003; ICH Q1A(R2), 2003; Eudralex, 2014; FDA Q1A(R2), 2003) thoroughly described the requirements for stability testing of finished drug product. The only statement in guidelines with regard to pharmaceutical bulk products or intermediates is that they have to be stored "in a suitable way".

In 2015, World Health Organization (WHO) released a final version of "General guidance on hold-time studies" as a part of WHO Technical Report Series No. 992 (WHO, 2015). Although this guidance describes the principles of establishing criteria for performing hold-time studies on coated tablets, those principles can be applied to other non-sterile dosage forms.

Recommendations for conducting hold-time studies

Hold time is a period of time during which materials can be stored under defined conditions and will retain their quality within the specified limits. This implies that hold times should be established for the materials at different phases of manufacturing, so one can be sure that holding didn't compromise the quality of the finished product.

Hold times should be established based on scientific data. It is recommended that the test is to be performed on one batch prior to registration of the product. All tests should be performed using validated stability-indicating methods. Storage conditions of samples are required to be the same as for the quarantine area or manufacture stage (WHO, 2015). Apart from that, sample should be stored in a simulated package mimicking the packaging of the bulk product (Huynh-Ba, 2009; WHO, 2015). This means that containers should be made of the same material and using same closure system as the system in the manufacturing stage. If there is a risk of substance's degradation as a result of oxidation processes, headspace in the containers should also be tested. It is necessary to determine the ratio of headspace to contents in the containers, so degradation of substance is unlikely to occur (WHO, 2015).

* evranic@yahoo.com

Data to support holding times could be collected during drug development, on a pilot or validation batches. If the product is already on the market, guidance (WHO, 2015) leaves a possibility to perform retrospective hold-time studies. Collected data should be processed statistically in order to note trends or to establish limits. Study should be performed through a period defined in the guidance, and not beyond that. It isn't necessary to prolong study until the lowest limit of quality is achieved. This approach is based on a "most probable" as opposed to "worst case" approach. For example, 90 days are enough time to keep core tablets as intermediates in the coated tablet manufacturing, to study hold-time stability (WHO, 2015).

General guidance on hold-time studies (WHO, 2015) provides examples of stages, tests and study times that may be performed for a coated tablet. Coated tablet have been chosen as an example, since tablets are the most widely used dosage form. Apart from that, manufacture of tablets and, especially, coated tablets can be a complex process, including a variety of manufacturing stages (Armstrong, 2007). Each stage can, in some way, influence the quality of the final product.

Once hold time is established, intermediates and bulk products should not be stored beyond this period.

Conclusion

Hold-time study plays an important role in the manufacturing of drug products in the GMP environment. The maximum hold time for intermediates and bulk pharmaceuticals should be established in order to continue their processing or start the packaging of the drug products before their quality becomes compromised.

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Macedonian pharmaceutical bulletin, **62** (suppl) 437 - 438 (2016)
ISSN 1409 - 8695
UDC: 615.215.24.015
Short communication

Comparison between some methods for solubility enhancement of lorazepam

Jasmina Hadžiabdić[1], Edina Vranić[1]*, Ognjenka Rahić[1], Alisa Elezović[1], Marija Glavas Dodov[2]

[1]*Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina*
[2]*Institute of Pharmaceutical Technology, Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Lorazepam has anxiolytic, sedative, hypnotic, and anticonvulsant as well as muscle relaxant properties. It is a highly lipophilic compound whose partition coefficient is 2.36 when measured at 20 °C. Lorazepam, as an amphiphilic substance, has two pKa values (1.3 and 11.5) determined spectrophotometrically in aqueous buffers. It is mainly used in different dosage forms as an active substance. The injectable solution comes in 1 mL ampoules containing 2 or 4 mg lorazepam. As derivative of 1,4-benzodiazepines, has certain limitations in pharmaceutical technology due to its poor solubility in water. The aqueous solubility of lorazepam, particle size 8.9 μm, is 0.0485 mg/mL (Hadžiabdić and Hadžović, 2005-2006). Often, in a certain volume of water, an adequate concentration of the drug cannot be achieved during formulation of a liquid dosage form due to the low solubility of the drug (Jinal et al., 2012).

This study investigated the use of the traditional solubilization approaches to increase the solubility of lorazepam. Our aim was to investigate the solubility of lorazepam in phosphate buffer solutions, in water/cosolvents mixtures (cosolvents: ethanol, propylene glycol, polyethylene glycol 200 and 400) (Corrigan and Healy, 2007; Vemula et al., 2010), and in water/surfactants mixtures [surfactants: Tween 80, Tween 20, Brij 35, sodium cholate, sodium deoxycholate, sodium taurocholate] (Corrigan and Healy, 2007; Rangel-Yagui et al., 2005) as well as its solubility in water/cyclodextrins mixtures [cyclodextrins: α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (2-HP-βCD)] (Loftsson and Brewster, 2010; Sathesh Babu et al., 2008). The main objective was to find the most suitable method for providing good solubility of lorazepam and thus its formulation in a liquid dosage form.

Materials and methods

Materials

Used chemicals were obtained from: Lorazepam (LZ, Sigma-Aldrich, Germany); Acidum sulfuricum 95-97% and Methanolum (Kemika, Croatia); Acidum hydrochloricum 37%, Disodium hydrogen phosphate and Potassium dihydrogen phosphate (Alkaloid, Macedonia); Acidum phosphoricum 85% (Fluka, Chemika, Switzerland); ethanol 96%, Polyethylene glycol 200, Polyethylene glycol 400 and Propylene glycol (Sigma-Aldrich, Germany); Tween 20, Tween 80 and Brij 35 (Merck, Germany); Sodium cholate, Sodium deoxycholate, Sodium taurocholate, α-CD, β-CD and 2-HP-βCD (Fluka, Chemika, Switzerland).

Solubility studies

The solubility of LZ was estimated by the solubility method of Higuchi and Connors (1965). Solubility measurements and the determination of saturation concentrations were carried out by adding excess amounts of lorazepam to phosphate buffer solutions (pH 2.0-8.0), water/cosolvent mixtures (concentrations 1-60% w/w), water/surfactants mixtures (concentrations 1-20% w/w, for anionic surfactants and 1-35% w/w, for non-ionic surfactants). Concentrations of cyclodextrins were selected based on

* evranic@yahoo.com

their solubility in water. Testing of LZ solubility with the addition of α-CD in aqueous solutions was carried out by its addition in concentrations up to 14%. The testing of LZ solubility was carried out in concentrations up to 1.8% of aqueous solutions of β-CD. The testing of LZ solubility with the addition of 2-HP-β-CD in aqueous solutions was carried out by its addition in concentrations up to 40%. The concentrations of dissolved LZ in tested solutions were determined using a Shimadzu UV-1601, UV VIS spectrophotometer (Shimadzu, Japan).

Results and discussion

It was found that LZ solubility was decreased with an increasing pH. When the pH value is lower, the percentage of protonation of lorazepam is higher. Protonation at low pH occurs at the nitrogen atom in position 4. Deprotonation occurs at a high pH with the loss of the hydrogen atom from the 3-hydroxyl group (Barrett et al., 1973).

To prepare the aqueous solution of LZ (2 mg/mL), it is necessary to have more than 50% (w/w) of propylene glycol, or more than 40% (w/w) of polyethylene glycol 400, polyethylene glycol 200 or ethanol. To completely dissolve 4 mg/mL LZ in water more than 50% (w/w) of polyethylene glycol 400 or polyethylene glycol 200 or less than 50% (w/w) of ethanol is needed. It is evident that LZ molecules preferably solubilize in a nonpolar environment rather than polar (aqueous) surroundings. The concentration of the solvents that enable this drug to be solubilized in water is far higher than that allowed for daily intake.

Increase of surfactants concentration in water leads to LZ solubility enhancement linearly. The best solubility was achieved with sodium taurocholate, of all the tested bile salts, while the best LZ solubility in water was achieved with Brij 35, of all the tested non-ionic surfactants (Alkhamis et al., 2003; Rangel-Yagui et al., 2005).

To dissolve 2 mg/mL of LZ in water, more than 15% of sodium cholate, more than 5% of sodium deoxycholate, or less than 10% of sodium taurocholate concentrations are needed. To make a LZ concentration in liquid form of 2 mg LZ/mL of water, more than 5% of Tween 80, more than 7% of Tween 20, or less than 3% Brij 35 is needed. To dissolve 4 mg/mL of LZ in water, more than 20% of sodium cholate, ~15% of sodium deoxycholate or less than 15% of sodium taurocholate is needed. To dissolve 4 mg of LZ/mL of water, 20% of Tween 80, more than 15% of Tween 20 or less than 7% Brij 35 should be used. The surfactants with higher HLB values were better solubilizers.

The increase of LZ solubility in aqueous solutions of chosen cyclodextrins, compared to its solubility in water. The solubility of LZ is increased 6.5-fold at 14% of α-CD, 4.38-fold at 1.8% β-CD, and 170.1-fold at 40% of 2-HP-β-CD. The changes in the solubility of LZ resulting from the addition of various concentrations of α-CD, β-CD and 2-HP-β-CD were used to plot phase solubility diagrams and to evaluate the stoichiometry and stability constant of the resultant complex. The phase solubility diagrams obtained are linear and show characteristics of AL-type of solubility curve. This is attributed to the formation of a soluble complex. The apparent stability constants (K1:1) were estimated from the straight line of the phase solubility diagrams according to equation of Higuchi and Connors (Higuchi and Connors, 1965; Mosher and Thompson, 2007). In this case, the K1:1 values obtained followed the order 2-HP-β-CD > β-CD > α-CD, reflecting the greater affinity of modified cyclodextrin for LZ compared with their parent α-CD and β-CD.

Conclusion

Based on the results, the changes in the pH value of the media do not lead to a greater solubility of lorazepam. Of the cosolvents used, the greatest increase in solubility of lorazepam in water was achieved with ethanol. Of the bile salts used, sodium taurocholate showed the best solubilization ability, while Brij 35 was the best of the non-ionic surfactants. The solubility of lorazepam with the cyclodextrin derivative, 2-hydroxypropyl-β-cyclodextrin was better than natural cyclodextrins. Surfactants have the highest ability of solubilization of lorazepam in water. Thus, the study generates an array of data for solubilization of lorazepam using various pharmaceutically accepted techniques which could be useful while formulating liquid dosage forms of lorazepam.

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Macedonian pharmaceutical bulletin, **62** (suppl) 439 - 440 (2016)
ISSN 1409 - 8695
UDC: 615.246:616.345
Short communication

Comparison of biopharmaceutical properties of 5-FU loaded TEOS and TEOS/APTES microparticles for colon targeting

Beti Djurdjic[1,2]*, Nikola Geskovski[1], Simona Dimchevska[1], Katerina Goracinova[1]

[1]*Institute of Pharmaceutical technology, Faculty of Pharmacy, University Ss Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Macedonia*
[2]*Department of Pharmacy, Faculty of Medicine, University of Montenegro, Kruševac bb, 81000 Podgorica, Montenegro*

Introduction

Colon drug targeting is valuable approach for the treatment of serious GIT diseases like ulcerative colitis, Chron's disease, colon carcinomas and infections; considering that high local concentration at the site of action can be achieved, and systemic exposure to the active substance as well as serious side effects can be avoided (Chourasia and Jain, 2003). Different studies point to the connection of the inflammation and tumorigenesis and the presence of immunoinflammatory mediators and inflammation in virtually all steps of colon cancer development, including initiation, promotion, progression, and metastasis. Therefore, the design concept of increased localization of micro and/or nanoparticles at the site of inflammation/cancer due to enhanced permeability and retention in the tissue damaged by inflammation/cancer may also be favourable for colon cancer targeting. Additionally, prolonged intimate contact with the epithelial membranes at the site of action, which may be enhanced by incorporation of bioadhesive polymers into the carriers is crucial for improvement of specific interaction with mucin and cell surfaces as well as for improved localization of the drug delivery systems at the site of action. Whence, the aim of this study was to design 5-fluorouracil loaded organomodified silica microparticles as systems for controlled and site specific colon delivery as well as to investigate the influence of the concentration of the silane coupling agent 3-aminopropyltriethoxysilane (APTES) upon the microparticle properties.

Materials and methods

Materials

Silica microparticles were prepared using the following reagents: tetraethoxysilane (TEOS; Sigma, Germany), 3-aminopropyltriethoxysilane (APTES; Sigma, Germany), ethanol 96% (v/v) (Merck, Germany), acetic acid (Merck, Germany), 5-Fluorouracil (5-FU; EBEWE Pharma, Austria), and deionised water. All other used chemicals and reagents were of analytical grade.

Preparation of silica microparticles

Silica microparticles were prepared from tetraethoxysilane (samples MP1) and tetraethoxysilane co-hydrolyzed with 3-aminopropyltriethoxysilane by combining sol-gel technology with spray-drying. TEOS-based silica microparticles (MP1) were synthesized by sol-gel method at room temperature using a one-step acid-catalized hydrolysis. Silica sol was prepared by hydrolysis and polycondensation of TEOS with deionised water, ethanol and acetic acid as a catalyst. The molar ratio of the silica sol was TEOS: water : ethanol : acetic acid = 0.01 : 1.39 : 0.43 : 0.009. Hydrolised silica sol was spray dried with a mini spray dryer (B-290, Büchi Labortechik AG, Switzerland). 5-FU was added after completed hydrolysis and incorporated in microparticles in network synthesis phase at concentration 12 wt%. For partial substitution of TEOS, 2.5 mol% (MP2) and 5 mol% (MP3) organomodified alkoxide was used. 5-FU was dissolved in the hydrolised sol before spray drying at concentration of 12 wt%.

Characterization of silica microparticles

Prepared silica microparticles were characterized in a terms of mean particle size and particle size distribution

* betibd@t-com.me

(Mastersizer 2000, Malvern Instruments Ltd., UK), surface morphology (SEM; Jeol-SEM6400, Japan) and drug loading efficiency (HPLC Agilent 1200 with UV detector).

Ex vivo mucoadhesion studies was performed and the percent of mucoadhesion was determined with modified everted sac method (Santos et al., 1999).

In vitro drug release studies

The dissolution test from prepared silica microparticles were carried out in closed glass tubes at 37 °C and 50 horizontal strikes/min (horizontal shaker; Shaker Unitronik OR, Selecta. Spain). To compare the drug release under different pH conditions, 50 mg of the microparticles mixed with 10 ml buffer solution with pH 1.2 and 10 ml buffer solution with pH 7.4. At appropriate intervals, 2 ml of samples were withdrawn, filtered through 0.45 μm membrane filter, and assayed by HPLC method.

In order to investigate the possible drug release mechanisms, data obtained from *in vitro* drug release studies were analyzed using different kinetic models (Higuchi equation, the traditional power law and modified power law) (Viitala et al., 2007). Determined *n* value indicated most probable drug release mechanisam, while goodness of fit was evaluated using the *r* (correlation coefficient) values.

Results and discussion

Prepared particles were spherical with smooth surfaces and unimodal narrow size distribution. Median volume diameter of microparticles was 1.3876 μm (SPAN factor 1.745), 2.1405 μm (SPAN factor 1.133) and 3.3763 μm (SPAN factor 1.713) for MP1, MP2 and MP3, respectively. The content of 5-fluorouracil was 89.5 mg (MP1), 145.71 mg (MP2) and 160.78 mg 5-FU/g microparticles (MP3). The experimental results suggest that addition of precursor 3-aminopropyltriethoxysilane in the silica sol influenced the inner structure of the matrix, resulting with increased porosity and hydrophilicity of the network, resulting in increased average paricle size and drug loading efficiency.

The results from the *in vitro* drug release studies of TEOS based microparticles showed that the silica matrices are capable for controlled release of 5-FU during prolonged time periods. However, the dissolution rate was proportional to 5-FU loading, most probably because of possible strong 5-FU – silica interactions that could initiate formation of matrix microdeformations during the sol-gel process, resulting in overall increase of matrix porosity (Djurdjic et al., 2011). pH dependent drug release was noticed from TEOS based particles with lower percent of 5-FU (2 and 6wt%) (Djurdjic et al., 2010, 2011), but pH dependency was lost when higher percent of 5-FU (12 wt%) was incorporated in the processed silica sol during production of the microparticles, most probably because of increased matrix porosity. The drug release data showed the best fit to modified power law equation; r^2 were 0.967 and 0.963 at pH 1.2 and pH 7.4, respectively for sample MP1. Values *n* for sample MP1 (0.07 and 0.09 at pH 1.2 and pH 7.4, respectively) point to drug diffusion through very porous matrix without capacity for control of the release during time.

Organically modified microparticles showed increased burst and increased drug release rate compared to TEOS based microparticles. Burst release varied accordingly to the percent of APTES. For formulations with highest percent of APTES and drug loading, the total ammount of incorporated 5-FU was released within few hours. The drug release data of organically modified microparticles show the best fit to modified power law model and r^2 were 0.859 and 0.955 (at pH 1.2 and pH 7.4, respectively) for MP2. Values *n* for sample MP2 (0.16 and 0.13 at pH 1.2 and pH 7.4, respectively) indicating to drug diffusion through highly porous system. Formulations with high percent of APTES were unable to control the 5-FU release in time.

The presence of -amino groups at the surface of APTES particles affected the intensity of interaction with cell membranes and lead to increased muco/bioadhesive potential relative to the unmodified TEOS particles (Djurdjic et al., 2011). Percent of mucoadhesion for organically modified microparticles increased with increased proportion of APTES (55.7% of mucoadhesion for MP3).

Conclusion

The addition of precursor APTES modifies the morphology of silica microparticles, resulting in increased particle size, drug loading efficiency, muco/bioadhesive potential and 5-FU release rate compared to TEOS based particles. Mucoadhesive potential, burst release and dissolution rate for organically modified particles varied accordingly to the percent of APTES.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 441 - 442 (2016)
ISSN 1409 - 8695
UDC: 615.33:616.314.17
616.314.17-085.33
Short communication

Doxycycline hyclate-enriched gelatine nanoparticles for periodontal disease treatment: preparation and evaluation study

Selestina Gorgieva[1], Vanja Kokol[1], Nikola Geskovski[2], Simona Dimchevska[2] and Katerina Goracinova[2]*

[1]*University of Maribor, Institute of Engineering Materials and Design, Smetanova ulica 17, 2000 Maribor, Slovenia*
[2]*Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Periodontal disease is chronic inflammatory disorder, believed to be generally caused by pathogenic microorganisms colonising the toot surface, amongst which the most popular are *Actonobacilius actinomyycetem comitans, Bacteroides forsitus, Porphyromonas gingivalis*, etc., being ~75% GRAM- anaerobic type. This disease affect the structural organs supporting the teeth, causing gingiva detachment from the tooth and periodontal pockets formation as an ideal ecological niche for the bacteria proliferation. Doxycycline (DOXY) is a wide spectrum antibacterial, bacteriostatic drug, effective against the aerob-/anaerobic type of GRAM+ and GRAM- bacteria as well as protozoa. It is a member of the tetracycline group, which is frequently used in dental treatments due to matrix metallo-proteinase- inhibitory effect and strong activity against periodontal pathogens (Tamimi et al., 2008). DOXY is almost completely absorbed in duodenum with a bioavailability of more than 80% with an average of 95%, and half-life of absorption is 0.85±0.41h.

Various drug delivery systems have been trailed in periodontitis treatments, such as fibres, gels, injectable systems, micro-spheres/particles, strips, compacts, films, and nanoparticles (NPs) (Raheja et al., 2013). Use of the later was found advantageous over the others due to their size allowing penetration in extra- and intracellular areas, such as gingival fluid, bacterial cells, from the gingival sulcus to the underlying connective tissue and to periodontal pocket areas below the gum line, being otherwise hardly accessible by different systems (Segundo-Pinon et al., 2000). Variety of materials have been proposed for drug-NPs processing; the important biomimetic merits of parent biopolymer (the biocompatibility and presence of multiple functional moieties) makes the gelatin (GEL) NPs effective delivery vehicles to be applicable for diverse therapeutics (Khan and Schneider 2013).

By this respect, presented study examines the applicability of nanoprecipitation as preferable methodology for DOXY-enriched GNPs processing. The variations range of processing factors, being selected within primary, trial-error experiments (i.e. the pH of GEL solution, DOXY concentration and EDC/NHS concentration) were established with utilization of ″one factor each time″ experimental approach. The limiting factor in all cases was drug/biopolymer precipitation. The influence of processing and formulation factors upon the particle size, drug content and encapsulation efficiency were estimate for processed formulation using MODDE software- generated experimental design. Chemical modification (crosslinking degree) and (in vitro) dissolution behaviour of selected DOXY-GNP formulation will be also discussed in this paper.

Materials and methods

Materials. GEL type B from bovine skin Bloom 225 (Sigma Aldrich, Germany), Doxycicline hyclate (DOXI) (Tocris, UK), Lutrol F-127 was kindly donated by BASF, Germany. 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich, Germany. All other chemicals were of analytical grade and were used as received, without additional purification.

GNPs preparation. 0.5% w/v GEL/H2O solutions were prepared at 50°C under moderate stirring for 1h. The

* kago@ff.ukim.edu.mk

two separate solutions were prepared in parallel, and subsequently adjusted to pH 7.5 and pH 9.5. 1mL of selected solution was added dropwise to 10mL of 7% w/v Pluronic F-127 solution in 96% EtOH, latter being vigorously stirred onto magnetic stirred with 700rpm. Additional 10mL EtOH were added afterwards, just prior addition of different concentrations of cross linkers (20-25% w/w, relative to gelatin), being dissolved in 1.250 mL deionised water just prior experiments. One set of samples were removed from the magnetic stirred, while the other half were subjected to mild stirring for 24h. In parallel with DOXY-free HNPs, the DOXY-loaded one slight modification of above procedure, by simple mixing of pre- prepared DOXY and GEL solutions in quantities described within experimental design.

Characterization. Scanning Electron Microscopy / SEM/ imaging and Differential Light Scattering /DLS/ analysis were performed to evaluate particle size, PDI and aggregation behaviour in different physiological media and particle morphology. Drug content (%), encapsulation efficiency (%) and drug release (%) were quantified by optimised HPLC method (according to European Pharmacopoeia 5.0). Uv-Vis Spectroscopy (using tri nitro-benzene-sulfonic acid /TNBS/ reagent) and Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy / ATR-FTIR/ method were used to identify the crosslinking efficiency. Dissolution test was performed in relevant (saliva-like) conditions (phosphate buffer pH 7.4, 37°C, horizontal mixing) using cellulose ester- based Float a Lyser device (MWCO 100kDa) (at particular time point samples were withdrawn and analysed by HPLC means and Uv-detection /254nm/ of DOXY component).

Results and discussion

Physiologically stable drug-free, as well as DOXY-enriched GNPs were successfully processed by optimised nanoprecipitation method, where EDC/NHS chemistry was introduced in final processing phase. This chemical stabilization results in up to 70% crosslinking degree, identified by means of NH_2 group's reduction. Due to the presence of suitable chemical moieties and their respective pKa values, we assume that cross-linking occur not only between GEL molecules, but also between GEL and DOXY, and potentially, between the GEL and the Pluronic F-127. DOXI chemical ″arrestment″ was found to affect its dissolution profile, which, for particular formulation was estimate to 35% in 24 h period. Moreover, we observe significant effect of processing conditions, i.e. the mixing of DOXY-GNPs dispersions after nanoprecipitation influence on their aggregation behaviour, giving a clue for more complex interactions within examined, multicomponent system.

Resulting DOXY- integrated GNPs where visualised mainly as individual, spherically shaped NPs in 150-240 nm size range, while, relatively high encapsulation efficiency (up to ~40%) and drug content (25%) were identified for selected factors combination.

Conclusion

DOXY-enriched GNPs may be further applied as single formulation or included in other delivery system (e.g. Guided Tissue/Bone Regeneration /GTR, GBR/ membrane) and used in periodontal disease treatment. Nanoprecipitation methodology can be further explored in processing of GNPs as carriers of different types of drugs, thus serving as a processing platform for facile, yet controlled engineering of drug delivery carriers.

Acknowledgement

This work was financially supported by the ERASMUS MUNDUS mobility program- Join EU-SEE>PENTA (Action 2).

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Macedonian pharmaceutical bulletin, **62** (suppl) 443 - 444 (2016)
ISSN 1409 - 8695
UDC: 615.849.2(497.7)
Short communication

Prospective of PET radiopharmaceutical development –new approach and strategy for their application

Katerina Kolevska[1]*, Maja Velickovska[2], Marija Atanasova[1], Filip Jolevski[1], Maja Chochevska[1], Emilija Janevik-Ivanovska[1]

[1]*Faculty of Medical Sciences, Goce Delcev University Stip, Republic of Macedonia*
[2]*Project Unit for implementation of Positron Emission Tomography - PET Center Skopje in Republic of Macedonia, Ministry of Health of the Republic of Macedonia*

Introduction

The University Institute for Positron Emission Tomography of the Republic of Macedonia is the first center in our country with opportunity for production of PET radiopharmaceuticals, which are prepared as sterile solutions for parenteral use. In the first line of production are included the most used radiopharmaceuticals for PET imaging, such as: [18F] FDG; Na18F; [13N] NH3; [11C] Choline and 68Ga labelled somatostatin analogs.

Pharmacological approach of the PET radiopharmaceutical drugs – first line of production of The University Institute for PET of Republic of Macedonia

[18F] FDG

[18F] FDG is the most widely used PET radiopharmaceutical in clinical practice, especially in oncology, neurology and cardiology, providing functional information based on tissue metabolism. This radiopharmaceutical is a structural analogue of 2-deoxy-D-glucose labelled with positron-emitting isotope 189F in the position 2 of the glucose core structure. Widespread use of [18F] FDG is based on the principle of 'metabolic trapping' in the cancer cells.

After intravenous administration, [18F] FDG through the bloodstream is distributed into cells with the same mechanism as plasma glucose. It is actively transported into the cell mediated by a group of structurally related glucose transport proteins (GLUT). Tumor cells have an overexpression of GLUT compared to a normal cell, and therefore the uptake of [18F] FDG is increased. When the [18F] FDG is transported in the cells, it is phosphorylated in [18F] FDG-6-phosphate under the catalytic action of hexokinase and it remains metabolically trapped intracellularly, because [18F] FDG-6-phosphate is not a substrate for glucose-6-phosphate isomerase, the enzyme that metabolizes glucose (Scott et al., 2012).

The accumulation of [18F] FDG in the malignant cells generally is proportional to the metabolic activity of the cancer cells, which enables their detection by the PET scanner. [18F] FDG is non-specific cancer radiopharmaceutical. Increased accumulation of [18F] FDG occurs in processes such as inflammation, infection, especially sarcoidosis, tuberculosis, fungal infections, and pneumonia (Jacobson et al., 2012).

Normally increased accumulation of [18F] FDG is observed in the brain because the glucose is the main energy source for the brain. Also very important is the concentration of glucose in the blood of the patient and it should be evaluated prior to administration of [18F] FDG. The increased concentration of blood glucose causes elevated levels of insulin, which increases biodistribution of [18F] FDG in muscle and adipose tissue (Lindholm et al., 1993).

[18F] Na18F

[18F] Na18F PET/CT is diagnostic tool for imaging benign and malignant bone diseases. Fluoride ions are incorporated into the bone matrix at the bone surface preferably in sites of newly mineralizing bone, such as during growth, infection, malignancy (primary or secondary), after trauma or during inflammation.

The initial [18F] Na18F distribution represents blood flow that varies among different bones. Almost all deliv-

* katerina.kolevska@ugd.edu.mk

ered [18F] Na18F is retained by bone after a single pass of blood. The uptake of fluoride ions is better in osteoblastic processes, while purely osteolytic processes have a lower uptake or even no uptake at all. [18F] Na18F is rapidly cleared from plasma and excreted by the kidneys. The target organ is bone, but approximately 20% is excreted through the kidney in the urine in the first 1-2 hours (Czernin et al., 2010).

[13N] NH3

The main clinical application of [13N] NH3 radiopharmaceutical is assessment of myocardial perfusion in patients with suspected or known coronary heart disease.

Following intravenous injection, [13N] NH3 rapidly clears from the circulation. It is taken up mainly by the myocardium, brain, liver, kidneys, and skeletal muscle. [13N] NH3 is extracted from the capillaries through the ammonia transporter. The accumulation in tissue is in proportion to blood perfusion of the tissue.

In the cells, it is converted to glutamine and can diffuse out of the cell or be metabolized to glutamate and retained within the cell. [13N] NH3 undergoes a five-enzyme step metabolism in the liver to yield [13N] Urea, the main circulating metabolite, which is eliminated from the body by the urinary excretion (Adeva et al., 2012).

[11C] Choline

[11C] Choline is radiopharmaceutical for oncological PET imaging of tumors which overexpress choline kinase. Result of overexpression of choline kinase is increased level of phosphorylcholine by accumulation of free choline for cell membrane synthesis. [11C] Choline is accumulated preferentially within prostate cancer tissue, for that reason the most important application of this radiopharmaceutical in clinical practice is in visualization of this type of tumor. [18F] FDG is not first choice in visualization of the tumors in pelvis area, because of the low uptake, related to lower expression of glucose transport proteins and to the huge [18F] FDG urinary excretion (Lodi et al., 2012).

The maximal tumoral[11C] Choline uptake is related to primary tumor stage (Reske et al., 2006).After intravenous administration, the peak of uptake is reached by five minutes and the activity is retained over the subsequent 30 minute scanning period. The distribution of [11C] Choline is mainly to the pancreas, kidneys, liver, spleen and colon. The major metabolite detected in blood is [11C] Betaine (Roivainen et al., 2000).

68Ga labelled somatostatin analogs: [68Ga] DOTATOC, [68Ga] DOTANOC and [68Ga] DOTATATE

The use of [68Ga] DOTATOC, [68Ga] DOTANOC and [68Ga] DOTATATE radiopharmaceuticals for visualization of neuroendocrine tumors is based on high affinity of the biological ligand to somatostatin receptors (SSTR). Natural somatostatin has low metabolic stability, therefore synthetic analogues with high affinity for SSTR and resistant to enzymatic degradation are developed. (Velykian, 2014).

[68Ga] DOTA SST analogues show a rapid localization of the target site, fast blood and renal clearance. Radioactive metabolites are not detected in serum or urine after 4 hours. The maximum accumulation of activity in the tumor is reached 70 ± 20 min after injection. Excretion is almost entirely by the kidneys.

Conclusion

The mapping of the radiopharmaceutical distribution in vivo provides images of functional morphology of organs in a non-invasive manner and plays an important role in the diagnosis of many common diseases associated with the malfunctioning of organs in the body as well as in the detection of certain type of cancers (IAEA, 2006).

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Macedonian pharmaceutical bulletin, **62** (suppl) 445 - 446 (2016)
ISSN 1409 - 8695
UDC: 665.58
Short communication

Cosmetovigilance

Irina Dukovska

Faculty of Pharmacy, University Skopje "Ss. Cyril and Methodius",
Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Definition of a cosmetic

The human need for improvement of the physical appearance is in constant increase year after year. This is revealed by various types of tests and ongoing market research. Hence, the production, the application and the consumption of products for personal hygiene as well as cosmetic products for care, protection and enhancement of the beauty are becoming more and more popular among large number of consumers.

A cosmetic product is classically defined as any preparation that is applied to the skin, eyes, mouth, hair or nails for the purpose of cleansing, appearance enhancement, protection and/or for pleasant smell. Unlike drugs, which are used for treatment or prevention of a disease in the body, cosmetics are not considered to change or affect the body's structure or functions. However, the distinction between drugs and cosmetics is sometimes not well defined. In spite of the considerable safety of and the high skin tolerance for the cosmetic products, adverse effects still occur from their use, which might be a result from the use of an inappropriately chosen product. Most often these adverse effects are of small or medium intensity and appear usually on the skin (Evaluation of the Cosmetics and Explosives Directives, 2007; Regulation (EC) N0 1223/2009). The research subject of this work is cosmetovigilance, which refers to a series of defined actions that have the purpose of revealing, estimating, monitoring, recording and preventing the potential undesirable reactions that appear as a consequence of the application of a cosmetic product.

The concept of cosmetovigilance is recently added to the European Regulation for cosmetic products. It is a form for objective monitoring of the unwanted effects of the cosmetic products, which are of public health interest. In contrast to the monitoring carried out by the industry, the aim of which is the safety of its own market for commercial purposes, cosmetovigilance has strictly medical aim (Vigan and Castelain, 2014).

The European regulation

The European resolution (ResAP (2006)1) from 2006 laid the foundations of the cosmetovigilance system which is based on the reports from the recorded cases. The European Regulation (EU) 1223/2009 for cosmetic products, which was officially introduced on 11 July 2013, requires from all manufacturers to appoint a responsible person who would constantly monitor and record the implementation of the cosmetovigilance for the products available on the market. From 2013 onwards, the new European regulation demands that the serious undesirable effects which are reported to the competent authority to be also delivered to the competent authority of the other member states as well as to the responsible person for a certain cosmetic product in the company. The regulation for cosmetic products primarily addresses the safety of the products that are used by large populations of healthy consumers. However, the efficacy and the safety of cosmetic products are not reviewed or approved by national competent authorities before they are sold to the public. The identification and the analysis of the adverse effects related to cosmetic products is a process that is still to a large extent industry-driven. It is a manufacturers' responsibility to determine that the product and its ingredients are safe before they are put on the market. Moreover, manufacturers have the responsibility of collecting the reports for all the recorded adverse effects. Although manufacturers do their best to ensure safety of their products, it should be taken into consideration that a potential conflict of interests is always present (Moretti and Velo, 2008).

The aim of this work is to present the implementation of the cosmetovigilance system in the countries in Europe

* idukovska@gmail.com

and how it is employed in practice. Also this work would attempt to resolve two major problems: evaluation of the causes and the types of reports. At the same time it would present data gathered from conducted studies the aim of which was to estimate the appearance of adverse effects from the use of a cosmetic product, utilizing different methods for their detection, monitoring and prevention, as well as establishing a standard cosmetovigilance system.

Causality assessment

The definition of causality assessment is slightly different for French Health Products Safety Agency (AFSSAPS) and European Cosmetic, Toiletry and Perfumery association (Colipa). For AFSSAPS, causality "assesses the cause and effect relationship between a cosmetic product and a specific clinical and/or paraclinical manifestation" (2010). Causality must be established for each product individually. For Colipa "Causality assessment is particularly useful when the same product is involved in the occurrence of several cases of undesirable effects, when it makes it possible to determine the extent of a link of cause and effect between the cosmetic product and the undesirable effects observed and then to take these effects into account in the subsequent drawing-up of corrective measures such as investigations, recommendations on the proper use of the product, or regulations at national or European level (restrictions on use, warnings on packaging labels, limited concentration or prohibition) (Zweers et al., 2012).

Several methods have been published. They are based on the analysis of evolving chronological and semiological elements. The results of relevant tests or of re-challenge tests can alter causality, for instance as regards contact allergy; appropriate patch testing provides a certain degree of causality. The AFSSAPS method is based on 6 criteria, divided into two groups, which are used to calculate a chronological score and a semiological score. The level of causality is determined using a decision table in which the scores are combined. The method has five levels of causality assessment: very likely, likely, not clearly attributable, unlikely and excluded. The causality assessment method by Colipa is based on three major criteria: symptomatology, chronology and results of specific tests. This method offers 3 levels of causality on the basis of a decision tree in which these criteria are combined: questionable, likely and very likely. Another method uses a flow chart as soon as the case has been reported, following a PLM (product lifecycle management) call approach. It is also based on chronological and semiological criteria and all the notifications can be analysed using 6 levels: irrelevant, not enough information, unlikely, possible, probable and certain. (The SCCPS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 2006).

The cosmetovigilance system is the right means of obtaining information on the safety of cosmetic products and their ingredients. It can be used by the competent authorities in Europe, which would confirm that the new directives ensure a high level of safety. Cosmetovigilance makes it possible to exclude and control potentially hazardous ingredients which are included in the product content, and thus develops the costumers' trust and reassures them about the safety of the products available on the market.

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Macedonian pharmaceutical bulletin, **62** (suppl) 447 - 448 (2016)
ISSN 1409 - 8695
UDC: 615.2:658.86/.87
Short communication

Good Distribution Practice for medicinal products

Fjola Hadjihamza[1]*, Eleonora Pandova[2], Violeta Bozinova[1]

[1]*Medika Internacional DOO, St. 1732 No 4 Lamela V 1/1-5 Skopje, Macedonia*
[2]*Malmed, Agency of medicines and medical devices "St. Cyril and Methodius" No. 54, 1000 Skopje, Macedonia*

Good distribution practice (GDP), as part of quality assurance system, for medicinal products for human use includes myriad requirements for purchase, receiving, storage and export of drugs intended for human consumption. GDP regulates the division and movement of pharmaceutical products from the premises of the manufacturer of medicinal products, or another central point, to the end user thereof, or to an intermediate point by means of various transport methods, via various storage and/or health establishments.

The wholesale distribution of medicinal products is an important activity in integrated supply chain management. Today's distribution network for medicinal products is increasingly complex and involves a lot of people from different professional profiles.

The GDP Guidelines establish appropriate tools to assist wholesale distributors in conducting their activities and to prevent falsified medicines from entering the legal supply chain. Compliance with these Guidelines will ensure control of the distribution chain and consequently maintain the quality and the integrity of medicinal products (GGDP, 2103).

The GDP Guidelines are intended to be applicable to all persons and outlets involved in any aspect of the storage and distribution of medicines from the premises of the manufacturer of the product to the person dispensing or providing medicines directly to a patient or his/her agent. This includes all parties involved in trade and distribution of medicines, including the manufacturers of bulk, finished products, wholesalers, as well as others such as suppliers, distributors, Government institutions, international procurement organization, logistic providers, traders, transport companies and forwarding agents and their employees as well as health workers. The main parts of GDP are: 1) Organization and management; 2) Quality management; 3) Personnel; 4) Equipment and premises; 5) Documentation; 6) Operations; 7) Outsourced activities; 8) Self inspections and 9) Transportation. In fact, the parts mentioned above form the GDP network. They interfere between each other forming a complex system in order to provide quality assurance that ensures that the quality of pharmaceutical products is maintained through adequate control throughout the numerous activities which occur during the distribution process. Every part of the GDP system is important to maintain the quality of the distribution chain. Regarding Organization and management, an adequate organizational structure for each entity in the chain of distribution should be defined with the aid of an organizational chart. The aim of this organizational chart is to have a clear view of the duties and responsibilities of each person involved in the process (GGDP, 2103; WHO, GDP, 2005).

Every individual involved in the process of distribution should have a written job description and at every level of the supply chain, employees should be fully informed and trained in their duties and responsibilities.

Training should be based on written standard operating procedures (SOPs). Personnel should receive initial and continuing training relevant to their tasks, and be assessed as applicable, in accordance with a written training program.

The system for managing quality should encompass the organisational structure, procedures, processes and resources, as well as activities necessary to ensure confidence that the product delivered maintains its quality and integrity and remains within the legal supply chain during storage and/or transportation. The quality system should be fully documented and its effectiveness monitored. All quality-system-related activities should be defined and documented. A quality manual or equivalent documentation approach should be established. A responsible person should

* f.haxhihamza@live.com

be appointed by the management, who should have clearly specified authority and responsibility for ensuring that a quality system is implemented and maintained.

This responsible person is designated by the wholesale distributor and should meet the qualifications and all conditions provided for by the legislation of the Member State concerned. A degree in pharmacy is desirable. The responsible person should have appropriate competence and experience as well as knowledge of and training in GDP (GGDP, 2103; GGDPBP, 2012).

Besides the responsible person there should be an adequate number of competent personnel involved in all stages of the wholesale distribution activities of medicinal products. The number of personnel required will depend on the volume and scope of activities.

Wholesale distributors must have suitable and adequate premises, installations and equipment, so as to ensure proper storage and distribution of medicinal products. In particular, the premises should be clean, dry and maintained within acceptable temperature limits.

Different medicines require different storage conditions. There are medicines that should be stored following the cold chain regime and medicines that should be stored on room temperature, 2-8 °C and 15 -25 °C respectively (Commission Directive 2003/94/EC, 2003; WHO, GDP, 2005).

Equipment and processes should be respectively qualified and/or validated before commencing use and after any significant changes, e.g. repair or maintenance.

There are a lot of SOPs (standard operative procedures) that determine every step of the processes that are conducted during the reception, storage and dispatch of the medicines.

All actions taken by wholesale distributors should ensure that the identity of the medicinal product is not lost and that the wholesale distribution of medicinal products is performed according to the information on the outer packaging. The wholesale distributor should use all means available to minimise the risk of falsified medicinal products entering the legal supply chain.

Also in compliance with the GDP standard qualification of the supplier and costumers must be done.

Regarding the situations that can occur after the patient has received the medicines, there must also be a SOP on how to handle a complaint from the costumer as well as a SOP for medicines that are returned for some reason.

In order to provide a continuous quality management system, self-inspections must be conducted in an impartial and detailed way by designated competent company personnel. Audits by independent external experts may also be useful but may not be used as a substitute for self-inspection.

The transportation of the medicines is a very important part of the distribution process, that is why the personnel in charge of transportation must be trained how to operate with the medicines that should be distributed and the vehicles involved in the process must be suitably calibrated and mapped as well.

Considering all the facts mentioned above a conclusion can be made that the Good Distribution Practice (GDP) system is a very important tool in order to provide a better medicine quality regarding distribution factors and an improved health care system as well, which will contribute for a higher safety of the patients (Commission Directive 2003/94/EC, 2003; GGDP, 2103).

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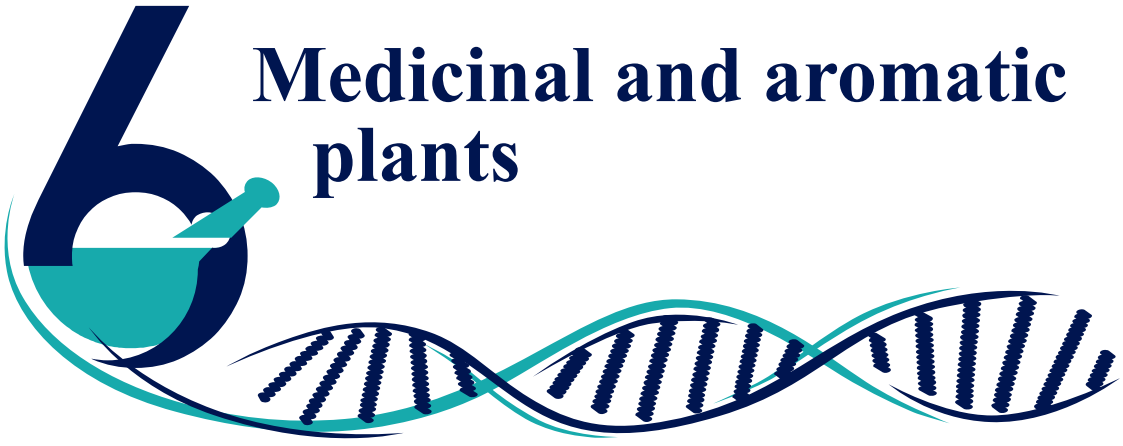
Medicinal and aromatic plants



Macedonian pharmaceutical bulletin, **62** (suppl) 451 - 452 (2016)
ISSN 1409 - 8695
UDC: 615.322.076
Short communication

How to include DNA-based authentication in quality control of medicinal plants and phytomedicines?

Johannes Novak

Institute for Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Wien, Austria

Introduction

The correct botanical identification of plant raw materials is a basic requirement to guarantee safety and efficacy of phytomedicines. The principal identification strategies are based on macro- and microscopic analysis, complemented by phytochemical methods. The methods are defined in national and supranational pharmacopoeias. Besides these methods, identification by DNA sequences is in fast development and supplements classical methods in difficult matrices like e.g. powdered roots or even extracts (Novak et al., 2007).

The DNA-sequence as tool to identify species

The sequence of the nucleotides in the DNA ('DNA sequence') is unique for each individual. However, related individuals share their DNA sequence to a big extent and the closer the relationship the higher the degree of sequence consensus. With a well-defined set of reference samples, mutations in short DNA pieces can be identified that are variable between taxa (e.g. species) but stable within the taxon. Based on these informative mutations, unknown samples can be assigned to a specific taxon (Kress et al., 2005). Right now, it is not possible to evaluate and compare the whole genome of each sample with each other in cheap and fast routine analysis. Therefore, routine methods are developed based on small informative sequence parts, which are mostly in the size range of a few hundred base pairs.

Since molecular biology has very wide application area from biology to medicine and forensics the development of new technologies is very fast. Therefore, also possible molecular biological methods for identifying medicinal plants and biological contaminations in plant material are numerous as exemplarily listed below:

- *Hybridization-based methods* (examples: dynamic allele-specific hybridization, molecular beacons, single nucleotide polymorphism (SNP) microarrays)
- *Enzyme-based methods* (examples: restriction fragment length polymorphism (RFLP), PCR-based methods (e.g. amplification refractory mutation system (ARMS))
- *Post-PCR methods based on physical properties of the DNA* (examples: Single strand conformation polymorphism, denaturing HPLC, high-resolution melting of the entire amplicon (HRM), use of DNA mismatch-binding proteins, SNPlex)

This presentation will focus on a few methodological examples, namely DNA sequencing, ARMS and HRM.

'Classical' DNA sequencing (Sanger sequencing)

The *'classical' DNA sequencing* delivers complete sequence information of short DNA pieces (amplicons). Comparing a sample sequence with the reference sequences (e.g. with phylogenetic methods), the sample can be identified (Kress et al., 2005). However, the classical assessment by DNA sequencing is still time-consuming and relatively expensive, a fact that can be overcome by other DNA-based methods better suitable for routine analysis.

Amplification-Refractory Mutation System (ARMS)

One method is the ARMS in which alternative PCR primers are developed in a way to bind only to the informative mutation (Little, 2001). Therefore, a PCR-product is only obtained if the primer fits to the desired mutation.

* Johannes.Novak@vetmeduni.ac.at

In gel electrophoresis following PCR, the presence or absence of the PCR product can be detected. This method was used to identify *Valeriana officinalis* increasing routine efficiency by combining several PCR reactions in one tube (Ruzicka et al., 2016).

High-Resolution-Melting-Analysis (HRMA)

Another method is HRMA. The principle of this method is the separation of the two DNA-strands ('melting' of the DNA) by slowly increasing the temperature at highly constant rates ('high-resolution'). The 'melting' can be measured by the decreasing intensity of a fluorescence dye intercalating the DNA resulting in a melting curve that is specific for a DNA sequence (Ruzicka et al., 2016). The elegance of this method besides its reliability in measurement is that the fluorescence measurement takes place immediately after PCR in the same machine and in the same tube as the PCR reaction. Sample manipulation is therefore limited to a minimum, which makes this method especially attractive for routine analysis (Schmiderer et al., 2010, Ruzicka et al., 2016). Another aspect of medicinal plant quality control is adulteration. HRM can be also used to detect adulterations in complex mixtures (Mader et al., 2011, Schmiderer et al., 2015).

Conclusion

In the last years, many different methods to identify medicinal plants using different technology were developed and their stability and efficiency proofed. Therefore, the way is open to consider them for their inclusion in pharmacopoeias as official testing methods in order to supplement classical identification methods in difficult species or matrices.

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Macedonian pharmaceutical bulletin, **62** (suppl) 453 - 454 (2016)
ISSN 1409 - 8695
UDC: 615.322.074
Short communication

High-content screening for identification of bioactive compounds in plant extracts

Laco Kacani*

ADSI - Austrian Drug Screening Institute Ltd., Innrain 66a, A-6020 Innsbruck, Austria

Introduction

The Austrian Drug Screening Institute (ADSI) is a research enterprise owned by the University of Innsbruck that offers screening services for pharmaceutical companies and academic institutions. The focus of the ADSI is on the hit-to-lead development, toxicology and "mode of action" studies *in vitro*. Focused libraries of natural or synthetic compounds are tested using an innovative high-content screening (HCS) approach that allows for the reliable identification of bioactive compounds or natural products.

Development of screening models

The main goal is on the development and uses of *in vitro* assays where cultivated cells have similarly to the patient's tissue *in vivo*. These *in vivo*-like *in vitro* models are used for the high-content screening of focused libraries of natural or synthetic compounds. Automated microscopy, multiplexing readouts, metabolomics and proteomics allow the detection of molecules of interest, their changes during incubation with active substances and give a hint on the "mode of action" of particular substance in biological pathways.

Primary cell co-culture systems *in vitro*

The use of human primary cell based culture systems provides for more predictable and clinically relevant results that facilitate new insights into the biology and cellular processes in the presence of tested compounds. Primary cells have much more delicate growth requirements and are often more sensitive to toxic agents than long-term cell lines. Synthetic and natural compounds are typically screened for toxicity with high-throughput assays that utilize tumor cell lines and simple endpoints like cell death or inhibition of cell proliferation. Although these methods have allowed the screening of huge numbers of compounds, many of the lead compounds screened by such practices have experienced late drug failure due to unacceptable toxicity in the clinic. In our hands, functional assays using primary cells from particular tissues deliver more predictive data on efficacy and actual toxic effects on the given tissues and organs than assays using cultured tumor cell lines. Toxicity tests developed in the ADSI enable early detection of potential side effects before the lead compounds reach pre-clinical tests in animal models. Following cell culture systems using human primary cells are available: mesenchymal stem cells (MSC), MSC derived adipocytes, MSC derived skeletal muscle myotubes, primary hepatocytes, monocytes and macrophages.

Laboratory automation

Robotics and automation are applied in an appropriate manner to achieve maximal information gain from a single experiment. Continuous monitoring with state-of-the art analytical and optical methods enables measurement of multiple cellular parameters modified by test substances including cell communication and transmission of signals.

3D cell culture

The pharma and biotech industry recognizes the importance of moving from 2D cell culture towards 3D tissue culture platforms to increase the *in vivo* relevance

* Laco.Kacani@adsi.ac.at

of *in vitro*-based assays for drug discovery. Synthetic hydrogels constitute the first choice to cultivate cells in a 3D environment when high levels of versatility, reproducibility and biological relevance are requested. Thanks to the possibility to engineer desired physical and biochemical properties, these hydrogels are capable of supporting the growth and differentiation of a wide range of cell types and tissue models in 3D. The Automated Screening Assay Platform was developed in order to automate cellular and tissue culture assays for 3D-cell-assay analyses in a time-resolved manner. 24/7 automation is achieved using robotic plate handling system, enabling users to schedule multiple runs of up to 40 cell culture plates in 96-well format.

Metabolomics, secretomics and proteomics

The analytical division focuses on the identification of compounds and pathways causing or mediating the biological activity in the cellular test systems. Advanced liquid chromatography separation techniques coupled to mass spectrometry provide the basis for analyses of test substances by means of metabolomics and proteomics approaches. Separation of samples is performed using analytical and preparative UPLC-systems either directly hyphenated to detection systems, sample spotters (MALDI-analysis) or fraction collectors. Mass spectrometry is used for the detection of small molecules by means of untargeted metabolomics in supernatants of cell co cultures, as well as in context of secretomics (proteins in cell supernatants) and or proteomics (proteins in cell lysates). Data mining and detailed analysis of acquired data facilitate the identification of compounds and pathways causing or mediating the biological activity in the cellular test systems.

Biostatistics & bioinformatics

All described biological and analytical methods produce huge amounts of raw data that have to be analysed properly. Acquired raw data from all systems are stored in the databank for further analyses. Biological and analytical data are processed using sophisticated biostatistics & bioinformatics tools and are then combined into comprehensive data sets providing reliable basis for the selection of drug candidates for later stages of drug development.

Extraction & fractionation

ADSI developed an expertise in the systematic variation of extraction procedures that allows for the development of pharmacologically active substance mixtures, such as plant extracts, that are optimized for their content of pharmacologically active ingredients. The fractionation of the samples using chromatographic techniques allows the rapid isolation of molecules of interest and the elucidation without dilation of their chemical structure. Special extraction procedures and chemical analytics allow optimization of the pharmacologically active substance mixtures in plant extracts. Further tailor-made assays and analyses are under development according to the demands of customers.

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Macedonian pharmaceutical bulletin, **62** (suppl) 455 - 456
(2016)
ISSN 1409 - 8695
UDC: 615.322.074:543.51
Short communication

The application of mass spectrometry and pathway analysis in understanding the biochemistry of medicinal plants

Shaun Bilsborough[1]* and Zoran Nastov[2]

[1]*Agilent Technologies, Cheadle Royal Business Park, Stockport, SK8 3GR, United Kingdom*
[2]*Farmahem, Kicevska 1, 1060, Skopje, R. Macedonia*

Introduction

Mass spectrometry profiling is used to determine the changes occurring in a biological system in response to different conditions in order to better understand aberrations in the biochemical pathways involved (Rojo et al., 2012). The same methodology can be applied to the understanding of the biochemistry of plants that show medicinal properties (Avula et al., 2014). When the investigation focusses upon the small molecule level, then different methods, mass spectrometry platforms and analytical conditions are required due to the various chemical properties of the compounds of interest, leading to numerous data files for each biological replicate. More recently, the application of hyphenated techniques such as ion mobility have led to better separation of complex biological samples while the additional dimension has added another level of complexity to the data generated (May et al., 2015). Once the identity of metabolites of interest has been determined, a critical requirement for the understanding of the biochemistry is the ability to map results directly to biochemical pathways (Bhat et al., 2014). In this presentation, an overview of the analytical and software requirements will be discussed together with the application of biochemical pathway analysis in the interpretation of complex data sets, which can lead to a better understanding of the biology of medicinal plants.

Material and methods

Compounds were extracted through maceration of plant material in 50% (v/v) methanol:water. Samples were then filtered to remove the plant material and the extracted compounds separated directly into a 6550 quadrupole time of flight mass spectrometer using a 1290 ultra-high performance liquid chromatography system (Agilent Technologies). The compounds were ionized by both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) and spectra acquired in both positive and negative ionization mode. An Eclipse Plus C18 column (2.1 mm x 150 mm; 1.8 um) was used for the separation with a linear gradient of 0.1% formic acid in water against 0.1% formic acid in acetonitrile starting from 2% to 98% over 20 minutes at a flow rate of 250 µL/min. The resulting data was initially extracted using MassHunter ProFinder using batch recursive feature extraction and comparative analysis then performed using MassProfiler Professional. Significant, reproducible changes detected were identified using the METLIN PCDL. The results were then visualized by mapping onto biochemical pathways using MassHunter Pathway Analysis.

Results and discussion

A comprehensive approach to LC/MS analysis using ESI and APCI ionization and both positive and negative acquisition together with cross-platform investigations, such as GC/MS, significantly increased coverage of the metabolome. The addition of ion mobility separation further increased coverage through an orthogonal separation method based upon collision cross section of each compound. However, this approach also produced a high volume of data and complexity to the investigation. Initial recursive batch extraction of the data files allowed significant data reduction while also improving the quality of the data that was then used for biological profiling. The extracted features were initially aligned and normalized followed by hierarchical clustering to check the reproducibility of biological replicates. Significant and reproducible differences between test

* shaun_bilsborough@agilent.com

groups were determined by analysis of variance, principal component analysis and fold-change filtering. The resulting features were identified and annotated onto biochemical pathways to aid visualization. This was facilitated by the METLIN database of metabolites (Scripps Institute) locally installed on the instrument computer, which also allowed smaller subset databases to be generated based on the metabolites involved in biochemical pathways of interest. This provided an advantage in allowing such subset databases to be used for targeted data extraction of the metabolites involved in the pathways. These metabolites belonged to energy, fatty acid, amino acid, nucleotide, and secondary metabolic pathways. Multi-omic correlation analysis between genomic and metabolomics technologies allowed the identification of co-regulated entities, such as genes and metabolites involved with amino acid, carbohydrate and fatty acid metabolism. Such a multi-omic approach provided additional evidence of important pathways and increased understanding of specific pathways in the production of compounds of medicinal relevance.

Conclusion

LC/MS provides a reproducible, accurate method of analyzing complex samples to determine the compounds present. The challenge is in the interpretation of the high volume of complex data generated. Recursive batch extraction, biological profiling and pathway analysis allowed the data generated to be converted to knowledge in the form of identified metabolites, but more importantly, that knowledge could then be utilized to provide understanding of the important underlying biochemical pathways through pathway analysis.

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Macedonian pharmaceutical bulletin, **62** (suppl) 457 - 458 (2016)
ISSN 1409 - 8695
UDC: 633.888(497.7)
Short communication

Cannabis in R. Macedonia: present situation

Gjoshe Stefkov*, Ivana Cvetkovikj, Marija Karapandzova, Svetlana Kulevanova

Institute of Pharmacognosy, Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Cannabis or hemp (fam. Cannabinaceae) has three main types: *C. sativa, C. indica*, and *C. ruderalis*. It is a matter of divided opinion whether the genus consists of one or more species. Cannabis plants contain at least 489 distinct compounds distributed among 18 different chemical classes including cannabinoids, nitrogenous compounds, amino acids, proteins, enzymes, glycoproteins, hydrocarbons, simple alcohols, aldehydes, ketones and acids, fatty acids, simple esters and lactones, steroids, terpenes, non-cannabinoid phenols, flavonoids, vitamins, and pigments (Hillig and Mahlberg, 2004).

Nowadays, the pharmacologically active substance in cannabis - cannabinoids, comprises more than 70 different phytocannabinoids, with potentially significant applications in medicine and pharmacy. The principal cannabinoids appear to be delta-9-tetrahydrocannabinol (i.e. Δ9-THC, THC), cannabinol (CBN), and cannabidiol (CBD). Other cannabinoids found in cannabis are cannabigerol (CBG), cannabichromene (CBC), tetrahydrocannabivarin (THCV) and many others.

Therapeutic properties of cannabis have been known since ancient times and have been a source of fiber, food, oil, medicine, and inebriant substances. There are significant health benefits associated with the consumption of seeds and its derivatives, due to its well-balanced fatty acid spectrum and high value proteins. The therapeutic applications of cannabis and its derivatives have been studied by various world bodies, including the Scientific Committee of the House of Lords in Great Britain (1998), the Institute of Medicine in the United States (1999) and the Senate Special Committee on Illegal Drugs in Canada (Abramovici, 2013). In order to assess the current knowledge on the therapeutic potential of cannabinoids, a meta-analysis has been performed through Medline and PubMed up to July 1, 2005. Seventy-two controlled studies evaluating the therapeutic effects of cannabinoids were identified. Research studies have clearly shown that cannabinoids are highly efficient, primarily in the treatment of nausea and vomiting and the management of chronic pain. For patients who suffer from severe, chronic diseases, such as cancer and AIDS, cannabis has been shown to relieve several symptoms at the same time in more efficient way than some registered medicines. Besides, there is a great potential of cannabinoids to be applied for the treatment of many other pathological conditions, such as multiple sclerosis, Alzheimer's disease, spinal cord injuries, Tourette's syndrome, epilepsy, hypertension, glaucoma, although it is necessary to complete the last phases of clinical trials in order to register these compounds as drugs, and to assure safe therapy for these diseases (Ben Amar, 2006).

Cannabis is a well-known drug and a controlled substance, which possession and use are illegal in most countries of the world. Nowadays, the use of cannabis and its legalization for medical use has become a worldwide trend. Laws and attitudes toward cannabis are changing these days. Legalization of cannabis use for medical purposes is a hot topic at the global level and in most countries there have been initiatives to amend the existing laws in order to make drugs based on natural ingredients of cannabis, as well as other related products, synthetically produced, available to patients (Sarmento et al., 2015).

Therefore, due to the increased global necessity of research on the cannabis and for the sake of reinforcing the ability of the R. Macedonia to respond to the challenges arising from this issue, we have started a study for chemical characterization of various cannabis products, mainly cannabis oil, as well as plant material from indigenous and foreign origin.

* gstefkov@yahoo.com

Materials and methods

Dry overgorund parts, leaves and buds, from wild growing *Cannabis sativa* L., Cannabinaceae (tentative identification) were collected in central east part of R. Macedonia (RM), during two vegetative seasons, in 2014 and 2015. Additionally, coarsely grounded dry leaves and buds of *Cannabis indica* L., Cannabinaceae (tentative identification) obtained by donation from patient for scientific-research purposes were used.

Cannabis oil (CO): various samples were obtained by donations from patients for scientific-research purposes.

0.1 g dry plant material or oil were dissolved in 10 mL n-Hexane and placed in ultrasonic bath for 15 min. Afterword were filtrated through disposable Econofilter 25/0.45 μm RC pore size and injected in the GC system.

Standard substance solutions were made by dissolving standards of THC and CBD into methanol in following concentrations: 0.05 mg/mL and 0.02 mg/mL, respectively.

Samples were analysed with an Agilent 7890A Gas Chromatography system with FID detector and Agilent 5975C mass spectrometer. HP-5ms capillary column (30 m x 0.25 mm, 0.25 μm) was used. Adams' analytical method was used prolonged for 10 min at the end (Adams, 2007). The components were identified by comparing the mass spectra of components present in the samples with reference spectra obtained from NIST, Wiley and Adams' mass spectra libraries. Quantification of the THC and CBD was done according corresponding standard substances.

Results and discussion

In the analyzed samples (CO and dry plant) the following six cannabinoids were identified: THCV, CBD, CBC, THC, CBG, CBN and delta-6-tetrahydrocannabinol (Δ6-THC). Five terpenoids compounds (α-pinene, camphene, limonene, *trans*-E-caryophyllene and germacrene B) were also detected with percentage share 1-6%. THC and CBD were predominant cannabinoids in all analyzed samples. Two types of samples can be distinguished: type 1-high in THC (73-95%) and low in CBD (0-16%) and type 2- low in THC (2-4%) and high in CBD (48-84%).

The analysis of the wild growing cannabis from RM, revealed low THC (3.02-4.13%), followed by CBC (4.48-6.88%) and high CBD content (72.30-73.73%), thus can be classified as type 2. On the contrary, *C. indica* plant sample was classified as type 1, appearing to be high in THC (86.38%) and low in CBD (0.71%). The relative abundance of these and other cannabinoids can vary depending on a number of factors such as the Cannabis strain, the soil and climate conditions or the cultivation techniques. The content of THC in the indigenous cannabis was (0.159-0.587%) and 19.23% in *C. indica*. Quite the opposite the CBD content varied from 3.11-7.22% in the wild growing cannabis and 0.122% in the *C. indica* plant sample. From an average of 2 151 samples collected in Europe between 2006 and 2008, the average concentration of THC found was 0.075% (Sarmento et al., 2015). The analysis showed that most of the CO belongs to type 1 and the content of THC and CBD is very close to the distinguished percentage share.

Recently, changes of the Law for narcotic substances in RM were made concerning legalization of cannabis for medicinal and scientific purposes, in a way of cultivation, production of cannabis medical preparations as well as import of pharmaceuticals and cosmetics, containing cannabis or cannabinoids.

Conclusion

Due to the legal constraints on the possession and use of cannabis, relatively little research on the medicinal qualities of this plant has been conducted. Recently, due to the changes of the Law for narcotic substances in RM, an opportunity, for starting a scientific-research work on this plant and related products, has appeared. Preliminary results of the study for chemical characterization of various cannabis products, CO and cannabis plant material from indigenous and foreign origin are reported.

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Macedonian pharmaceutical bulletin, **62** (suppl) 459 - 460 (2016)
ISSN 1409 - 8695
UDC: 633.75(497.7)
Short communication

ALKMAF – Breeding opium poppy for improved alkaloid content

Gjoshe Stefkov[1], Jelena Acevska[2], Mirjana Jankulovska[3], Marija Karapandzova[1], Aneta Dimitrovska[2], Svetlana Kulevanova[1], Sonja Ugarkovik[4], Igor Mickovski[4], Natasha Nasteva[4], Sonja Ivanovska[3]*

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, University Ss. Cyril and Methodius, MajkaTereza 47, Skopje 1000, Republic of Macedonia*
[2] *Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje 1000, Republic of Macedonia*
[3] *Institute of Plant Biotechnology, Faculty of Agricultural Sciences and Food, University Ss. Cyril and Methodius, Aleksandar Makedonski bb, Skopje 1000, Republic of Macedonia*
[4] *Alkaloid AD, Skopje, Alexandar Makedonski 12, 1000 Skopje, Republic of Macedonia*

Introduction

According to the International Narcotics Control Board of UN (Technical report 2015: estimated world requirements for 2016 and statistics for 2014), in the preceding decades, the demand for natural poppy alkaloids (morphine, codeine, thebaine and oripavine) is continually growing, which consequently implies on the necessity for advanced production. Opium poppy (*Papaver somniferum* L.) is a valuable source of therapeutic agents and has great historical and socio-economic importance for Republic of Macedonia. The excessive and continuously growing need for natural alkaloids from opium poppy was the main reason to launch a project for reviving the growth and utilization of opium poppy in Republic of Macedonia.

One of the goals of this project is to increase the ultimate yield of opium poppy alkaloids. The production of opium poppy in Macedonia is mainly based on local landraces and one commercial cultivar. The need to improve existing germplasm became apparent in the recent years. Consequently, the renewal and upgrade of the poppy breeding program was necessary. The first step was to establish a germplasm collection out of which promising lines could be selected. The genotypes in the starting collection should be grouped according to their characteristics. Therefore, analyses of the genetic diversity among poppy landraces and breeding lines from Macedonia and introduced poppy genotypes based on various morphological traits and determination of the extent of genetic diversity for proper utilization in the breeding program were performed (Ivanovska et al., 2012; Jankulovska et al., 2012, 2013; Stefkov et al., 2012). The overview of the advancements in the newly established opium poppy breeding program is presented.

Materials and methods

The field experiments were performed every year (2010-2015) near Skopje in R. Macedonia. Each year, number of genotypes decreased as a result of positive selection. During the vegetation different growth stages were observed, as well as the morphology of the genotypes. The agro-morphological characterization and assessment of the genetic diversity of opium poppy genotypes was based on 11 quantitative and 10 qualitative traits. Agro-morphological traits were described according to UPOV descriptor (1999). The GC/FID/MS and the HPLC/DAD analysis were performed on poppy straw methanolic extracts, obtained by optimized ultrasonic agitation, for determination of the content of six alkaloids: morphine,

* s.ivanovska@zf.ukim.edu.mk

codeine, thebaine, oripavine, papaverine and noscapine (Acevska et al., 2012a, 2012b).

Principal component (PCA) and cluster analyses (CA) were applied for variation evaluation of the most important quantitative characters. Positive selection of genotypes with desirable traits was continuously conducted (Ivanovska et al., 2012; Jankulovska et al., 2012, 2013; Stefkov et al., 2012).

Results and discussion

The pattern of morphological and productive variation between all accessions was evaluated through the qualitative (shape of capsule base, capsule ribbing, shape of capsule, capsule dehiscence, shape of stigmatic disc, surface of lobes, shape of lobes apex, seed color, petal color, petal base color) and quantitative (plant height, number of capsules per plant, number of stigma lobes, diameter of stigmatic disc, diameter of capsule, length of capsule, weight of main capsule, weight of seed of main capsule, seed yield per plant, capsule yield per plant, total yield per plant) characters. The analyzed opium poppy collection expressed broad variability for all investigated traits. Principal component (PCA) and cluster analyses (CA) served as effective tools for identification of genotypes with a combination of positive characteristics. The most variable traits were seed yield per plant and number of capsules per plant.

Extraction procedure (liquid extraction using methanol as an extraction solvent, under reflux and ultrasonic agitation) followed by HPLC/DAD were developed and optimized for fast screening and in same time accurate assessment of the content of six alkaloids in the poppy straw samples using design of experiments (DoE). Chemical characterisation of the samples revealed high variability in the alkaloid content between the analyzed genotypes e.g.: morphine (0.07-1.65%); codeine (0.01-0.66%); thebaine (0.05-0.17%); oripavine (0.001-0.07%); papaverine (0.007-0.62%); noscapine (0.005-0.63%).

The starting germplasm collection represented a good basis for successful selection of promising opium poppy lines with a combination of high seed and capsule yield as well as high alkaloid content.

Conclusion

The characterization of opium poppy germplasm revealed high variability among the evaluated genotypes considering agromorphological traits and alkaloid content. Genotypes which expressed high seed and capsule yield per plant and high morphine content were successfully identified. These genotypes will be considered for variety registration and production in R. Macedonia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 461 - 462 (2016)
ISSN 1409 - 8695
UDC: 582.929.4
Short communication

Possible health benefits of pine nuts as a source of omega fatty acids

Marija Karapandzova*, Ivana Cvetkovikj, Gjoshe Stefkov, Svetlana Kulevanova

University "SS. Cyril and Methodius", Institute of Pharmacognosy, Faculty of Pharmacy, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

In the public's mind, the word "dietary fat" has become synonymous with obesity and heart disease. Consequently, as nuts are typically high in fat, they are traditionally avoided in an attempt to low blood cholesterol and the risk of coronary heart disease (CHD). However, there is increasing evidence that diets that include nuts may elicit cardio protective effects (Ryan et al., 2006). Different heath studies reported that women who consumed nuts five or more times a week have a 35% lower risk of total CHD compared with women who rarely eat nuts (Hu et al., 1998) and strongly support the link between consumption of nuts and the reduced risk of heart disease (Albert et al., 2002; Fraser et al., 1992). Additionally, several clinical studies have evaluated the effect of diets high in nuts on blood lipids level. In a randomized controlled study, Sabate et al. (1993) reported that men on nut diet had a 12% and 16% decrease in total and in low density lipoprotein (LDL) cholesterol levels, respectively, compared to normal diet. Furthermore, the beneficial effects of nut consumption observed in epidemiological and clinical studies underscore the importance of distinguishing different types of fat and have long established that the type of fat, but not the total amount of fat, predicts serum cholesterol levels. Although, nuts are high in fat, the predominant types are "healthy or good fats" such as mono and polyunsaturated fatty acids which lower the LDL cholesterol and hence the CHD risk (Hu et al., 2001).

In many cultures, pine nuts are traditionally used as ingredients in sauces, pastries or in desserts and are added to savory foods. The main species consumed in Europe are *Pinus pinea, Pinus koraiensis, Pinus sybirica* and *Pinus gerardiana* (Destaillats et al., 2011). Nowadays, very little is known about the consumption of pine nuts from Macedonian species probably due to the lack of data related to their chemical composition as well as fatty acids profile. Thus, the aim of the present study was to determine total oil content and fatty acid composition of these conifers nuts in order to obtain any information that will help to disclose their possible usage.

Materials and methods

Plant materials (pine nuts) of two pine species (*Pinus peuce* and *Pinus nigra*) were collected from two localities in R. Macedonia (Pelister and Berovo, respectively). Before oil extraction, plant material was dried and properly homogenized.

A known weight of sample (30 g) was extracted in a Soxhlet apparatus using petroleum ether as a solvent for 36 hours. After isolation, the samples were evaporated to remove residual traces of petroleum ether (Trajkovic et al., 1983).

Fatty acid methyl esters (FAME) were prepared from extracted oil with potassium hydroxide in methanol according to method described by Trajkovic et al. (1983) and were analyzed on Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector. For that purpose, HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm) was used. Operating conditions were as follows: oven temperature at 60 °C (2 min), 10 °C/min to 200 °C (2 min) and 5 °C/min to 240 °C (7 min); helium as carrier gas at a flow rate of 1 ml/min; injector temperature 250 °C and that of the FID detector 250 °C. One μl of each sample was injected at split ratio 1:1.

The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 230 °C, transfer line temperature 280 °C and mass range from 50 - 500 Da. The

* marijakarapandzova@yahoo.com

MS was operated in scan mode.

Identification of FAME was made by comparison of their retention times and mass spectral data with those obtained from standards and from Nist, Wiley and Adams mass spectra libraries. The percentage ratio of FAME was computed by the normalization method of the GC/FID peak areas without any correction factors.

Results and discussion

The content of total oil isolated from pine nuts of Macedonian species yielded from 32.61% to 38.56% in nuts of *Pinus nigra* and *Pinus peuce*, respectively.

Data analysis of the chemical composition of examined oil samples revealed three different classes of fatty acids: saturated, monounsaturated and polyunsaturated fatty acids. The last one was dominant fraction in both species (65.25% in nuts of *Pinus peuce* and 58.65% in nuts of *Pinus nigra*), while the fraction of saturated and monounsaturated fatty acids were present in smaller amount (7.15% and 26.10% in *Pinus peuce* and 14.70% and 25.56% in *Pinus nigra*, respectively).

Total of 21 fatty acids were identified in the investigated oil samples isolated from *Pinus peuce* nuts which represented 97.79% of total oil. The most abundant fatty acids were linoleic acid (61.95%) which is classified as polyunsaturated ω-6 fatty acid and oleic acid (24.98%) known as monounsaturated ω-9 fatty acid. Palmitic acid (3.51%) was dominant in the class of saturated fatty acids. Similarly 28 fatty acids were identified in the oil samples isolated from nuts of *Pinus nigra* which represented 98.60% of total oil. Predominant fatty acids were the same linoleic (54.81%), oleic (22.80%) and palmitic acid (9.07%).

In terms of data concerning the chemical composition of oil isolated from pine nuts, only one study could be found regarding chemical composition of fatty oil isolated from black pine or *Pinus nigra* but still there is no available published data about the composition of oil obtained from nuts of *Pinus peuce* or Macedonian pine. According Bagci and Karaagacli (2004), the oil isolated from nuts of black pine from Turkey contains linoleic (47.10%), oleic (17.80%) and palmitic acid (4.70%) as major constituents what correlates to our findings.

Conclusion

Summarizing the obtained results, it is evident that pine nuts collected from Macedonian species are rich in oil that contain significant amount of unsaturated ω-6 as well as ω-9 fatty acids thus could be considered as their potential source. Moreover further investigations of chemical composition should be made in order to provide more data for their possible medicinal, pharmaceutical or commercial utilization.

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Macedonian pharmaceutical bulletin, **62** (suppl) 463 - 464 (2016)
ISSN 1409 - 8695
UDC: 663.253:543.544.5.068.7
Short communication

Biogenic amines in red and white wines determined by HPTLC method

Igno Tasev[1]*, Jasmina Tonic Ribarska[2], Jürgen Fröhlich3, Donka Doneva-Sapceska[4]

[1] *Tochem Dooel, Atinska 10, Skopje, Macedonia*
[2] *Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, Skopje, Macedonia*
[3]*Erbslöh Geisenheim AG, Erbslöhstraße 1, Geisenheim, Germany*
[4]*Faculty of Technology and Metallurgy, University "Ss Cyril and Methodius", Ruger Boskovic 16, 1000 Skopje, Macedonia*

Introduction

Biogenic amines (BA) are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones which in low concentration are part of metabolic and physiological processes of animals, plants and microorganisms. They are organic bases with aliphatic (putrescine (Put), cadaverine (Cad), spermine (Spm), spermidine (Spd)), aromatic (tyramine (Tyr), phenylethylamine (Phe)) or heterocyclic (histamine (His), tryptamine (Trp)) structures, can be classified as monoamines (Phe and Tyr), diamines (His, Cad, Put) or such as volatile (Phe) and nonvolatile (His, Cad, Put, Tyr, Spm, agmatine (Agm), Trp) biogenic amines. The main factors for formation of biogenic amines in foods or fermented foods are following: the availability of free amino acids, the existence of decarboxylase positive microorganisms and the presence of suitable pre-conditions that allow bacterial growth. Present in higher concentration in foods, poses health risk for sensitive peoples and are cause for a number of adverse effects on human health such as rash, edema, headache, urticaria, rhinitis, respiratory and digestive problems (Ladero et al., 2010). Some pharmacological effects of biogenic amines which have appropriate precursors are: liberates adrenaline and noradrenaline, stimulates both sensory and motor neurons, controls gastric acid secretion (histamine); increases the cardiac output, causes lacrimation and salivation, increases respiration (tyramine); hypotension, depreciation of the wine aroma, potentiate the toxicity of other amines (putrescine and cadaverine); releases noradrenaline from the sympathetic nervous system, increases the blood pressure, causes migraine (phenylethylamine) etc. (EFSA, 2011; Shalabi, 1996).

During wine production biogenic amines are already present in must obtained from sunny or rotten grape, because from vineyard it is not possible to get grape which will be free of biogenic amines (Gloria et al., 1998). Additionally the biogenic amines are accumulated during fermentation, from yeast (alcoholic fermentation) or bacteria (malolactic fermentation) or from other naturally present microorganisms. According to that, the wines where the amino acids are present are ideal environment for production of biogenic amines. The negative influence of biogenic amines on human health is the reason why the world with high velocity works on detection and determination the level of biogenic amines in different foods and wines.

Although, different methods such as high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) are usually used for separation and detection of biogenic amines in foods, fermented foods and wines, the aim of this study is to develop and optimise the HPTLC method for qualitative and quantitative analysis of five biogenic amines in few red and white wines as an alternative method with several advantages.

Materials and methods

Samples and chemicals: A total 12 wine samples (six red wines and six white wines) were collected from different winery and different aging period for determination of five biogenic amines. All chemicals and reagents were ana-

* igno_tasev@tochem.com

lytical grade. The hydrochloride forms of biogenic amines were used as standards and purchased from Sigma, Merck and VWR from Germany. Stock solution of His, Cad, Put, Phe and Tyr dissolved in 10% EtOH was prepared (500 ppm) and then for determination of calibration curve were prepared standard biogenic amines solutions: 0.1 mg/L; 0.5 mg/L; 1 mg/L; 5 mg/L; 10 mg/L; 15 mg/L in which 1.6 diaminohexan was added as internal standard.

Preparation of wine samples for HPTLC analysis: liquid-liquid extraction of biogenic amines from wines was made by following procedure: 5 mL of wine sample was mixed with 1 mL 5 M NaOH and 2 mL 5 M Na_2CO_3. After that were added successively three times butanol (6 Ml + 3 mL + 3 ml), and upper butanol layer was segregate and mixed 4 min. with 5 mL 0.1 M HCl. Separated lower layer was derivatised as followed: 200 µL sample was mixed in plastic tubes (2 mL) with 400 µL Dansyl chloride (50 mg/ 10 mL acetone) and 200 µL $NaHCO_3$ (1.5 g/ 10 mL H_2O). Derivatisation was conducted at 60 °C on dark place for 60 min. and after that in sample was added 500 µL toluene. The above layer was separated and put in vials for automatic ATS4 application of the sample on the HPTLC plate. For elution of biogenic amines was used 10 mL mobile phase: Toluene:Chloroform:Trietylamine (10:6:5 by volume) and 0.5% Tween 80. Saturation time was 40 min. at room temperature, on dark, avoiding air movements. Overall elution time was 20 min.

Instrumentation and conditions: HPTLC plates precoated with silica gel 60F254 (20 cm x 10 cm) from Merck (Germany) were used as the stationary phase. The samples were sprayed on the HPTLC plate (spray-on technique) with the help of a sample applicator AUTOMATIC TLC SAMPLER 4 (ATS4, CAMAG). The chromatogram was developed in horizontal developing chamber (CAMAG). Detection of components from developed HPTLC plates was made with fluorescence Vilber Lourmat detector. Obtained chromatograms were analyzed by software packages Infinity 15.01 and Tina.

Results and discussion

Biogenic amines are of increasing interest to the wine industry due to proposed regulatory issues. Although HPTLC technique is relatively simple, it is difficult to make directly separations of biogenic amines from complex wine matrix. For effectively separation of biogenic amines and solve the problem with effect of wine matrix into qualitative and quantitative routine analysis two derivatisation agents FMOC-CL (9-fluoroenylmethyl chloroformate) and DAN-CL (dansyl chloride) were investigated. After derivatisations and liquid-liquid extraction, biogenic amines in wine samples were simultaneously analysed by using CAMAG ATS 4 automatic sampler on precoated silica gel 60 F254 plate and Vilber Louromat fluorescent detector. After analysis of obtained results was concludes that second development derivatisation and modificated liquid-liquid extraction protocol allowed effectively HPTLC separations and identification of biogenic amines in complex wine (red and white) matrix in concentration from 0.1 to 15 µg/mL. Results of analysis shown that all red wine samples had higher level of biogenic amines than white wines. The validated method displayed excellent selectivity, sensitivity, linearity, precision, accuracy and robustness.

Conclusion

According to the known literature data, analysis of biogenic amines by HPTLC method in wines has been used for the first time in Republic of Macedonia. The optimised HPTLC method compared with HPLC is less expensive for routine analyses of wines and enables reducing the use of organic solvents for derivatisation and for mobile phase, and provides an opportunity for determination of biogenic amines without higher interference with the compounds from the complex red or white wine matrix.

In conclusion, the proposed HPTLC method is suitable for application in analysis of biogenic amines in different wines and could be considered as an alternative method and an important tool in routine analysis of biogenic amines in wine.

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Macedonian pharmaceutical bulletin, **62** (suppl) 465 - 466 (2016)
ISSN 1409 - 8695
UDC: 664.9:633.8
Short communication

Herbal additives for extended shell-life of processed meat products

Ivana Cvetkovikj[1]*, Gjoshe Stefkov[1], Marija Karapandzova[1], Marija Glavash-Dodov[2], Maja Simonovska Carcarevska[2], Vesna Kotevska[3], Ana Kaftandzieva[3], Svetlana Kulevanova[1]

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, "Majka Tereza" 47, 1000 Skopje, Macedonia*
[2] *Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril and Methodius University, "Majka Tereza" 47, 1000 Skopje, Macedonia*
[3] *Institute of Microbiology with Parasitology, Medical Faculty, Ss. Cyril and Methodius University, "50 Divizija" 6, 1000 Skopje, Macedonia*

Introduction

An expanding tendency of global scale is the commitment of food processors and consumers to reduce the use of synthetic chemicals in food preservation. Recently, there has been a considerable interest in extracts and essential oils from common culinary herbs, spices and aromatic plants with established antimicrobial and antioxidant potency.

Among all, some representatives of the Lamiaceae family (oregano, marjoram, savory, sage, rosemary, thyme, basil ect.), possessing high polyphenols content and volatile compounds, are often used to prevent different meet products from food born microorganisms or to stabilize fat and fat-containing foods.

The direct addition of spices to food is the most common technological method, in order to improve its taste, smell and organoleptic effect, to increase the freshness of products and at least to preserve the products in a proper way (Kostic-Nikolic, 2013). However, these days numerous efforts have been made to find alternative solutions to the aim of avoiding undesirable inactivation, and adulteration of the smell and taste. Spraying, dipping, and coating treatment of food with active natural principles are currently applied to product prior to packaging as valid options (Ahmed and Ismail, 2010).

Therefore the aim of this study was to assess the usage of some Lamiaceae spices and its respective preparations and formulations as natural preservatives in treatment of processed meat products, as well as developing novel technological formulation (active packing) to maintain the food quality and to prolong their shelf-life.

Materials and methods

Dry leaves of *Salvia officinalis* and *Rosmarinus officinalis* were purchased from Herbal Pharmacy-Alkaloid AD, Skopje.

Dry leaves, essential oils (EO) and dry extracts (water, alcoholic) were prepared according to the methods listed in the monographs in the European pharmacopoeia or in the Cvetkovikj et al. (2013) paper, and added in different meet products (minced meat, kebabs, and sausages) as preserving agents.

The different plant preparations were chemically characterized by Agilent HPLC-DAD and HPLC–DAD–ESI-MS^n to examine the polyphenols and for the volatile compounds characterization Agilent GC-FID-MS system was used.

Chitosan/sodium-triphosphate/pectin microparticles containing extract from sage were prepared using spray drying technique (Büchi B-290, Flawil, Switzerland).

Microbial strains: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Clostridium sporogenes* ATCC 19404, *Escherichia coli* ATCC 25992, *Klebsiella pneumoniae* ATCC 700603 and clinical

* ivanacvetkovikj@gmail.com;
ivanacvetkovikj@ff.ukim.edu.mk

isolates from patients (*Listeria monocytogenes, Salmonella enteritidis*) were used.

Disc diffusion (the zone of inhibition was determined, expressed in mm) and disk dilution (the minimal inhibitory concentration-MIC was determinate) methods were used in order to investigate the antimicrobial activity of the preparations.

To assess the antioxidant potential of the plant preparations and formulation, β-carotene linoleic acid assay was performed according to Wettasinghe and Shahidi (1996), with small modifications, while the thiobarbituric acid (TBA) test for secondary products of lipid oxidation suggested by Tarladgis et al. (1960), and modified by Shahidi et al. (1987) was used.

Results and discussion

With HPLC–DAD–ESI-MS^n qualitative analysis of the extracts were performed and 27 different compounds were detected. The identified compounds were classified in four groups: nine hydroxycinnamic acid derivates, 10 flavone glycosides of luteolin and apigenin, two flavanone glycosides of hispidulin and six phenolic diterpenes, derivates of carnosol. Rosmarinic acid was predominant compound, present with more than 40% in the analysed samples. On the other hand, the ratio of the flavanone glycosides and the phenolic diterpenes was almost equal.

The EO yield was 1.65% and complies with the minimal requirements of the European Pharmacopoeia. With GC/FID/MS in total 32 constituents were detected. The identified compounds were classified in three groups: oxygenated monoterpenes (68.76%), followed by the monoterpenes fraction (12.91%) and oxygenated sesquiterpenes (7.36%). The sesquiterepene fraction and the diterepenes were detected in amounts < 3.00%. As principal EO constituents were considered: camphor, 1,8-cineole, *cis*-thujone, *trans*-thujone, camphene and viridiforol. The chemical composition of the EO did not comply with the ISO 9909 and GDC standards.

The gas chromatographic analysis of the dry extract revealed four different classes of terpenes compounds (oxygenated monoterpenes, diterpens, oxygenated sesquterepenes and sesquterepenes). When compared to the EO it is evident that in the methanolic extracts the highly volatile monoterepenes were not detected.

The activity of the preserving additives (dry minced leaves, dry extracts, essential oil and microparticles loaded with extract) – was evaluated by the growth inhibition and MIC for food borne microorganisms (four Gram-positive and three Gram-negative bacteria). Generally all additives showed better activity towards Gram-positive bacteria that is in accordance with the literature. The most potent antimicrobial activity posses the EO, with lowest MIC (mg/mL) determinate towards five microorganisms (*S. aureus, S. epidermidis, L. monocytogenes, E. coli* and *K. pneumonia*) that can be attributed to the presence of oxygenated monoterpenes (60% of EO), which is in correlation with the online available data (Pierozan et al., 2009). The microparticles showed antimicrobial activity alike EO.

On the other hand, the extract incorporated into microparticles (1 mg/mL), showed strong antioxidant activity thus inhibiting 90% of the oxidation of the linoleic acid compared to the extracts that can be probably attributed to the synergistic action between the dry extract and the formulation properties of the designed carrier system. Additionally, performing the TBA test, this formulation possesses the potential to protect the product from lipid oxidation, and to provide longer shelf-life storage with low TBA number (0.2029) compared with the other investigated preparations (Valesco and Williams, 2011).

Conclusion

Different preparations of dry plant leaves from sage and rosemary, showed antimicrobial and antioxidant activity that have potential to be used as natural preservatives for processed meat products. Additionally, novel technological approach was investigated in order to diminish the adulteration of the taste, smell and color on the meat as most common side effect of adding of natural additives. The obtained results suggest that the dry extract incorporated into microparticles can be considered as promising technological solution in preserving food.

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Macedonian pharmaceutical bulletin, **62** (suppl) 467 - 468 (2016)
ISSN 1409 - 8695
UDC: 633.8:581.135.5(497.115)
Short communication

Essential oils from Kosovar aromatic plants

Avni Hajdari[1, 2]*, Behxhet Mustafa[1,2]

[1] *Department of Biology, Faculty of Mathematical and Natural Science, University of Prishtina "Hasan Prishtina", Mother Theresa St.,10000 Prishtinë, Kosovo.*
[2] *Institute for Biological and Environmental Researches, Faculty of Mathematical and Natural Science, University of Prishtina "Hasan Prishtina", Mother Theresa St.,10000 Prishtinë, Kosovo.*

Introduction

Kosovo retains a great biological diversity, currently around 1 800 plant species known to make up the flora of Kosovo, and these are deposited at the Herbarium of the Faculty of Mathematical and Natural Science of the University of Prishtina. However, the actual total flora of Kosovo is estimated to comprise more than 2 500 plant species (Krasniqi, 1998), which accounts for about 30% of the entire Balkan flora and 16% of the European flora, although Kosovo covers only 2.3% of the Balkan land area (Mustafa, 1998). The diversity of aromatic plants in Kosovo isn't fully known, as up to now no detailed studies were carried in this regard. It is estimated that in Kosovo are present more than 100 aromatic plant species which can serve as a source of essential oils.

Materials and methods

This review focuses on the phytochemical composition of essential oils obtained from wild population of aromatic plats in Kosovo and on their biological activity (antimicrobial and antioxidant activity). For that, previously published papers related to the chemical composition of the essential oil originated from Kosovo and their biological activities were used to produce this review.

Results and discussion

Several researches on chemical composition and/or their biological activity of the essential oil originated from Kosovo were carried by different working groups. The investigated species belong to different family as follow: Cupressaceae (*Juniperus communis* L., *J. communis* subsp. *alpina* (Suter) Čelak and *J. oxycedrus* L.), Pinaceae (*Abies alba* Mill., *A. borisii-regis* Mattf., *Pinus mugo* Turra, *P. peuce* Griseb., *P. heldreichii* H. Christ, *P. sylvestris* L. and *P. nigra* J. F. Arnold, *Picea abies* (L.) H. Krasr.), Lamiaceae (*Betonica officinalis* L., *Hyssopus officinalis* L., *Micromeria albanica* (Griceb. ex K. Mal) Silić), *Origanum vulgare* L., *Satureja montana* L., *Stachys sylvatica* L., *Thymus jankae* Čelak., *Th. pulegioides* L. subsp. *montanus*, *Th. tosevii* Velen., *Th. longicaulis* Presl.), Hypericaceae (*Hypericum perforatum* L.), Anacardiaceae (*Pistacia terebinthus* L.), Asteraceae (*Achillea alexandri-regis* Bornm. & Rudsky, *A. chrysocoma* Friv., *A.millefolium* L., *A. holosericea* Sm., *A. lingulata* Waldst. & Kit., *Artemisia absinthium* L., *A. vulgaris* L., *A. alba* Turra., *Tanacetum larcatum* (Gris.) Kanitz.). Betulaceae (*Betula pendula* Roth), Tiliaceae (*Tilia cordata* Mill.).

Except chemical screening of the essential oil, in the plant species *J. communis, J. oxycedrus, Pinus mugo, P. peuce, B. officinalis, S. sylvatica, S. montana, H. perforatum, P. terebinthus* the chemical variability of the essential oil among different population were analysed too (Hajdari et al. 2012a; 2012b; 2014a; 2014b; 2015). Essential oil obtained from the following species: *J. communis, P. mugo P. peuce, P. heldreichii, P. sylvestris, P. nigra, T.s longicaulis, M. albanica* and *P. terebinthus* were tested for their antimicrobial, while essential oil obtained from *P. mugo P. peuce, P. heldreichii, P. sylvestris, P. nigra* were tested for their antioxidant activity too. In scientific aspect the most interesting analysed taxa for their essential oils are *M. albanica* and *A. alexandri-regis*, which are local endemic of Kosovo and are characterized with limited population size. Other threatens aromatic plant species are *A. alba, P. peuce, S.*

* avni.hajdari@uni-pr.edu

scardica and *T. cordata*, because of their uses as biological resource (listed in the IUCN Red List).

Conclusion

The group of aromatic plant species in Kosovo represents important natural resources in term of economical aspects, in other hand aromatic plants represent another group of plants that are particularly threatened by overexploitation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 469 - 470 (2016)
ISSN 1409 - 8695
UDC: 615.85.015.32
Short communication

Homeopathic remedies - classical and complex homeopathy in Serbia

Snezana Cupara*, Olivera Milovanovic, Ana Barjaktarevic

Pharmacy Department, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, 34000 Kragujevac, Serbia

Homeopathy uses medicines made of plants, animals or minerals. During preparation these remedies are repeatedly diluted and vigorously shaken at each stage of dilution. Their final potency is described in decimal (D) or centesimal (C) scale of dilution. There are low, medium and high potencies within each scale, while all D potencies are weaker then C potencies. Though, opinions on categorization of potencies may differ, potencies up to six are generally considered low potencies (6D or 6C).

Classical homeopathic treatment involves taking a history of the case and a detailed examination. It differs from conventional treatment since it uses principle of treating the whole patient rather than individual symptoms. Improvement is seen as bothering symptoms are diminished or absent and patient reports more energy, better sleep and mood. Benefits of using homeopathic remedies are multiple: they are not teratogen, non-toxic and do not produce side effects, in acute cases they act faster than conventional drugs, they may be effective in the cases where conventional medicine has nothing to offer, their shelf-life is very long and possible unlimited, they are well tolerated by patients of all ages, their application is not invasive in conditions where swallowing is difficult (e.g. vomiting, coma, epilepsy) and they have very low cost (Vithoulkas, 2008).

There are two main approaches in use of homeopathic remedies – the use of single remedy made in single potency and only of one substance (classical homeopathic remedies) and the use of several substances in different potencies combined together in such a way to cover specific disturbing symptoms (complex homeopathic remedies). In both situations the use of homeopathic remedies may or may not be associated with the use of conventional, mostly chemical drugs. This paper discusses specificity of these approaches within Serbian settings.

In classical homeopathy whole patient is treated by taking in account his mental (e.g. disposition, attitude etc.), general (e.g. tolerance to coldness, heat, damp, wind etc.) and particular or peculiar symptoms (worse from movement, better by hard pressure, throbbing pain, pulsating pain, etc.). This information helps determine one homeopathic remedy that is prescribed in one potency, in a posology that is adjusted to the individual patient's health state. This remedy may be so compatible with constitution of the patient that outcome is amelioration or complete disappearing of the troubling symptoms, while at the same time patient feels better on a general level. That is the main aim for patients that choose homeopathy– either because the conventional medicine has failed to help them or they prefer it as the gentlest way of healing during acute or chronic ailments.

Complex homeopathy, on the other hand, tries to bridge classical homeopathy and conventional medicine offering partial benefits of both. A complex homeopathic remedy, made of several different substances, all in low potencies and combined with the aim that each is targeting different symptoms, can be used independently of a homeopathic consultation – either by advice of a conventional doctor, a pharmacist or as a part of self-medication. Complex homeopathic remedy of different content may be commercialized under a brand name and can undergo research that proves its efficacy (Thinesse-Mallwitz et al., 2015). A complex remedy cannot offer constitutional healing to the patient, but one of its benefits is that it attempts to achieve clinical efficacy in cases when a classical homeopathic consultation is not available (Malapane et al., 2014). Still, many classical homeopaths never prescribe complex homeopathic remedies. Their negative attitude is based on the premise that use of complex homeopathic medicine compromises effects of classical prescribing and spoils the case.

* snezanacupara@yahoo.com

Homeopathic remedies, either classical or complex, can be used in conjunction with conventional medicine, although their action may be less effective in that case due to the difference in the mechanism of action. Homeopathic medicine acts on body's auto regulatory mechanisms. If these have already been influenced by powerful drugs, the action of homeopathic remedy may be impaired. However, in the case of the maintenance therapy for deficiency diseases (e.g. insulin, thyroxine, etc.) conventional medicine must not be discontinued during the homeopathic treatment. A homeopathy is an effective additional form of therapy, but in a multi-medicated patient it should be used with high doctor's prudence and experience.

There are several specificities in homeopathy in Serbia. One-component remedies for classical homeopathy are not imported and readily available in pharmacies since they are not registered. Patients self supply themselves in a difficult manner - mostly from abroad (Cupara et al., 2013). Moreover, legislation and education of homeopaths are roughly regulated and there are practical inadequacies. Many homeopaths due to the above mentioned problems have small volume of consultations on annual level, which consequently lead to less experienced practice. There is a lack of competent structural information about practitioners and patients have obstacles in choosing and reaching a homeopath. The additional difficulty is the self-medication that patients practice, which is also a driving force for unjustified use of many conventional drugs, even in a case when a proper pharmacist's advice is given.

In this specific situation, in which patient cannot easily reach a competent homeopath of his choice or cannot easily obtain the prescribed remedy, together with tendency for unjustified self medication by conventional drugs and slow, but steadily growing number of patients that require alternative to conventional drug, a complex homeopathic remedy seems to be a possible choice that harms the least.

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Macedonian pharmaceutical bulletin, **62** (suppl) 471 - 472 (2016)
ISSN 1409 - 8695
UDC: 633.888:615.212.7
Short communication

Is cannabis addictive?

Svetlana Golocorbin-Kon[1]*, Nebojša Pavlovic[1], Maja Đanić[2], Mladena Lalic-Popovic[1], Slobodan Gigov[3], Nikola Jojic[3], Momir Mikov[2]

[1] *Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, Novi Sad, Serbia*
[2] *Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, Novi Sad, Serbia*
[3] *Faculty of Pharmacy, University Business Academy in Novi Sad, Trg Mladenaca 5, Novi Sad, Serbia*

Cannabis is a tall plant with a stiff upright stem, divided serrated leaves, and glandular hairs. Cannabis has an extremely long history of use. Although cannabis is indigenous to Central and South Asia, it spread all over the world very early in human history. While humans in Taiwan were growing hemp as early as 8 000 BCE, the historical use of cannabis as a physical and psychological medicine began over 5 000 years ago. Therapeutic properties of cannabis have been known since ancient times, although the application has been limited mainly due to narcotic characteristics of this plant. Cannabis is also known as marijuana and numerous other street names such as ganja, pot, astro turf, grass, hemp, dagga, kiff, weed, hash, purple, etc. Hashish is related form of the drug made from the resins of the Indian hemp plant. Also called chocolate, shit or hash, it is on average six times stronger than marijuana (Mikuriya, 1969).

Nowadays, the pharmacologically active substances in cannabis - cannabinoids, are very interesting group of compounds with potentially significant applications in medicine and pharmacy. Research studies have clearly shown that cannabinoids are highly efficient, primarily in the treatment of nausea and vomiting and the management of chronic pain. For patients who suffer from severe, chronic diseases, such as cancer and AIDS, cannabis has been shown to relieve several symptoms at the same time, in more efficient way than some registered medicines. Besides, there is a great potential of cannabinoids to be applied for the treatment of many other pathological conditions, such as multiple sclerosis, Alzheimer's disease, epilepsy, hypertension, glaucoma, although it is necessary to complete the last phases of clinical trials in order to register these compounds as drugs, i.e. in order to assure the safe therapy for these diseases (Grotenhermen and Russo, 2002). Although tetrahydrocannabinol (THC) proved to be very efficient in the treatment of numerous diseases, new drugs have been intensively developing that tend to possess improved selectivity, better pharmacokinetic properties, and favorable relationship between desired pharmacological effect and side effects. Laws and attitudes toward cannabis are changing these days. Legalization of cannabis use for medical purposes is a hot topic at the global level and in most countries there have been initiatives to amend the existing laws in order to make drugs based on natural ingredients of cannabis, as well as other related products, synthetically produced, available to patients, that would certainly, based on the results of investigations, contribute to a better quality of patients' lives (Clark et al., 2004). On the other hands, there is a question: "Is cannabis addictive?" What would be a proper answer? Cannabis, like other analgesics, can cause dependence and addiction. Over time, the persistent overstimulation of the endocannabinoid system can cause changes in the brain that result in addiction. This is much more likely in people who start using cannabis (marijuana) when young and who are heavy users. An estimated 9% of people who use cannabis become dependent on the drug. Teenage users have a 17% risk of becoming addicted, and 25-50% of regular, daily, users become addicted. Short term effects of cannabis are both pleasant and unpleasant and short term cannabis effects are not the same for everyone. Short term cannabis effects vary depending on the person's size, experience with the drug, the amount of drug consumed and individual physiology. Desirable short term effects of cannabis are generally characterized as "high". Pleasant short term cannabis effects include: euphoria, intoxication, relaxation, detachment, decreased anxiety and

* magistrakon@orcon.net.nz

alertness, altered perception of time and space, intensified experiences, laughter, talkativeness. However, while those short term effects of cannabis are pleasant, short term effects of cannabis also include: depression, anxiety, panic, paranoia, amnesia, confusion, delusions, hallucinations, psychosis, mania, short term memory impairment, sudden increase in heart rate, at risk for heart complications, dizziness, lack of coordination and muscle strength, lethargy, decreased concentration, slurred speech. It is also known that mental illness and cannabis use are linked, particularly to schizophrenia, but at this time it's not clear whether cannabis causes, exacerbates or is simply a predictor of mental illness. Even the short term effects of cannabis can include an increase in the severity of existing mental illnesses. Long term effects of cannabis tend to be more negative than the short term effects. This is primarily because tolerance builds to the drug's effects and the user takes greater doses of cannabis, increasing the short and long term effects of cannabis as well as its potential for abuse. Once tolerance to the drug is achieved, one of the long term effects of cannabis becomes cannabis withdrawal after using it and during periods of abstinence (http //www.drugfreeworld.org/drugfacts/marijuana/short-and-long-term-effects.html). The withdrawal syndrome begins on the second day of stopping and may persist for two weeks. Discontinuation symptoms include anxiety, irritability, insomnia, stomach pain and decreased appetite. Sleep problems can potentially persist beyond that time frame. Cannabis is an illicit drug that has the potential to lead to abuse and addiction. Despite the commonly held belief that herbal cannabis is harmless, its use is not without adverse effects and undesirable outcomes. The full extent of long-term health risks of chronic cannabis use is currently unknown. It is also important to note that "synthetic cannabis" is not actually cannabis (http://www.addictions.com/marijuana/). There are many research projects that are examining the medical benefits of individual cannabinoid chemicals derived from or related to those in the marijuana plant, not the plant itself, although a few use unprocessed plant material. Individual cannabinoid chemicals may be isolated and purified from the marijuana plant or synthesized in the laboratory, or they may be naturally occurring (endogenous) cannabinoids found in the body and modified using other, non-cannabinoid chemicals (Boyd, 2013).

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Macedonian pharmaceutical bulletin, **62** (suppl) 473 - 474 (2016)
ISSN 1409 - 8695
UDC: 635.65(497.7)
Short communication

Macedonian bean diversity and its health benefits potential

Sonja Ivanovska[1]*, Mirjana Jankulovska[1], Gjoshe Stefkov[2]

[1]*Faculty of Agricultural Sciences and Food, University "Ss. Cyril and Methodius", Aleksandar Makedonski bb, Skopje 1000, Republic of Macedonia*
[2]*Institute of Pharmacognosy, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Majka Tereza 47, Skopje 1000, Republic of Macedonia*

Introduction

Common bean (*Phaseolus vulgaris* L.) is a legume crop native to Central America and Mexico. As huge diversity of bean varieties and landraces are cultivated at the Balkan countries, this region is considered as secondary centre of origin. Cooked bean's seed is traditional dish in Macedonia and although the white seeded varieties are more popular, farmers also preserve landraces varied in shape, size, color and pattern of seeds. This valuable diversity is passed through generations for more than two centuries (Ivanovska et al., 2013; Maras et al., 2009).

Lately, the trend of balanced diet essential for healthy life is increasing and the consumption of many agricultural crops is recommended. In this sense, bean is considered as healthy food due to its nutritional and health benefits (Mesina, 2014). Its main nutritional component is starch, predominantly consisted of amylose and amylopectin. The specific ratio of these two polysaccharides along with the slow digestion of amylose raises blood sugar more gradually and keeps it lower, compared to other types of starch. Thus, bean ranks very low according to the glycemic index and is beneficial particularly for diabetics (Barrett and Udani, 2011; Hutchins et al., 2012). Beans are one of the richest sources of plant proteins, out of which widely studied are phaseolin, lectins and protease inhibitors. In particular, they are among the only plant foods that provide significant amounts of the indispensable amino acid lysine. It also contains insoluble fibers alpha-galactosides that cause flatulence in some individuals and substantial quantity of resistant starch which may be used in weight control. Both substances are fermented in the colon by beneficial bacteria, stimulating their growth and function as prebiotics. Beside, as a result of the fermentation short-chain fatty acids are formed, such as butyrate, acetate and propionate that may reduce the risk of colon cancer (Havenaar, 2011). Beans are also rich in various vitamins and minerals, like molybdenum, vitamin B9, iron, copper, manganese, potassium, vitamin K1 and phosphorus. Other positive bioactive compounds of beans are the antioxidants isoflavones, known as phytoestrogens (Konar et al., 2012) and anthocyanins found in the colored seed coat (Lin et al., 2008). Bean also contains negative compounds such as phytic acid (found in all edible seeds) which reduces the absorption of minerals and can be eliminated by soaking and fermenting the beans, and a toxic lectin phytohaemagglutinin which is eliminated with cooking (Queiroz et al., 2002).

Beside its nutritional value, several studies have linked bean consumption with reducing the LDL cholesterol and systolic blood pressure, as well as the risk of overweight and obesity (Papanikolaou and Fulgoni, 2008), metabolic syndrome and ischemic heart disease (Mesina, 2014). As starch blockers are completely inactivated when seeds are boiled at 100 ºC for 10 min., they are extracted from raw white kidney beans and sold like a weight loss supplement (Grube et al., 2014).

Having all this in mind the aim of this study was to explore the existing bean diversity in Macedonia starting with creation of a landrace germplasm collection. Further on, they will be tested for different plant characteristics including their nutritive value. The final goal of this initiative is to promote bean consumption in everyday diet and to explore the potential of different landraces as raw material for production of supplements.

Materials and methods

In order to assess the existing bean diversity in Macedonia, a collection mission was initiated in 2015 by the re-

* s_ivanovska@yahoo.com

searchers of the Department of Genetics and Plant Breeding, Faculty of Agricultural Sciences and Food in Skopje. Seed samples of landraces and relevant information were collected in 237 villages from 58 municipalities and preserved at the Faculty's Gene bank. Considering that the activities are continuous, until now a total number of 1025 samples are collected. They will be analyzed in a field experiment to eliminate duplicates and to characterize and evaluate the landraces.

Results and discussion

The collected 1025 samples belong to three types of beans. Two types are common bean belonging to the species *Phasolus vulgaris* L.: ssp. *nanus* or bush bean (BB) and ssp. *volubilis* or pole bean (PB). The third one is runner bean (RB) belonging to the species *Phaseolus coccineus* L. Regarding to the type, distribution of samples is 590 BB, 395 PB and 40 RB, indicating that the last type is facing extinction. The analysis based on seed color only, showed that white seeded beans share 46.9% of the collection with 310 samples of BB, 155 of PB and 16 of RB. Further division for the white seeded beans only has been done according to the seed shape on 4 general categories: kidney, round, oval and compressed. Highest amount (123) of PB samples was with compressed shape (like the famous local landrace Tetovski grav). Within the BB collection, three types of samples are almost equally distributed: oval (120), kidney (101) and round (79). Out of the 177 samples of mono-colored seeds, landraces with brown seeds prevail (30; 25), followed by cream (19; 30) and green (2; 35) of PB and BB, respectively. Other colors were much less present with 21 black, 10 dark red, 3 dark lilac and 2 dark green-blue seeded samples.

A category bicolored seed (26 samples in total) refers to seeds with oval or round shape that have one half white and one half colored seed. In this category, the colored half has various colors and often an additional pattern.

The rest of the collected samples (341) belong to the colored seeds with pattern. Many different combinations of size, shape, color and pattern type (speckles, stripes etc.) are registered. Most distributed are cream seeds with dark red or brown speckles, as well as brown seeds with dark brown or black stripes or speckles. If additional categories are added in the analysis in total, like size and shape of the seeds, color and type of the pattern, as well as vegetative characteristics of the plants, it is obvious that diversity of landraces is huge.

Conclusion

From the preliminary analysis of the bean collection based on the seed color only with several parameters it may be predicted that more than 300 different landraces are collected. Thus, the collection presents a valuable gene pool which will be further analyzed for all traits according to bean descriptors. Landraces with colored seeds will be particularly promoted for their nutritional and medicinal value.

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Macedonian pharmaceutical bulletin, **62** (suppl) 475 - 476 (2016)
ISSN 1409 - 8695
UDC: 615.277.015.4
Short communication

Apoptotic and antioxidant activity of *Centaurea depressa* Bieb. (Asteraceae) extracts on colon colorectal adenocarcinoma (Caco-2) cell lines

Özge Tarançı[1], Selcen Babaoğlu Aydaş[2]*, Belma Aslim[1]

[1]*Gazi University, Faculty of Science, Department of Biology, 06500, Teknikokullar, Ankara, Turkey.*
[2]*Gazi University, Vocational High School of Health Services, 06830, Ankara/Gölbaşı, Turkey*

Introduction

Adenocarcinomas are the cancer of intestinal gland cells and the representative of more than 95% of colon and rectal cancers. "Adeno" is the prefix for gland and the intestinal gland cells that line the inside of the colon and/or rectum, where the adenocarcinomas typically start. A reduction in the incidence of colorectal cancer is possible although the screening techniques are imperfect (CTCA, 2016).

Flavonoids exert their anticarcinogenic effects by modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis. Flavonoids are proved to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals, which are possibly involved in DNA damage and tumor promotion (Sandhar et al., 2011). Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death. So, one of the useful strategies for anticancer drug development is the induction of apoptosis in cancer cells (Kwon et al., 2006).

It has been reported by pharmacological trials that some members of the genus *Centaurea* possess antiinflammatory, antimicrobial, antipyretic, cytotoxic, and immunological activities (Zengin et al., 2010). The present study was carried out to evaluate the apoptotic and antioxidant effects and to determine the possible mechanisms of cell death elicited by the extracts of *Centaurea depressa* on human colorectal adenocarcinoma cell line Caco-2.

Materials and methods

Plant material and extract preparation

C. depressa was harvested from field in Gölbaşı, Ankara, Turkey. The identification of plant materials was confirmed by plant taxonomist, Prof. Dr. Zeki AYTAC, at the Department of Biology, Gazi University, Ankara, Turkey. A voucher specimen was deposited at the Herbarium of Gazi University, Ankara, Turkey. The aboveground tissues were shade dried, powdered and 30 g samples were separately extracted with either methanol or water, using Soxhlet apparatus for 6 h and the extracts were filtered and concentrated in rotary evaporator.

Cell lines and culture medium

Caco-2 cell lines obtained from ATCC were cultured in DMEM with 20% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. The cells were incubated at 37 ˚C with 5% CO_2 (Hadjiakhoondi et al., 2013). The cells were planted in microtiter plates at an initial density of 1 x 104 cells/well. After optimum confluence cells were exposed to CME or CWE (500 µg/mL) as determined to be the effective dose in former antiproliferation experiments for 48 h. Untreated cells were considered as controls.

Determination of the total antioxidant capacity

Cayman's antioxidant assay was used to determine the total antioxidant capacity. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2'-Azino-di(3-ethylbenzothiazoline-sulphonate) to $ABTS^{\bullet+}$ by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents. The ABTS produced by the cells after the extract treatment were measured according to the kit's protocol.

Determination of superoxide dismutase (SOD) activity

Cayman's superoxide dismutase assay kit was used. The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxan-

* selcenb@gmail.com

thine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The measurements were achieved according to the kit's protocol.

Cell death detection assay

Apoptosis was determined by a Cell Death Detection ELISAPLUS assay. This kit is based on a photometric enzyme immunoassay and is used to detect DNA fragmentation; mono- and oligonucleosomes released into the cytoplasm by biotinylated anti-histone- and peroxidase-coupled anti-DNA antibodies, following the manufacturer's protocol. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample (A405) by the absorbance of the controls without treatment (A490) which was equal to 1.

Detection of apoptosis associated morphological changes with Hoechst 33342.

Apoptotic effects of plant extracts were evaluated with Hoechst 33342 staining assay determining the morphological alterations in the cells. Propidiumiodide (PI) is used to detect the necrotic cells. Cells were imaged with fluorescence microscope (Türk et al., 2011). Apoptotic index was defined as the percentage (%) of apoptotic cells and calculated with the formula; number of apoptotic cells / total number of cells x 100 (Jackisch et al., 2000).

Statistics

Results are expressed as means ± Standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using the Tukey's test, with $P<0.05$ considered as statistically significant. All experiments were independently repeated at least three times, with triplicate samples for each treatment.

Results and discussion

In our former experiments investigating the antiproliferation activity of the *C. depressa* methanol (CME) and water extracts (CWE) in varying concentrations (100, 250 and 500 µg/mL), the results exhibited a dose dependent trend and both methanol and water extracts showed a significant antiproliferative activity on Caco-2 cells with 500 µg/mL concentration (63%, 69% cell death, respectively). So this study was carried out to evaluate the apoptotic and antioxidant effects of the extracts and to determine the possible mechanisms of cell death elicited by the extracts of *C. depressa* (500 µg/mL) on Caco-2 cell line. Activities of total antioxidant enzymes were analysed using Caymans's kit, however no detectable change were observed as compared with the control cells (0.31±0.02 mM Trolox) ($p<0.5$). Similar results were also found in the SOD activity (0.8±0.03 U/mL) ($p<0.5$) and SOD activity also did not vary according to the extract type.

Apoptotic effect of CME and CWE (500 µg/mL) on the Caco-2 cells were determined by cell death detection after 48 hours incubation. Apoptotic effect of the CME and CWE are higher as compared to the control group with the enrichment factor of 1.69-fold and 1.98-fold respectively and the CWE has higher apoptotic effect than CME.

Hoechst 33342 staining revealed the morphological changes including cell shrinkage and nuclear condensation occurred with *C. depressa*; producing a more brighter blue light in apoptotic cells compared to normal cells that showed normal nuclear morphology. Dead cells had violet-red fluorescence and showed no signs of chromatin condensation and apoptotic bodies. PI dye cannot cross cell membrane of dead cells, as opposed to apoptotic cells. The extracts may have inhibited cell growth via inducing apoptosis. Therefore apoptotic index was defined as the percentage (%) of apoptotic cells stained with Hoechst 33342 dye and the results indicate that CME and CWE exhibited significant high apoptotic index following 48 hours treatment in favor of CWE (47% and 60% respectively).

Conclusion

C. depressa exhibited considerable apoptotic effects on Caco-2 cancer cell lines. Studies investigating the bioactive ingredients of these herbal extracts would pave the way for mechanistic and translational studies in efforts to design novel anticancer drugs to be used alone or concomitant with other therapies to prevent progression of tumors and/or to treat them.

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Macedonian pharmaceutical bulletin, **62** (suppl) 477 - 478 (2016)
ISSN 1409 - 8695
UDC: 633.888(091)
Short communication

Cannabis history and timeline

Biljana Bauer[1]*, Vesna Kostic[2], Svetlana Cekovska[3], Zoran Kavrakovski[1]

[1]*Faculty of Pharmacy, SS Cyril and Methodius University, Majka Tereza No 47, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Public Health, Faculty of Medicine, 50 Divizija 6, 1000 Skopje, Republic of Macedonia*
[3]*Department of Medical and experimental Biochemistry, Faculty of Medicine, 50 Divizija 6, 1000 Skopje, Republic of Macedonia*

Introduction

Cannabis or hemp (*Cannabis sativa* L. Fam. Cannabinaceae) is very popular nowadays because of its medical use. The plant Cannabis sativa L. is a source of a number of drug products. Marijuana represents the dried top parts of female hemp plant in flower. The main ingredient in hashish is the resin secreted by the glandular hairs found all over the plant but mainly around flowers. In addition, the cannabis plant can be used as a source hemp fibers, as well as hemp seeds and fatty oil (Kovacevic, 2000). Throughout human history cannabis has been used for many purposes such as recreation, therapy, art, religion, food, medicine, as a textile. This plant was also used for treating insomnia, healing and also as painkiller. Each culture and subculture from prehistory up to now use this plant because it causes selective changes in consciousness of its consumers strictly dosing what is beyond reality, and also for medical reasons. Today, cannabis is forbidden in many countries because of its narcotic and negative influence to the nerve system. In some cultures cannabis is a protected mark, other cultures are its big admirers and third do not know or do not look on that way about cannabis (Tyler et al., 1988). Canada is first country in the world that has offers medical marijuana to its patients since 2003. Therefore, there is an increased necessity of research on the history of cannabis for the sake of reinforcing the ability of pharmacists and physicians to respond to the challenges arising in the provision of professional services in order to facilitate human life.

Chronology from the past to the present

History of cannabis

Cannabis is one of the oldest psychoactive plants known to humanity. It grows as weed and cultivated plant all over the world in a variety of climates and soils. Historical records and archaeological materials date from 6000 BC pointing out the use of cannabis seeds for food in China. In 4000 BC textiles made of cannabis are used in China. Remains have been found of cannabis fibers from this period and a century later. First recorded use of cannabis as a medicine was in 2727 BC in Chinese pharmacopoeia. In 1500 BC cannabis is cultivated in China for food and fiber. Scythians cultivated cannabis and use it to weave fine hemp cloth. According to Herodotus they introduced cannabis into Europe (Tucakov, 1948). Bhang, composed from dried cannabis leaves, seeds and stems is mentioned in the Hindu sacred text Atharva veda as "Sacred Grass", one of the five sacred plants of India. It is used medicinally and ritually as an offering to Shiva. Scythian tribes leave cannabis seeds as offerings in royal tombs. This closely matches the Herodotus reports on both ritual and recreation use of cannabis by the Scythians. The psychotropic properties of cannabis are mentioned in the newly compiled herbal Pent Ts'ao Ching which is attributed to an emperor Shen Nung. He recommended it against malaria, constipation, rheumatic pains, and absence of the spirit, female problems and a mixture of cannabis, resin and wine as an analgesic during surgery (Booth, 2003). Cannabis as psychoactive plant is respected from Buddhists. In an old testament is mentioned as a sacred plant in holy anointing oil prepared from myrrh, laurel, cinnamon and "kaneh bosm"- identified by linguists as cannabis (Teodorova et al., 2005). Dioscorides

* biba@ff.ukim.edu.mk

in 70 AD mentions the use of cannabis as a Roman medicament while Galen in 170 AD alludes to the psycho activity of cannabis seed confections. The Jewish Talmud mentions the euphoria properties of cannabis. Ancient Greeks and Romans were also familiar with cannabis, while in the Middle East, use spread throughout the Islamic empire to North Africa. In the early 12th century hashish smoking is very popular throughout the Middle East. Cannabis is introduced in Egypt during the reign of the Ayyubid dynasty in 12th century. Arab traders bring cannabis to the Mozambique coast of Africa in 13th century. Marco Polo during his journeys from 1254-1324 gives reports that Assassins are using hashish (Mez-Mangold, 1971). In 1545 cannabis spread to the western hemisphere where Spaniards imported it to Chile for its use as fiber. In North America cannabis, in the form of hemp, was grown on many plantations for use as rope, clothing and paper. In the late 17th century hashish becomes a major trade item between Central Asia and South Asia. Napoleon discovers that much of the Egyptians habitually used hashish. His soldiers in their returning to France bring the tradition with them in 1798 year (Tucakov, 1948).

Medicinal properties of cannabis

Medicinal use of marijuana arrived in Europe from the East during the 18th century. It was brought to Europe much later, but it was not less popular. It reaches the high society very soon. In Paris, a club was open where many famous people, even Balzac, enjoyed marijuana. The first comprehensive description of the medical use of Indian hemp in Europe was written in 1830 by the German pharmacist Friedrich Ludwig Nees von Esenbeck. Until that point in time, use of marijuana for medical purposes had remained at a low level. Thanks mainly to the work of W.B.O'Shaugnessy in 1839 marijuana become recognized within European – school medicine. He used various hemp compounds in his investigations, partly with great success, against rheumatism, rabies, cholera, tetanus, convulsions and delirium tremens. In America in 1840 medicinal preparations with cannabis base are available in Persian pharmacies. Hashish's eater's club is established in Paris in 1843 and after 1850 hashish appears in Greece (Booth, 2003). Cannabis begins to prohibit for nonmedical use in the US during 1915-1927. The prestigious US Institute of Medicine published its report Medical Use of Marijuana in 1999 (Weiner, 1990). Recent studies reviewed by Park et al. 2004 that marijuana, THC and other exogenous cannabinoids exert potent effects on the endocannabinoid system in both the gonads and during pregnancy (Frankhauser, 2008).

Conclusion

Cannabis preparations have been used as remedies for thousands of years and the active ingredients of the plant can be put to use in a multitude of medical conditions. Cannabis has been used throughout history in many different cultures to change mood, perception and consciousness. Its effects range from increasing creativity to provoking mystical experiences, to heightening the capacity to feel sense and share. The reports established the evidence base support for the further examination of cannabis products for medical use.

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Macedonian pharmaceutical bulletin, **62** (suppl) 479 - 480 (2016)
ISSN 1409 - 8695
UDC: 582.477-113.55
Short communication

Chemical composition of the essential oils of *Juniperus communis* subsp. *alpina* (Suter) Čelak (Cupressaceae)

Behxhet Mustafa[1], Dashnor Nebija[2]*, Avni Hajdari[1]

[1]*University of Prishtina, Faculty of Mathematics and Natural Sciences, Department of Biology, Mother Theresa n.n. 10000 Prishtina, Kosovo*
[2]*University of Prishtina, Faculty of Medicine, Department of Pharmacy, Bulevardi i Deshmoreve n.n. 10000 Prishtina, Kosovo*

Introduction

Juniperus communis subsp. *alpina* (Suter) Čelak (Cupressaceae) grows mainly in the high-mountain regions of the in the Dinarides, northern Scardo-Pindic mountain system, Rhodopes and the Balkan mountains (Filipowicz et al., 2006; Lakusic and Lakusic, 2011). Juniper cones have long been used in medicine, food and the cosmetic industry. In the traditional medicine, preparations of juniper cones are used as folk remedy mostly against rheumatic complaints (Mustafa et al., 2012). In addition to the manifold and diverse chemical composition of secondary metabolites found in Juniper cone oil, it also has different biological activities, including hypoglycemic, carminative, antioxidant, antiseptic, and antibacterial activity (Hajdari et al., 2015). Chemical composition of essential oils of *J. nana* has been reported by several authors (Filipowicz et al., 2006; Proenca da-Cunhaa and Roquea, 1989).

Previously we studied the composition of essential oils in two *Juniperus* species *J. communis* and *J. oxycedrus* respectively growing wild in Kosovo (Hajdari et al., 2015). On the other hand, to the best of our knowledge, there are no published studies that address the composition of essential oils obtained from *J. nana*, therefore the principal aim of this study was to investigate the chemical composition of the essential oils obtained from ripe cones, branches and leaves of this plant harvested from wild population in Kosovo.

Materials and methods

Plant materials (ripe cones) of *Juniperus nana* were collected in wild populations in locality Hajle, Bjeshket e Nemuna, Kosovo (Latitude, 42° 45' 12'' N; Longitude, 20° 07' 59'' E; Altitude, 2029 m). Voucher specimens of each population were deposited with the Herbarium of the Department of Biology, University of Prishtina. For the essential oil extraction the plant material was air-dried in the shade at room temperature, prior to processing and chemical analysis. The ripe cones were chopped up in small pieces, and the essential oils were obtained by hydrodistillation using a Clevenger apparatus for 3 hours. The samples were stored in the dark at -18 °C in a freezer until further analysis. The yield of essential oil is expressed as the volume percentage of the dry mass of the air-dried plant material. GC/FID analyses were performed using an Agilent 7890A GC system equipped with an FID detector (Agilent Technologies). The separation was conducted on a HP-5MS column (30 m × 0.25 mm with a 0.25 μm film thickness). Helium was used as the carrier gas with an initial flow rate of 0.6 ml/min and then at a constant pressure of 50.0 psi. The front inlet was maintained at 250 °C in a split ratio of 50:1. The GC oven temperature was increased from 60 °C to 260 °C at a rate of 5 °C/min, and the FID was operated at 250 °C with an air flow of 350 mL/min and a hydrogen flow of 35 mL/min. The injection volume was 1.0 μL.

GC/MS analyses were performed using an Agilent 7890A GC system coupled to a 5975C MSD (Agilent Technologies). The ionisation energy was 70 eV with a mass range of 40 - 400 m/z. The separation was conduct-

* dashnor.nebija@uni-pr.edu

ed using the same column and temperature program as for the analytical GC.

The identification of each of the components of the essential oil was performed by comparing their Kovat's retention indices with those from the literature. The Kovat's index was calculated based on a linear interpolation of the retention times of a homologous series of n-alkanes (C_9-C_{28}) under the same operating conditions. The components were also identified by comparing the mass spectra of each constituent with those stored in the NIST 08.L and WILEY MS 9th databases and with mass spectra from the literature. Furthermore, some of the main peaks were identified by comparing the retention times and mass spectra with those of authentic constituents. The percentage composition of the oils was computed using the normalization method from the GC peak areas, calculated as the mean of three samples, without correction factors.

Results and discussion

In total 75 constituents were identifies in the essential oils obtained from the juniper cones, leaves and branches. Principal constituents were monoterpenes: α-pinene (24.7-36.7%); sabinene (8.2-22.9%); myrcene (4.3-1.6%); D-limonene (2.4-4.4%); β-pinene (1.6-2.4%); terpinolene (1.0-1.9%). Concentration of oxygenated monoterpene, terpinene-4-ol was 1.4-7.5%. Monoterpenes were followed by sesquiterpenes: germacrene D (2.0-7.9%); α-caryophyllene (0.4-2.1%); bicyclogermacrene (0.3-2.2%); β-caryophyllene (0.1-1.9%); zonarene (0.5-3.1%); δ-cadinene (0.7-3.9%); germacrene B (0.2-1.1%); β-elemene (0.3-1.9%), whereas concentration of the oxygenated sesquiterpene, 1-epi-α-cadinol, ranged from 0.7-4.1%. In regard to the percentages of essential oil constituents in different drug organs, experimental data revealed that monoterpenes were the most abundant constituents in berries, whereas the highest concentration of sesquiterpenes and oxygenated sesquiterpenes was in branches. Results are in the accordance with previously published data where α-pinene and sabinene were principal components of *J. nana* (Filipowicz et al., 2006). In Portugese sample, the major hydrocarbon components of the oil were α-pinene (20.0%), δ-cadinene (10.4%), limonene (8.7%), and myrcene (8.5%) (Proenca da-Cunhaa and Roquea, 1989).

Conclusion

The study of chemical composition essential oils obtained from berries, branches and leaves of *J. nana* growing wild in the Bjeshket e Nemuna region in Kosovo represents a contribution in the field of the essential oil constituents of the genus *Juniperus*. The main essential oil components were a-pinene, followed by sabinene, myrcene and germacrene D.

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Macedonian pharmaceutical bulletin, **62** (suppl) 481 - 482 (2016)
ISSN 1409 - 8695
UDC: 615.322:582.929.4-119.2
Short communication

Chemical characterization and determination of antioxidant activity of basil (*Ocimum basilicum* L.) extracts using different types of *in vitro* tests

Branislava Rakić[1]*, Nevena Grujić-Letić[1], Svetlana Goločorbin-Kon[1], Zorica Mrkonjić[2], Jovana Drljača[1], Aleksandar Rašković[1]

[1]*Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia*
[2]*Faculty of Science, University of Novi Sad, Trg Dositeja Obradovica 3, 21000 Novi Sad, Serbia*

Introduction

The interest in a natural and healthy lifestyle has moved the functional food under the spotlight. Functional food provides the health advantages because of their physiologically active substances (Maskovicet al., 2015). Phenolics and flavonoids isolated from plants are one of the groups of functional ingredient which can enhance health, increase psychical and mental well-being and scavenge free radicals (Menrad, 2003). It is known that family *Lamiaceae*, where sweet basil (*Ocimum basilicum*, L.) belongs to, has a strong antioxidant activity because of great amount of phenolic and flavonoid components. Phenolic antioxidants prevent free radicals from the substrate by donating hydrogen atoms or electrons (Kaurinovic et al., 2011). Sweet basil is widely used plant which could be used in the form of teas, essential oils, liquid extracts, and as a spice and has an important application in the food, pharmaceutical and cosmetic industries. It is mostly used in a treatment of inflammatory diseases, headaches, constipation, respiratory infections, flu, cough, insomnia and also as sedative and anticonvulsant. It is estimated that about 60% of the total population of the world uses herbs and natural products and basil is recognized as important drug in their nutrition (Harvey, 2000).

Materials and methods

Different extracts were obtained by various types of extraction: maceration with ethanol (30%, v/v) and distilled water (30 minutes and 24 hours), infusion (10 minutes), microwave (4 minutes) and ultrasound extraction (10 minutes). The amount of total phenolic compounds in the extracts was determined colorimetrically with the Folin–Ciocalteu (FC) reagent (Božin et al., 2008). The absorbance of the resulting solution was measured at 760 nm on a UV/Vis spectrophotometer. The concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per g of dried extract (DE), using a standard curve of gallic acid. Measurement of total flavonoid content in the investigated extracts was determined spectrophotometrically, using a method based on the formation of Al-flavonoid complex with the absorbance maximum at 430 nm. The flavonoids content was expressed as l g of quercetin equivalents (QE) per g of dried extract (DE), by using a standard line. Antioxidant activity of extracts was determined using the DPPH method (Grujić et al., 2012). Absorbance was measured at 515 nm. The IC_{50} value, defined as the concentration of the test sample leading to 50% reduction of the free radical concentration, was calculated graphically and expressed as μg of the extract/mL of the final solution in measuring cell. Testing of the neutralization of peroxide and hydroxide radical were carried out. RSC value represents the percentage of neutralization of peroxide or hydroxide radical. The IC_{50} value which defined as the concentration of the test sample leading to 50% reduction of the radical was tested. Absorbance was measured at 515 and 562 nm. Lipid peroxidation intensity was also tested and absorbance was measured at 532 nm. The IC_{50} values represented the concentration of the tested extract are responsible for 50% inhibition of lipid peroxidation. Chemical characterization of basil extracts was performed by high performance liquid chromatography (HPLC) using UV/Vis and fluorescence detectors.

* branislava.rakic@faculty-pharmacy.com

Results and discussion

Basil extracts contained chlorogenic, *p*-hydroxybenzoic, caffeic, ferulic, vanillic, rosmarinic and cinnamic acid, rutin, quercetin, naringenin i epicatechin. From phenolic acids, the most common in the extracts are rosmarinic and ferulic acid which is in agreement with similar research (Lee and Scael, 2009). Using a fluorescent (FAD) detector, epicatechin was identified. Based on these results, it can be concluded that the FAD is sensitive for the determination of certain flavonoids (epicatechin) compared to UV/Vis detector which is in accordance with the literature (Donovan et al., 1999). Total phenolic content ranged from 0.53-118.19 mg GAE/g DE, and the highest yield was determined in extract obtained as infusion after 10 minutes. Flavonoid content ranged from 0.42-2.84 mg KE/g, with peak in microwave extract after 4 minutes. IC_{50} values (concentration of analyzed sample that is required for 50% inhibition) varied from 11.72-210.39 μg/mL for DPPH radical. The highest antioxidant capacity showed a sample obtained with 30% ethanol after 30 minutes. Testing the neutralization of the hydroxyl radicals, results showed that the IC_{50} values ranged from 11.44-57.86 μg/mL. The highest scavenging activity showed a sample obtained by ultrasound by extraction after 10 minutes. Examination of antioxidant activity by neutralization hydrogen peroxide indicates that the samples obtained by different extraction methods have shown IC_{50} values ranging from 0.41-52.45 μg/mL. The highest antioxidant activity, showed sample obtained by extraction with 30% ethanol for 30 minutes. Results of examination of antioxidant activity by the ability of inhibition of lipid peroxidase are summarized and IC_{50} values ranged from 1.41-46.99 μg/mL. Lowest IC_{50} (the highest antioxidant potential) corresponded to the sample obtained by the extraction with water for 30 minutes.

Conclusion

Basil extracts showed significant antioxidant activity. Sweet basil is a rich source of antioxidants that could contribute to its disease-fighting potential. Extracts with the strongest antioxidant capacity were obtained by ethanol (30%, v/v) maceration during 30 minutes and infusion during 10 minutes. Fluorescence detector of HPLC showed higher sensitivity for epicatechin determination than UV/Vis detector.

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Macedonian pharmaceutical bulletin, **62** (suppl) 483 - 484 (2016)
ISSN 1409 - 8695
UDC: 616-006.6-085.322
615.322:616-006.6
Short communication

Evidence-based research of plants used in cancer prevention or treatment

Snezana Cupara*, Ana Barjaktarevic, Olivera Milovanovic

Faculty of Medical Sciences, Pharmacy Department, University of Kragujevac, Svetozara Markovica 69, 34 0000 Kragujevac, Serbia

Introduction

Many plants have been traditionally used in folk medicine for cancer prevention or treatment. They may belong to food or medicinal plants. The research in last 10 years has concentrated efforts to identify molecular targets for these natural components. Several major cell-signaling pathways have been identified by which plant derived components influence carcinogenesis.

Carcinogenesis may affect: transcription factors (nuclear factor-kapa B (NF-kB), activator protein-1 (AP-1) and signal transducer and activator of transcription (STAT3), anti-apoptotic proteins (Bcl-2, Bcl-X1), pro-apoptotic proteins (caspases), protein kinases (mitogen-activated protein kinases (MAP)), cell cycle proteins (cyclins), cell adhesion molecules, cyclooxigenase-2 (COX-2), lipoxygenase (LOX) and growth factor pathway. A big variety of plants employs modulation of NF-kB activity as the main pathway for anticancer effect. NF-kB can be activated by free radicals, inflammatory stimuli, cytokines, endotoxins, radiation etc. This paper presents natural occurring components for which evidence-based research identified some molecular targets in cancer prevention and therapy (anethole capsaicin, curcumin, diosgenin, gambogic acid, gingerol, resveratrol, thymoquinone, ursolic acid, zerumbone) (Aggarwal and Shishodia, 2006; Aggarwal et al., 2008; Nobili, 2009; Rajput and Mandal, 2012).

Selected plants

Anethole and derivatives (dithiolethione, eugenol, isoeugenol) present in fennel, anise, and camphor increase intracellular level of glutathione and glutathione S-transferase. Eugenol and isoeugenol are antioxidants, affect lipid peroxidation and inhibit arachidonic acid - induced tromboxane B2, due to which these components are used in adenocarcinomas (Aggarwal and Shishodia, 2006). Anethole blocks NF-kB activation and affects inhibitory kappa B alpha kinase (IkBα). The suppression of carcinogenesis by anethole and derivatives is proposed to be by mediation of tumor necrosis factor (TNF) induced cellular responses (Aggarwal and Shishodia, 2006; Aggarwal et al., 2008).

Capsaicin presents anticancer effects in culture cells (human tumor cells) and in animal models (skin, colon, lung, tongue and prostate cancer). Capsaicin lead to accumulation of cells in G1 phase induced apoptosis and inhibited proliferation. The molecular mechanism included blocking TNF-mediated activation of NF-kB. In human multiple myeloma cells it also blocked the STAT3 activation pathway (Aggarwal and Shishodia, 2006; Aggarwal et al., 2008).

Active compound of curcumin is polyphenol diferuloylmethane. It down regulates COX-2 and LOX expression, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF), chemokines, cell surface adhesion molecules, growth factor receptors (epidermal growth factor receptor - EGFR and human epidermal growth factor receptor – HER2) and inhibits protein kinases (Aggarwal and Shishodia, 2006; Aggarwal et al., 2008).

Diosgenin, as a steroid saponin, is common for many different plants. It inhibits proliferation and straightens apoptosis in cell cultures (human colon cancer, osteosarcoma, leukemia, rheumatoid arthritis). The proposed molecular mechanisms are disruption of Ca2+ homeostasis, release of apoptosis-inducing factors, modulation of caspase-3, COX-2 and LOX activity, binding to chemokine receptor. It can suppress osteoclastogenesis through inhibition of TNF-mediated activation of NF-kB (Aggarwal et al., 2008).

* snezanacupara@yahoo.com

Gambogic acid derived from resin of *Garcinia indica* inhibited gastric, hepatic breast and lung cancer. Some of the specific molecular mechanisms include inhibition of telomerase and telomerase reverse transcriptase mRNA expression and interaction with transferrin receptor. Since it inhibited the expression of gene products in apoptosis, proliferation and angiogenesis, which are all regulated by NF-kB it modulates on general level NF-kB pathway (Aggarwal et al., 2008).

Gingerol has been tested mostly in prostate, gastric and breast cancer. Topically applied it inhibited COX-2 expression by suppression of NF-kB (Aggarwal and Shishodia, 2006).

Resveratrol from grapes uses many different general molecular targets in apoptosis among which modulates activity of NF-kB and MAP kinases (Aggarwal and Shishodia, 2006).

Thymoquinone, a quinone from black cumin, suppresses proliferation of colorectal, ovarian, pancreatic and breast cancer, osteosarcoma, myeloblastic leukemia. It down regulates COX-2 and 5-LOX activity, iNOS, TNF and cyclin D1 which are all known to be regulated by NF-kB. Thymoquinone prevented tumor angiogenesis both in vitro and in vivo (Aggarwal et al., 2008).

Ursolic acid is a triterpenoid present in many plants in different amounts, but considerably in rosemary. It suppresses expression of genes regulated by NF-kB, COX-2, LOX, iNOS, matrix metaloproteinase. It induces apoptosis by inhibition of DNA replication, inhibition of protein tyrosine kinases, activation of caspases and induction of Ca^{2+} release (Aggarwal et al., 2008).

Zerumbone, isolated from essential oil of wild ginger, has been effective in colon, breast and skin cancer. It inhibits activation of NF-kB and NF-kB-regulated gene expression. It also suppresses superoxide and nitric oxide generation and down-regulates COX-2, interleukin (IL-1) and TNF (Aggarwal et al., 2008).

Conclusion

Plants known as spices, general food or medicinal plants have been investigated for anticancer activity. It was found that they target different molecules in carcinogenesis. Therefore, the contribution to antitumor effect of a single plant may be due to synergic action of all active signaling pathways that the plant exert.

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Macedonian pharmaceutical bulletin, **62** (suppl) 485 - 486 (2016)
ISSN 1409 - 8695
UDC: 582.771.714:615.272(497.11)"2013"
Short communication

Chemical profiling and antioxidant activity of *Sorbus intermedia* (Ehrh.) Pers fruit extracts and jam

Zorica Mrkonjić[1]*, Jelena Nađpal[1], Branislava Rakić[2], Marija Lesjak[1], Ivana Beara[1]

[1]*University of Novi Sad, Faculty of Science, Trg Dositeja Obradovica 3, 21000 Novi Sad, Serbia*
[2]*University of Novi Sad, Faculty of Medicine, Hajduk Veljkova 3, 21000 Novi Sad, Serbia*

Introduction

Nowadays, food industry has a dual role: to provide required nutrients and to have favorable impact on humans' health, protecting them from many chronic diseases. In the lights of that, biological active compounds deriving from plants are attracting more and more attention. Fruits from *Sorbus* genus are commonly consumed fresh or as traditionally prepared preservatives, such as jam, juice, jelly or wine. The same are also widely used as remedy in a treatment of anemia, dyspepsia rheumatism and many digestive disorders. In spite of the widespread use in diet, preservatives preparation, beverage manufacture and the traditional medicine, there are still very few reports concerning their detailed phenolic profile and biological activities. Namely, within *Sorbus* genus, the most renowned species are *S. aucuparia* and *S. domestica* which are the most utilized as food. On the other hand, other species from *Sorbus* genus, such as *S. intermedia* (Swedish Whitebeam) are quite unexplored and the literature data are also very limited. Overall, plant phenolics are recognized as phytonutrients with significant biological activities which are generally based on their ability to prevent oxidative stress and minimize oxidative cell injury. Thus, the aim of this study was a detailed examination of 44 plant phenolics using LC-MS/MS technique and antioxidant potential of *S. intermedia*.

Materials and methods

Plant material was collected in November 2013, in Novi Sad, Republic of Serbia. Five extracts of fruits were prepared: water extract of fresh (WF) and air-dried sample (WD), methanol extract of fresh (MF) and air-dried sample (MD) as well as extract of traditionally prepared jam (J). For preparation of methanol extracts, 30 g fresh or air-dried fruits were grinded and extracted by maceration with 80% aqueous methanol (1 mL of solvent/ 0.1 g of plant material), constantly shaken at 120 rpm/min during 72 h at room temperature. For preparation of water extracts, 30 g fresh or air-dried fruits were grinded and extracted by maceration with boiling, distilled water (1 mL of solvent/ 0.1 g of plant material), constantly shaken at 120 rpm/min during 1 h at room temperature. After filtration, the solvents were evaporated in vacuum at 40 °C. Crude residues were dissolved in hot, distilled water (10 mL/g). In order to remove the non-polar compounds, the extracts were washed exhaustively with petroleum ether (fraction 40−60 °C) and concentrated to dryness under vacuum, yielding 9.84%, 26.87%, 6.84% and 31.59% for MF, MD, WF and WD extracts of *S. intermedia* fruits, respectively. Jam was prepared according to Serbian traditional recipe by cooking fruits in boiled water and crushing them during stirring. Afterwards, cooked fruits were sieved in order to remove seeds. The obtained product corresponds to definition of fruit purée. Further, in order to prepare jam the purée was boiled and 350 g of sugar per 1 L of purée was added. The mixture was stirred until appropriate consistency of jam. In order to prepare jam extracts, jam of *S. intermedia*, was weighed out (10 g) and evaporated in vacuum at 40 °C. Crude residues of jam extract were dissolved in hot, distilled water (10 mL of water/ 1g of crude residue). Extract was then filtered and evaporated in vacuum at 40 °C yielding 33.08%. Dried extracts of methanol, water and jam were dissolved in distilled water to obtain 300 mg/mL stock solutions for evaluation of the total phenolic and flavonoid content, as well as antioxidant activity. Additionally, dried extracts were dissolved in distilled water for LC-MS/MS analysis to obtain 20 mg/mL stock solutions. Determination of selected phenolics in ex-

* zoricamrkonjic@gmail.com

tracts of *S. intermedia*, was performed according to previously reported procedure (Orčić et al., 2014). For estimation of total phenolic and total flavonoid content method reported by Beara et al., (2014) was conducted. In order to examine antioxidant activity of *S. intermedia* several different *in vitro* assays were performed. The scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH), super oxide anion ($O_2^{\bullet-}$), nitric oxide (NO) and hydroxyl ($HO^{\bullet}$) radical, reducing power (FRAP assay) were tested according Beara et al. (2014).

Results and discussion

Presence of seven phenolics was confirmed using LC-MS/MS analysis, with chlorogenic acid being the most abundant compound particularly in MF extracts. Furthermore, we detected presence of selected flavonols such as, amentoflavone, epicatechin and quercetin-3-*O*-glucoside in *S. intermedia* fruit extracts, while there was no prior study which specifically identified mentioned compounds. The total phenolic content in extracts of *S. intermedia* fruits ranged from 0.10-5.28 mg GAE/g of dw. It could be generally concluded that the highest content of total phenolics were detected in air-dried extracts. Concerning the overall flavonoid content, similar amounts were found in all extracts. Having in mind that this is the first comprehensive study of phenolic compounds in *S. intermedia* fruits, the obtained results are significant for further research and could support their usage as food supplement with heath benefits.

Generally, the methanol extracts, especially fresh fruits showed the highest antiradical activity in all tests performed. Testing the neutralization of the hydroxyl radicals, results showed that the IC_{50} values ranged from 0.18-0.88 mg/mL. Methanol extract of fresh and air-dried fruits demonstrated a significant, same as standard antioxidant BHT, $HO^{\bullet}$ scavenger activity. These facts are particularly important because $O_2^{\bullet-}$ react fast with other free radicals, such as $NO^{\bullet}$, and can cause biological damage occurring in many human diseases. Interestingly, jam extract was good as methanol extract of fresh fruit in neutralisation of DPPH radical, had a higher reducing power of extracts and was more active in neutralisation of $NO^{\bullet}$ radical than water extracts.

Conclusion

Overall, according to the results presented it could be noted that extracts exhibited some antioxidant activity and jam showed to be rich source of antioxidants in everyday diet. Also, presented results firmly support everyday consumption of *Sorbus intermedia* fruits as food with valuable health benefits.

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Macedonian pharmaceutical bulletin, **62** (suppl) 487 - 488 (2016)
ISSN 1409 - 8695
UDC: 582.929.4-119.2(497.7)
Short communication

Chemical composition of volatile aroma compounds in fresh and dried spontaneous and cultivated rosette leaves of *Sideritis scardica* from R. Macedonia

Bujar Qazimi*, Gjoshe Stefkov, Marija Karapandzova, Ivana Cvetkovikj, Svetlana Kulevanova

Institute of Pharmacognosy, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Mountain tea (*Sideritis scardica*) is endemic to the Balkan Peninsula and is distributed in R. Macedonia, Bulgaria, Turkey, Albania and Greece (Obon de Castro, 1994). Flowering parts of *S. scardica* are widely utilized in Mediterranean folk medicine in the form of a decoction or infusion. Various extracts of *S. scardica* are rich with flavonoids, phenolic acids, terpenes, phenylpropanoids (Karapandzova et al., 2013; Petreska et al., 2011) and show complex mineral composition (Karapandzova et al., 2013). However, the specific and particular aroma is maybe the most important reason for the wide use of the plant (Qazimi et al., 2014a). Rosette leaves of mountain tea traditionally are not used (Qazimi et al., 2014b), although characterized by specific aroma. There are no data about the volatile aroma compounds in the rosette leaves. To analyze these compounds a refined method of headspace sampling hyphenated with GC/FID/MS analysis can be utilized (Watson, 2005).

Therefore, the aim of this work was the determination of the volatile aroma compounds in the fresh and dried spontaneous and cultivated rosette leaves of *S. scardica* using a headspace (HS) method with GC/FID/MS.

Materials and methods

Plant material: The rosette leaves of *S. scardica* were collected in three different localities in R. Macedonia during the summer of 2012. The spontaneous samples were collected from Ljuboten (Shara Mountain) and Gurgurnica (Suva Gora). One cultivated sample was collected from Dren (near Prilep). The plant material was air dried, packed in paper bags and kept in a dark and cold place until analysis.

Analyses of aroma compounds

GC and GC-MS analyses: 0.3 g of fresh or dried rosette leaves was put in sealed vials, warmed for 5 min. and the gas phase (highly volatile compounds) was investigated on Agilent 7890A Gas Chromatography system equipped with flame ionization detector (FID) and Agilent 5975C Mass Quadrupole detector as well as capillary flow technology which enable simultaneous analysis of the sample on both detectors. HP-5ms (30 m x 0.25 mm, film thickness 0.25 μm) capillary column was used. Operating conditions were as follows: oven temperature 60 °C, 20 °C/min to 280 °C; helium as carrier gas at a flow rate of 1 mL/min; injector temperature 260 °C and FID temperature 270 °C. 1000 μL of gas phase was injected at split ratio 1:1. The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 230 °C, transfer line temperature 280 °C and mass range from 50-500 Da. The MS was operated in scan mode.

Headspace method: Incubation temperature 80 °C; incubation time 5 min; syringe temperature 85 °C; agitator speed 500 rpm.

Identification of components: Identification of the components was made by comparing the mass spectra of components with those from NIST, Wiley and Adams mass libraries, by AMDIS (Automated Mass Spectral Deconvo-

* bqazimi2003@yahoo.com

lution and Identification System) and by comparing literature and estimated Kovat's (retention) indices that were determined using a mixture of a homologous series of normal alkanes from C_9 to C_{25} in n-hexane, under the same conditions. The percentage ratio of the components was computed by the normalization method of the GC/FID peak areas and average values were taken.

Results and discussion

Total of 21 and 24 individual components were identified in the fresh spontaneous (f-Ss) and in the fresh cultivated (f-Sc) rosette leaves of *S. scardica*, representing 96.62-100% and 98.05% of the total content, respectively. Data analysis of the chemical composition revealed five different classes of components: monoterpene hydrocarbons (MH) 40.51-41.97% and 48.49%, oxygen containing monoterpenes (OM) 3.65-4.17% and 4.46%, sesquiterpene hydrocarbons (SH) 42.07-47.66% and 30.31%, oxygen containing sesquiterpenes (OS) 0.57-3.12% and 0.18%, alcohols (AL) 4.54-8.36% and 14.21%, respectively. The prevailing components in f-Ss and f-Sc samples were: β-pinene (21.15-24.79% and 27.63%), α-pinene (11.42-12.69% and 14.09%) and limonene (3.40-7.49% and 6.53%) from the MH fraction; followed by the SH: germacrene D (21.05-21.82% and 14.53%) and *trans*-caryophyllene (10.86-13.00% and 9.76%), and the alcohol, 1-octen-3-ol (4.54-8.36% and 14.21%). The representatives from the OM and OS fractions were considered as minor constituents (< 2%).

Total of 14 and 16 individual components were identified in the dried spontaneous (d-Ss) and in the dried cultivated (d-Sc) rosette leaves of *S. scardica*, representing 89.36-96.81% and 98.37% of the total content, respectively. MH formed 40.32-55.43% and 92.43%, OM 2.99-26.16% and 1.72%, SH 17.6-23.59% and 4.22%, respectively. The OS fraction was found only in the d-Ss samples, 10.35-12.73%. The most abundant components in d-Ss and d-Sc samples were monoterpene hydrocarbons: β-pinene (21.25-23.12% and 38.89%), α-pinene (19.07-19.81% and 33.05%) and limonene (up to 6.68% and 13.01%). The higher abundance of OM: *trans*-pinocarveol (2.99-10.03%), myrtenal (up to 6.37%), pinocarvone (up to 5.09%), SH: germacrene D (4.91-9.29%), *trans*-caryophyllene (4.34-5.90%), δ-cadinene (3.03-5.93%), α-copaene (2.47-5.32%) and OS: caryophyllene oxide (6.76-9.35%) was specific only for the dried spontaneous samples of rosette leaves. The number of components found in d-Sc was larger than in d-Ss. Also, the share of the classes of components in d-Sc (MH > SH > OM) differed from d-Ss (MH > OM > SH > OS).

Conclusion

Generally, f-Sc and d-Sc contain larger amount of monoterpenes, but smaller amount of sesquiterpenes than f-Ss and d-Ss. Only minor differences were revealed in the qualitative composition of the aroma volatiles between spontaneous and cultivated rosette leaves of *S. scardica*. Furthermore, there was almost no difference in the chemical profiles of the aroma components between fresh and dried rosette leaves, except 1-octen-3-ol, that was present only in fresh rosette leaves of *S. scardica*.

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Macedonian pharmaceutical bulletin, **62** (suppl) 489 - 490 (2016)
ISSN 1409 - 8695
UDC: 547.56:582.929.4(497.7)
Short communication

Comparison of phenolic compounds between spontaneous and cultivated flowering stems of mountain tea (*Sideritis scardica* Griseb.) from R. Macedonia

Bujar Qazimi[1]*, Jasmina Petreska-Stanoeva[2], Gjoshe Stefkov[1], Marina Stefova[2], Svetlana Kulevanova[1]

[1]*Institute of Pharmacognosy, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University Ss. Cyril and Methodius, Arhimedova 5, 1000 Skopje, R. Macedonia*

Introduction

Sideritis scardica Griseb. is wild, hardy flowering perennials, known as mountain tea and is often used for preparing teas to cure common cold, to alleviate sinus congestion, aches, pains and viruses, including flu (Karapandzova et al., 2013). The important role of *S. scardica* as traditional remedies tea and its conservation status has required its cultivation as market production (Kostadinova et al., 2008). Methanol and boiling water extracts (by domestic infusion procedure) of *S. scardica* showed that extracts were rich in bound forms of phenolics such as hydroxycinnamic acids, phenylethanoid glycosides and flavonoid glycosides, and showed very high antioxidant capacity (Petreska et al., 2011a). Phenolic compounds have several roles in the plants physiological processes and have demonstrated significant health beneficial effects (Petreska et al., 2011b).

Therefore, the aim of this work was the determination of the phenolic compounds in the methanolic extracts of spontaneous and cultivated flowering stems of *S. scardica* using LC/DAD/ESI-MS^n.

Materials and methods

Plant material: The flowering stems of *S. scardica* were collected in four different localities in R. Macedonia during the summer of 2013. The spontaneous samples (Ss) were collected from Ljuboten (Shara Mountain), Gurgurnica (Suva Gora) and Lazaropole (Bistra Mountain). One cultivated sample (Sc) was collected from Dren (near to Prilep). The plant material was air dried, packed in paper bags and kept in a dark and cold place until analysis.

Extraction of phenolic compounds: 0.2 g of powder plant material (homogenized samples from flower, leaf and stem) was extracted with 25 mL of 70% methanol, 30 min using US bath. The supernatant was filtered through 0.45 µm pore-size polyethersulfone filter before analysis.

LC/DAD/ESI-MS^n analysis: Chromatographic separations were carried out on 250 mm x 4.6 mm, 5 µm C18 Luna column (Phenomenix). The mobile phase consisted of two solvents: water - formic acid (1% v/v) (A) and methanol (B). A linear gradient starting with 25% B was installed to reach 30% B at 7 min, 45% B at 30 min, 50% B at 50 min and 100% B at from 55 to 60 min. The flow rate was 0.5 mL/min to 50 min and 0.8 mL/min from 50 min to 65 min, the injection volume 10 µL. The HPLC system was equipped with an Agilent 1100 series diode array and mass detector in series (Agilent Technologies, Waldbronn, Germany).

Spectral data from all peaks were accumulated in range 190-600 nm and chromatograms were recorded at 290 and 300 nm from glycosides and acylated derivatives and at 330 nm for phenylethanoid glycosides and hydroxycinnamic acid. The mass detector was a G2449A Ion-Trap Mass Spectrometar equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Ag-

* bqazimi2003@yahoo.com

ilent, v.6.1.). Nitrogen was used as nebulising gas at pressure of 65 psi and the flow was adjusted to 12 L/min. The heated capillary and the voltage were maintained at 325 ºC and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan covered the mass range at m/z 100-1200. Collision–induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 5, respectively.

The identification and peak assigmentation of all phenolic compounds was based on comparison of their retention times and mass spectral data with those of standards and published dates. Hydroxycinnamic acids were quantified using 5-caffeoylquinic acid external standard at 330 nm, phenylethanoid glycosides were quantified and expressed as verbascoside equivalent at 330 nm, hypolaetin glucosides were quantified with 4'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside at 290 nm, whereas isoscutellarein glucosides were quantified and expressed as isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2) glucoside equivalent at 300 nm. The stock solutions of phenolic standards were made up in 70% methanol to a concentration of 1000 µg/mL. The corresponding calibration curves were constructed with five dilutions of the stock solutions.

Results and discussion

Phenolic compounds in the Sideritis extracts were identified by their UV spectra, their deprotonated molecular ions and their corresponding ion fragments, by using LC/DAD/ESI-MSn. Total of 30 and 19 individual components were identified in the methanolic extracts of spontaneous (Ss) and in the cultivated (Sc) flowering stems of S. scardica, representing 45.71-100.97 mg/g DW and 49.46 mg/g DW of the total content, respectively. Phenolic compounds in Ss and Sc were classified into four groups: hydroxycinnamic acids derivatives (4 and 3), phenylethanoid glycosides (8 and 5), flavonoid 7-O-diglucosides (5 and 4) and flavonoid acetylglucosides aglycones (13 and 7).

The total amount of hydroxycinnamic acid derivatives in Ss and Sc extracts ranged from 2.80-5.31 mg/g and 2.28 mg/g, respectively. 5-Caffeoylquinic acid was found in all samples and it was dominant hydroxycinnamic acid. Phenylethanoid glycosides (PHEG) were the abundant group of polyphenols in the studied samples with the content ranging from 17.92-59.39 mg/g in Ss and 11.68 mg/g in Sc. Verbascoside (7.07-24.00 mg/g), lavandulfolioside (4.77-14.92 mg/g), jonaside A (0.75-6.54 mg/g), allysonoside (1.23-6.40 mg/g), forsythoside A (1.03-4.01 mg/g) and leucoseptoside A (1.32-3.00 mg/g) were the most abundant compounds in Ss samples and represent around 90% of total PHEG content. Verbascoside (6.77 mg/g) and lavandulfolioside (2.47 mg/g) were the major compounds in Sc sample. Total content of flavonoid glycosides (non acetylated and acetylated) in Ss and Sc ranged from 24.75-42.00 mg/g and 35.49 mg/g, respectively. The prevailing components in Ss and Sc samples were isoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside (2.00-5.47 mg/g and 12.89 mg/g), 3'-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)glucoside (2.89-10.76 mg/g and 10.34 mg/g), hypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2) glucoside (2.44-3.79 mg/g and 1.13 mg/g) and apigenin 7-(6"-p-coumaroylglucoside) (1.72-2.52 mg/g and 2.61 mg/g), respectively. 4'-*O*-Methylhypolaetin 7-*O*-[6‴-*O*-acetyl]-allosyl-(1→2)-[6″-*O*-acetyl]-glucoside (3.34-8.78 mg/g) was the major component only in Ss samples.

Conclusion

Generally, the number of phenolic components found in Ss was larger than in Sc. The differences in total phenolic content between spontaneous and cultivated samples are directly correlated with differences in phenylethanoid content.

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Macedonian pharmaceutical bulletin, **62** (suppl) 491 - 492 (2016)
ISSN 1409 - 8695
UDC: 635.649:543.422
Short communication

Spectral analysis of extracts from red hot pepper (*Capsicum annuum* L.)

Jana Simonovska[1], Denitsa Yancheva[2], Bozhana Mikhova[2]*, Žejko Knez[3], Mateja Primožić[3], Zoran Kavrakovski[4], Vesna Rafajlovska[1]

[1]*Institute of Organic Technology, Faculty of Technology and Metallurgy, University "Ss. Cyril and Methodius", Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev 9, 1113 Sofia, Bulgaria*
[3]*Laboratory of Separation Processes and product design, Faculty of Chemistry and Chemical Engineering, University in Maribor, Smetanova 17, 2000 Maribor, Slovenia*
[4]*Institute of applied chemistry and pharmaceutical analyses, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

Pepper is an excellent source of proteins, vitamins, minerals, fats and oils, phenolic compounds, aromatic substances and other biologically active compounds (Campos et al., 2013). The importance of the red hot pepper varieties and their oleoresin extracts in the food and pharmaceutical industry is caused by the characteristic compounds, capsaicinoids and carotenoids (Guzman et al., 2011). Mainly, for determination of the compounds in the sweet or hot pepper varieties, the chromatographic methods (TLC, HPLC and GC) and UV-VIS spectrometric method were used (Davis et al., 2008; Othman et al., 2011). On the other hand, ^{1}H NMR spectroscopy has been extensively used to provide information about the composition and relative content of fatty acid units in triglycerides (Barison et al., 2010). For analysis of the capsaicinoids and carotenoids, NMR spectroscopy is one of the most informative methods applied (Gómez-Calvario et al., 2015). The degree of unsaturation of vegetable oils can be effectively studied by IR spectroscopy based on the changes observed in the frequency data of some bands and in the ratios of absorbances of the IR spectra (Vlachos et al., 2006). Therefore, the aim of this study is to evaluate the possibility of applying spectroscopic techniques (NMR and IR) in the characterization of extracts obtained from red hot pepper.

Materials and methods

Extracts from the pericarp, placenta, seeds, and stalk of red hot pepper (*Capsicum annuum* L., ssp. microcarpum longum conoides, convar. Horgoshka) obtained by extraction with n-hexane using Soxhlet method and supercritical carbon dioxide were analyzed using NMR and ATR-IR spectroscopy. The NMR spectra were run on a Bruker AVANCE II+ 600 spectrometer at ambient temperature. About 15 mg of each sample were dissolved in CDCl3. TMS was used as an internal standard. The ATR-IR spectra were measured in the middle IR region 600-4000 cm^{-1} on a Brucker Tensor 27 FT spectrometer. The samples were directly deposed on diamond crystal ATR accessory and spectra were recorded by accumulating 64 scans at resolution of 2 cm^{-1}.

Results and discussion

NMR Spectra. It was shown that the samples of extracts from the seeds contain exclusively triglycerides (TG). The calculated percent of unsaturated fatty acids was estimated about 80%. The estimated linoleic:oleic acid ra-

* bozhana@orgchm.bas.bg

tio was 2:1. N-polyunsaturated fatty acids (linolenic acid) were not observed. In this way the composition of TG in the seeds was shown to be similar to the corn and soybean oil. Traces of capsaicinoids were detected, while quantitative determination was not possible. Carotenoids were not detected. The spectra of the samples from pericarp showed similar composition as these from the seeds. Additionally, the presence of about 25% of n-polyunsaturated fatty acids was detected. In the samples from the placenta a substantial amount of capsaicinoids was presented. The proportion of TG:capsaicinoids was 1:2. In the region delta about 1.2-1.4 ppm intense signals appear for long CH_2 chains, probably waxes. In the stalk samples intensive signals were obtained due to presence of waxes. The proportion of the TG:capsaicinoids was approximately 1:0.15.

ATR-IR Spectra. The oil composition affects the exact position of the band for the C-H stretching of the *cis*-double bond, and yields higher-frequency shift when the oil has higher content of polyunsaturated acyl groups (Vlachos et al., 2006). Furthermore, the ratio of the absorbance of the bands responsible for the C-H stretching of the *cis*-double bonds and the asymmetric C-H stretching the methylene bonds could be used for quantitative estimation of the degree of unsaturation (Vlachos et al., 2006). In the ATR-IR spectra of the extracts of *Capsicum annuum* the band for the C-H stretching of the *cis*-double bond typical for vegetable oils rich in linoleic acid, such as soybean and corn oil, was found at 3009 cm^{-1}. According to the ratio of the absorbance at 3009 and 2923 cm^{-1}, the highest degree of unsaturation was found for the seed extracts. Also, the ATR-IR technique provides a fast and reliable identification of capsaicinoids in *Capsicum annuum* extracts. The typical frequencies of the amide C=O stretching and amide N-H bending vibration of capsaicin do not overlap with the IR bands of triglycerides, and therefore allow identification of capsaicinoids even at low concentrations. Among the studied extracts, those obtained from placenta had the highest content of capsaicinoids. However, for exact determination of the capsaicinoids content a calibration curve based on a series of standard mixtures with known amount of triglycerides and capsaicin is required.

Conclusion

Extracts from the pericarp, placenta, seeds, and stalk of red hot pepper (*Capsicum annuum* L., ssp. microcarpum longum conoides, convar. Horgoshka) were studied by NMR and IR spectroscopy. It was shown that both spectral techniques provide useful information on the triglyceride content and degree of unsaturation of the red hot pepper extracts. The IR spectroscopy could serve as a fast tool for identification of capsaicinoids in the extracts, while the NMR analysis could be successfully applied for determination of the proportion tryglycerides:capsaicinoids.

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Macedonian pharmaceutical bulletin, **62** (suppl) 493 - 494 (2016)
ISSN 1409 - 8695
UDC: 635.649.076:581.192]:615.277
Short communication

Molecular mechanisms of capsaicin mediated cytotoxic activity

Viktorija Maksimova[1]*, Zorica Arsova Sarafinovska[2], Liljana Koleva Gudeva[3]

[1] *Faculty of Medical Sciences, University "Goce Delcev", Krste Misirkov bb, 2000, Stip, R. Macedonia*
[2] *Institute for Public Health of the Republic of Macedonia, 1000, Skopje, R. Macedonia*
[3] *Faculty of Agriculture, University "Goce Delcev", Krste Misirkov bb, 2000, Stip, R. Macedonia*

Introduction

In the last few decades, capsaicin has exerted potent anti-cancer properties by enouncing anti-proliferative, apoptotic and anti-metastatic activity. To the best of our knowledge, the specific cell targets and successive mechanism of cytotoxic activity of capsaicin in different type of malignant cells, still remains unclear and contradictory. Therefore, this mini review, explains some of the most significant chemopreventive mechanisms of the action of capsaicin at a cellular level, reported in the recent literature.

The specific cytotoxic activity of capsaicin is usually a result of targeting of capsaicin toward two aims: TRPV1 (transient receptor potential cation channel subfamily V member 1) and tumor associated NADPH-oxidase. Some researchers reported that capsaicin provokes its anticancer activity through the interaction with TRPV1 receptor (Kim et al., 2006). Amantini et al., (2007) reported that capsaicin elicits apoptosis in U373 glioma cells, because the TRPV1 receptor was highly expressed and oppositely the cytotoxic effect in U87 cell line in which the TRPV1 receptor was very low expressed, capsaicin did not show cytotoxic effects (Amantini et al., 2007). Hu et al., (2008) have shown that TRPV1 receptor is included in capsaicin induced Ca^{2+} influx, generation of reactive oxygen species (ROS), depolarization of the mitochondrial membrane, and ultimately cell death on the synovial fibroblasts in rats. On the other side, capsaicin is one of the molecules which could inhibit the activity of tumor associated NADPH-oxidase, which is related to the inhibition of proliferation of cancer cells (Hedges et al., 2003).

The ability of capsaicin to inhibit the growth of different cancer cells is primary mediated by its ability to induce apoptosis. Apoptosis represents a type of programmed cell death, which is one of the physiological mechanisms for maintaining the homeostasis in the organism. It has been reported that two different pathways are mainly mediating the process of activation of apoptosis. First one is extrinsic pathway which is accomplished by activating of the "death receptor", and the other is intrinsic pathway which is followed by activation of cascade of caspase enzymes (Chou et al., 2007).

Extrinsic pathway

The extrinsic mechanism of apoptosis is characterized by activation of the external cell surface receptors, namely TRAIL (Tumor necrosis factor (TNF)-related apoptosis-inducing ligand) and DR (death receptor, Fas/CD95), leading to downstream caspase-mediated apoptosis (Codesido et al., 2014). These receptors can be activated by a signal that activates the enzymes procaspase 8 and 3, and therefore consequently triggers the apoptosis of cells. The number of studies, which included the extrinsic pathway into the mechanism of capsaicin mediated apoptosis, is much lower than numbers of reported studies which indicated the intrinsic pathway as the main mechanism of apoptosis.

Moon et al., 2012 notified that capsaicin induced the surface expression of TRAIL-receptor D5 through the activation of SP1 due to a calcium influx-dependent SP1 (specific protein 1) in kidney cancer cells. In multiple malignant glioma cells, subtoxic concentrations of capsaicin sensitized TRAIL-induced apoptosis mediated through ER Stress proteins CHOP/GADD153. DR5 and surviving contribute to amplification of the caspase cascade, thus restoring TRAIL sensitivity (Kim et al., 2010).

* viktorija.maksimova@ugd.edu.mk

Intrinsic pathway

The mechanism of intrinsic pathway of apoptosis is distinguished by intracellular activation of apoptosis through intracellular stressors, in which the most common are reactive oxygen species (ROS) and excess intracellular calcium. Disruption of the mitochondrial membrane and alterations in bcl-2, Bax, bcl-xL, and bad proteins, can cause a release of cytochrome C in cell cytosol and subsequent activation of the caspase cascade. These processes are the initial triggers that are eventually leading to apoptosis (Zhang et al., 2008). Cytochrom C together with some other factors can induce activation of caspase-9. Activated capsasa-9 can lead to activation of caspasa 3 and 7, which cleaves the inhibitor of caspasa activated DNAase and results in DNA fragmentation. Many studies examining the cytotoxic effects of capsaicin on prostate and other malignant cell lines, have reported that capsaicin induced apoptosis is linked to intrinsic mechanisms.

Pramanik et al., (2011) have evaluated the mechanism of capsaicin-mediated ROS generation in pancreatic cancer cells and they suggested that mitochondrial complex-I and III are involved in capsaicin mediated ROS generation. They demonstrated that capsaicin inhibited the enzymatic activity of antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione reductase, which resulted in oxidative stress.

According to Kryston et al., (2011), the agonistic effect of capsaicin on TRPV1, can evoke intracellular influx of calcium, which leads to further intracellular stress, activating apoptosis in various cell lines, namely prostate cancer. They found that the generation of ROS induced by capsaicin correlated with the dissipation of the inner mitochondrial transmembrane potential and the release of cytochrome-c into the cytosol. Activation of the caspase-3 cascade resulted in the cleavage of poly(ADP-ribose)polymerase (PARP) and resultant apoptosis.

Conclusion

A large number of investigators clearly demonstrated that capsaicin inhibits the growth of cancer cells by inducing apoptosis and cell cycle arrest, but its molecular mechanisms in some types of cancers are not well understood. Therefore, additional studies are required to elucidate and to supplement the missing part of this data.

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Macedonian pharmaceutical bulletin, **62** (suppl) 495 - 496 (2016)
ISSN 1409 - 8695
UDC: 615.322: 582.736.3-119.2
Short communication

Review of critical points in quality assessment of red clover dry extract (*Trifolium pratense* extractum siccum): quantitative composition and providing of a representative sample

Veljko Petrović*, Nada Pavičić, Ivan Velikinac, Tamara Miladinović

Pharmanova d.o.o., Industrijska 8, 11500 Obrenovac, Serbia

Introduction

Dietary supplements containing red clover (*Trifolium pratense*) extract are used to reduce the symptoms of menopause, due to high isoflavone content with estrogenic effect: daidzein, genistein, formononetin and biochanin A (Boothet et al., 2006).

A large number of manufacturers and suppliers are present in the market, offering extracts of different quality. This paper presents the results obtained by Quality Control division of Pharmanova, during the qualification and selection process of raw material suppliers. The requirement specified for the quality of the extract used in Oestrofact E capsules is: at least 40% of total isoflavones, calculated on a dried basis. After reviewing the official scientific literature, the testing of samples, sent by the manufacturers/suppliers was performed. Monographs for the herbal drug (Powdered Red Clover), herbal extract (Powdered Red Clover Extract) and final product (Red Clover Tablets), that were consulted, have been published by the U.S. Pharmacopoeia (USP36/NF31, 2013). The monograph for Powdered Red Clover Extract states the isoflavone content should be not less than 90% and not more than 110% of the declared amount, calculated on the dried basis as the sum of daidzein, genistein, formononetin and biochanin A content.

Materials and methods

Test samples of red clover dry extracts with appropriate documentation (specifications, certificates, production flow charts) were collected from three manufacturers. The samples were tested for the individual content of each isoflavone (daidzein, genistein, formononetin and biochanin A) as well as the total isoflavone content. One of the samples was also tested for homogeneity dependant on the sampling position (bottom, top, middle, centre, left, and right).

The method used for the identification and determination of isoflavones in red clover extract was developed in physico-chemical laboratory of Quality Control (QC) Pharmanova, and validated according to ICH guidelines. The technique used is HPLC with UV-VIS detector (254 nm). Separation is achieved using a Phenomenex® Luna C18 (2), 250 x 4.6 mm, 5 µm particle column, at isocratic elution at 1 mL/min, using the following mobile phase: acetonitrile: 0.5% phosphoric acid (40:60, V/V).

Results and discussion

Individual and total isoflavone content was determined in three test samples. The results presented in percentage (%, m/m) represent the average of two consecutive results of the same sample:

1. Test sample I: daidzein 0.52%, genistein 0.51%, formononetin: 19.99%, biochianin A 11.31%, total isoflavones 32.33%
2. Test sample II: daidzein 9.26%, genistein 11.29%, formononetin: 17.80%, biochianin A 3.66%, total isoflavones 42.01%
3. Test sample III: daidzein 3.38%, genistein 8.72%, formononetin: 15.53%, biochianin A 18.25%, total isoflavones 45.80%

In order to get information on the homogeneity, one of the extract was sampled from different positions of the

* veljko.petrovic@pharmanova.com

bulk package. The total isoflavone content was determined from each of the samples and the results were as follows, top: 43.38%; bottom: 44.21%; middle center: 43.36%; middle left: 42.77%; middle right: 43.87%. When the results were statistically processed, the relative standard deviation (RSD) was found to be 0.54%, while the recovery percentage in relation to the declared value was in the range from 98.25% to 101.65%.

The estrogenic activity of red clover extract originates from its isoflavones: genistein and diadzein (called soy isoflavons, due to high contents in soy products), and two structurally similar, methylated isoflavons, biochanin A and formononetin. In fact, Biochanin A is methylated genistein, and formononetin methylated daidzein, so after oral application and demethylation in the digestive tract, only genistein and daidzein are found as active metabolites in the organism (Toleson et al., 2002). What remains the question is whether demethylation can occur during one of the dry extract production phases, as well as later during storage and sample manipulation. Thus, during the quality assessment of the extract, the ratio between individual isoflavones should be considered. The U.S. Pharmacopoeia, within HPLC identification, sets requirements for quantitative ratio between two 5,7-dihydroxyisoflavones (biochanin A and genistein) and two 7-hydroxyisoflavones, to be between 0.1 and 10.0.

The information collected, concerning the quality of world renowned brands used in clinical research and available in electronic databases indicate that in these products more than 75% of total isoflavone content comes from biochanin A and formononetin, while soy isoflavons (genistein and daidzein) represent a significantly smaller percentage:

- Promensil® capsule, 43.5 mg: biochanin A 26 mg (60%), formononetin 16 mg (37%), genistein 1 mg (2%) and daidzein 0.5 mg (1%) (Knight et al., 1999)
- Rimostil® capsule, 28.6 mg: formononetin 27 mg (87%), biochanin A 2 mg (7%), genistein and daidzein in trace amounts (Schult et al., 2004)

When all is considered, the ratio of peaks from the chromatogram could indicate a possible attempt to falsify the extract. It is preferable for the peaks of formononetin and biochanin A to stand out in relation to ones coming from genistein and daidzein.

Conclusion

Test sample I contains satisfactory levels of formononetin and biochanin, but low total isoflavone content (< 40%).

Test samples II and III comply with requirements for total isoflavone content (> 40%), but high levels of genistein and daidzein can indicate that the extracts were not pure red clover.

Requirements for the ratio between pairs of related isoflavones (B + G / D + F) were met by all of the extract samples, in accordance with USP/NF31 (0.1 to 10.0).

The total isoflavone content results in samples taken from different positions of the bulk package show no statistically significant difference (RSD < 2 and recovery < ± 3%), which confirms the homogeneity of the tested extract. The sampling principles in accordance with Ph.Eur.8.0, chapter 2.8.20. Herbal Drugs: Sampling and Sample Preparation should be applied whenever possible.

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Macedonian pharmaceutical bulletin, **62** (suppl) 497 - 498 (2016)
ISSN 1409 - 8695
UDC: 582.661.15-119.2:615.281(497.7)
Short communication

Chemical composition and antimicrobial activity of *Chenopodium botrys* L. (Amaranthaceae) from Macedonian flora

Ljubica Adji Andov[1]*, Marija Karapandzova[1], Ivana Cvetkovikj[1], Gjose Stefkov[1], Ana Kaftandzieva[2], Svetlana Kulevanova[1]

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, "Majka Tereza" 47, 1000 Skopje, R. Macedonia.*
[2] *Institute of Microbiology with Parasitology, Medical Faculty, Ss. Cyril and Methodius University," 50 Divizija" 6, 1000 Skopje, Macedonia*

Introduction

The genus *Chenopodium* (Amaranthaceae) comprises numerous species of perennial and annual plants known as goosefoots, which occur anywhere in the world. Only few species produce essential oil (glandular goosefoots) usually with characteristic chemical composition. In Europe, glandular goosefoot is *Chenopodium botrys* L. (syn. *Dysphania botrys* (L.) Mosyakin and Clemants) known as Jerusalem Oak Goosefoot, native to the Mediterranean region. The plant has been used for treatment of catarrh and humoral asthma and is known as a good substitute for the traditionally known medicinal plant *Chenopodium ambrosioides* (Yadav et al., 2007).

C. botrys contains flavonoids, alkaloids and terpenoids. The characteristic odor of the plant is due to some monoterpenes and sesquiterpenes which were found to be responsible for the specific aromatic and fetid scent. The presence of some oxygen-containing sesquiterpenes was correlated with pronounced antibacterial and antifungal activity (Kokanova-Nedialkova et al., 2009). Studies on the flavonoids revealed presence of flavonols: chrysoeriol, quercetin, kaempferol and herbacetin and flavones: hispidulin, salvigenin, 5-methylsalvigenin, 7-methyleupatulin, sinensetin and jaceosidin (Morteza-Semnani, 2015).

Dried herbal part of *C. botrys* is used from local people in Republic of Macedonia (RM) for preparing infusions or liquid extracts with diuretic, antispasmodic, carminative and antidiarrheal properties. The aim of this study was determination of the chemical composition of Macedonian *C. botrys* and evaluation of its antimicrobial capacity.

Materials and methods

The aerial flowering parts (herba) of *C. botrys*, were collected in the period from July to September in 2012, 2013 and 2014 from five localities in RM: 1. Kozuf, 2. Pretor, 3. Strumica, 4. Zletovo and 5. Radovish. Collected plant material was air-dried and preserved in paper bags until analysis, when it was minced and homogenised appropriately.

The essential oil was isolated with steam distillation using all-glass Clevenger type apparatus according to the method listed in the monograph in the European pharmacopoeia.

Essential oil samples were analysed on Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector using previously published method (Adji Andov et al., 2014).

Hydrolyzed extract of *C. botrys* herba was prepared according to the method listed in the monograph in the European pharmacopoeia and were analyzed using an Agilent 1200 series HPLC system equipped with a G1315D photo-diode array detector, controlled by ChemStation LC 3D software. Chromatographic separations were carried out on a XDB-C18 Eclipse column (150 mm x 4.6 mm, 5 µm, Agilent Technologies, USA). Spectral data for all peaks were accumulated in the range of 190-400 nm and chromatograms were recorded at 260, 280, 330 and 370 nm.

Antimicrobial activity. Microbial strains: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10259 and clinical isolates from patients (*Enterococcus, Streptococcus pyogenes, Proteus mirabilis, Salmonella enteriti-*

* bube.petrovska@yahoo.com

dis) were used. Method: Disc diffusion method was used in order to determine the growth inhibition zones of the microorganisms. The results were compared to the antimicrobial activity of several antibiotic drugs. Preparation of extracts: methanol extracts (1 g/mL) were prepared using ultrasonic assisted extraction procedure.

Results and discussion

The results of the GC/FID/MS analysis showed that the main compounds of the essential oil of C. botrys herba were the sesquiterpenes: elemol acetate, saline-11-en-4α-ol, selina-3,11-diene-6α-ol and elemol, followed by lower content of α-eudesmol acetate, α-henopodiol, botrydiol and α-chenopodiol-6-acetate. These compounds represented 62.74-81.21% of the oil. Additionally more than 50 other compounds were identified in very low quantities or in traces. The monoterpene ascaridol was absent.

In hydrolyzed extract of *C. botrys herba*, hydroxycinnamic acid, chlorogenic acid and caffeic acid and its derivate were identified together with quercetin and isorhamnetin. Tentatively apigenin and luteolin were identified, based on comparison of the Rt and UV-DAD spectra with those of the authentic substances of apigenin and luteolin, used as referent flavonoids. The component that appeared in the HPLC chromatogram on Rt 24.7 min has UV-DAD spectra with UVmax on 348 nm and was assume to be herbacetin.

Methanol extracts of *C. botrys herba* showed promising antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes*, and no activity against *Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa* and *Proteus mirabilis*. Additionally the extract showed promising antifungal activity against *Candida albicans*. Obtained results are in accordance with the literature data.

The essential oil isolated from *C. botrys* from different regions in the world contains varying chemical composition where dominant components are the sesquiterpenes, mainly α- and β-chenopodiol, eudesma-3,11-dien-6α-ol, botrydiol, elemol, elemol acetate, α- β- and γ-eudesmol and new identified compound guaia-3,9-dien-11-ol, that was also identified in our oil. Some authors point out on higher presence of other sesquiterpenes such as α-cadinol, epi-α-muurolol, cubenol and E-caryophyllene, while some other identified higher percentages of monoterpenes γ-terpineol, β-myrcene, p-cymene, α-terpinene, limonene, and especially ascaridole which were reported in essential oils of *C. botrys* from Spain and Slovakia (Morteza-Semnani, 2015).

According Kokanova-Nedialkova et al. (2009) *Chenopodium* species contain phenol derivatives such as alcohols, aldexydes and glycosides (vanillic alcohol and vanillic acid, phenolic glycoside named chenoalbuside, cinnamic, sinapic and ferulic acid and their derivatives, hydroxycinnamic acylglycosides and many other phenolic compounds. In the group of flavonoids, quercetin, isorhamnetin, kaempferol and herbacetin and their glycosides were the only flavonols isolated from *Chenopodium* species, including *C. botrys*, besides highly metylated flavons: salvigenin, sinensetin, hispidulin and their derivatives.

Up to now, antimicrobial activity was tested only on the essential oil of *C. botrys*, which expressed significant bactericidal activity against selected strains of Gram (+) and Gram (-) bacteria, comparable to that of the reference antibiotics amicacin and cephotaxim (Kokanova-Nedialkova et al. 2009) and significant fungicidal activity against selected strains of Aspergillus niger and *Candida albicans*, comparable to that of the reference antibiotics nystatine and amphotericin (Tzakou et al., 2007).

Conclusion

In the essential oil chemical composition of Macedonian *Chenopodium botrys* as predominant compounds were determinate the oxygen-containing sesquiterpenes: elemol, eudesmol, chenopodiol and seline alcohols and their esters. The hydrolysed extract contained few phenolic acids, predominantly, cinnamic and caffeic acid and their derivates and quercetin, isorhamnetin, apigenin, luteolin and probably herbacetin. Methanol extracts show promising antimicrobial and antifungal activity. Further investigations are required for complete chemical identification of phenolic and flavonoid compounds as well as to reveal the antimicrobial and antifungal capacity of the plant extract, for further medicinal or commercial use.

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Macedonian pharmaceutical bulletin, **62** (suppl) 499 - 500 (2016)
ISSN 1409 - 8695
UDC: 582.661.15-119.2(497.7)
Short communication

The content of some biogenic elements in *Chenopodium album* L. and *Chenopodium botrys* L. (Amaranthaceae) from Macedonian flora

Ljubica Adji Andov[1]*, Marija Karapandzova[1], Gjose Stefkov[1], Ivana Cvetkovikj[1], Katerina Baceva[2], Trajce Stafilov[2], Svetlana Kulevanova[1]

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, University 'Ss. Cyril and Methodius', Mother Teresa 47, 1000 Skopje, R. Macedonia*
[2] *Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University 'Ss. Cyril and Methodius', Arhimedova 5, POB 162, 1001 Skopje, R. Macedonia*

Introduction

The chemical compounds present in the living organisms are composed of chemical elements. In these days almost 120 chemical elements are known and 30 of them create the living matter, therefore are known as biogenic elements. Plants provide themselves with elements from air, soil and water. They are present within the compounds or in the form of ions and in some special cases they are unbound (e.g. oxygen). According to their representation in organisms, the biogenic elements are divided into three groups: macro-biogenic, micro-biogenic and trace elements. Macro-biogenic elements (C, O, H, N, S, P, Na, K, Ca, Mg, Cl, Fe) have a building function, micro-biogenic elements (Cu, I, Mo, Mn, Zn, Co) are parts of enzymes and their function is catalytic, while trace elements (Al, As, B, Br, F, Li, Ni, Se, Si, Ti, V) are presented less than 0.001% and their biological role still remain undiscovered. The content of these elements vary a lot depending on many factors. Nevertheless, edible plant represent biological source of these elements for other living organisms including humans. Thus, *Chenopodium* spp. (Amaranthaceae) is being used as a leafy vegetable and subsidiary grain crop in different parts of the world due to its rich nutritional quality and its ability to grow in stress conditions.

The *Chenopodium* genus comprises numerous species of perennial and annual plants that grow as herbaceous plant or as shrubs and small trees. In the flora of the Republic of Macedonia (RM), 15 species of this genus occur including *C. botrys* and *C. album*. Dried herbal parts of *C. botrys* are used from local people for preparing infusions or liquid extracts with diuretic, antispasmodic, carminative and antidiarrheal properties while fresh leaves of *C. album* are known as substitute of some green leafy vegetables such as spinach. These two plant species have not been issue of chemical characterisation so far. Therefore, the aim of this study was determination of the content of selected biogenic elements of wild samples of *C. botrys* and *C. album*, collected from different localities of the Republic of Macedonia.

Materials and methods

Plant material (25 specimens), consisting of aerial flowering part (*herba*) and dried roots (*radix*) of two *Chenopodium* species (*C. album* and *C. botrys*), was collected from wild specimens on different localities in R. Macedonia, during 2012 and 2013. Collected plant material was air-dried and preserved in paper bags until analysis, when it was minced and homogenised appropriately.

For mineralization purposes 0.5 g of plant material were placed in a Teflon digestion vessels (OMNI/xp 1500), 7 mL (69%, m/V) HNO_3 and 2 mL H_2O_2 (30%, m/V) were added, the vessels were capped closed, and after 24 hours were placed in the rotor of the Mars microwave digestion (CEM, USA). The digestion was carried out on 180 °C in

* bube.petrovska@yahoo.com

two steps, first 25 min and then additional 15 min. The digests were filtered on filter paper (Munktell, Sweden), quantitatively transferred to 25 mL calibrated flasks, diluted with demineralised water and analyzed for the selected elements.

All analyzed elements (Ca, K, Mg, P, Na, Cu, Fe, Mn and Zn) were determined by the application of inductively coupled plasma atomic emission spectrometer (ICP-AES) (Varian, 715-ES) applying an ultrasonic nebulizer CETAC (ICP/U-5000AT+) for better sensitivity. The optimal instrumental parameters for this technique were published previously (Balabanova et al., 2010). All results were calculated on a dry mass basis (mg/kg d.m.). Samples were made in triplicates. Each data represents the mean ± 2 standard deviation of three samples.

Results and discussion

The two analyzed species of *Chenopodium* contained large amounts of potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P), which ranged from 961 mg/kg for P to 40907 mg/kg for K, in dried aerial flowering part (*herba*) of *C. botrys* and *C. album*. The most abundant macro-biogenic element in all analysed samples of *herba* was K (14136-40907 mg/kg), followed by Ca (7405-14146 mg/kg), Mg (1082-5913 mg/kg) and P (961-2752 mg/kg). The content of micro-biogenic elements in *herba* of *C. album* and *C. botrys* was much lower, ranging from 4.0 mg/kg for Cu to 863.1 mg/kg for Fe. The most abundant micro-biogenic element in the investigated samples of *herba* was Fe (33.3-863.1 mg/kg), followed by Na (14.6-606.0 mg/kg), Mn (9.4-177.8 mg/kg), Zn (11.8-27.9 mg/kg) and Cu (4.0-15.6 mg/kg).

The content of macro-biogenic elements in roots (*radix*) of *C. album* and *C. botrys* was lower when compared with the *herba* of both species, ranging from 589 mg/kg for P to 15839 mg/kg for K. The most abundant macro-biogenic element in all analysed samples from *radix* was K (14136-40907 mg/kg), followed by Ca (7405-14146 mg/kg), Mg (1082-5913 mg/kg) and P (961-2752 mg/kg). The content of micro-biogenic elements in *radix* of *C. album* and *C. botrys* was lower than in *herba*, ranging from 4.0 mg/kg for Cu to 863.1 mg/kg for Fe. Fe was also the most abundant micro-biogenic element in all investigated specimens of *radix* (33.3-863.1 mg/kg), followed by Na (14.6-606.0 mg/kg), Mn (9.4-177.8 mg/kg), Zn (11.8-27.9 mg/kg) and Cu (4.0-15.6 mg/kg).

According to literature data Bomkazi et al. (2013) found very high amount of K in young shoots and mature plant-leaves of *C. album*, (45799.3 mg/kg and 49028.6 mg/kg, respectively), while the contents of Ca and Mg were lower. Comparing to our results, the content of K, Ca and Mg was higher than in our Macedonian samples from *herba* of *C. album*. Smaller amounts of Fe (218.1 and 120.4 mg/kg), Zn (26.2 and 23.0 mg/kg) and Cu (14.0 and 9.1 mg/kg) were found also in young shoots and mature plant-leaves, respectively. In leaves of Indian *C. album* 452 mg/100 g K was found, followed by 150 mg/100 g Ca, and smaller amounts of Na, Mg, Zn, and Fe (43.0, 34.0, 24.0 and 4.2 mg/100 g, respectively) (Dey et al., 2013). Obtained results for Macedonian *C. album* were in accordance with literature data. According to literature, *C. botrys* was found to be very good bio-accumulator of heavy metals, especially for Fe, Mn, Zn and Cu, which content rich up to 4145, 175, 276 and 56 mg/kg, respectively (Malayeri et al., 2008).

Conclusion

The content of biogenic elements in investigated *Chenopodium* species (*C. album* and *C. botrys*) from Macedonian flora, showed that in both species the most abundant macro-biogenic element was K, in the dried aerial flowering part (*herba*) as well as in the dried roots (*radix*), followed by high amounts of Ca, Mg and P. Fe was predominant micro-biogenic element, present in higher amounts in the *herba* of *C. botrys*. Both species represent biological source of Mn, Zn and Cu. Differences in the contents of biogenic elements were high, depending on the harvesting locations. Further investigations are needed for analyzing the environmental influence on the contents of biogenic elements in *Chenopodium* species.

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Macedonian pharmaceutical bulletin, **62** (suppl) 501 - 502 (2016)
ISSN 1409 - 8695
UDC: 615.322:582.712-119.2
Short communication

Phytochemical study and antioxydant properties of Tunisian *Zizyphus lotus* L. extracts

Simu Georgeta-Maria*, Rădulescu-Grad Maria, Anca Dragomirescu, Bouani Bouthaina, Dehelean Cristina

"Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania
Institute of Chemistry Timisoara of the Romanian Academy, 24 Mihai Viteazul, RO-300223, Timisoara, Romania

Introduction

Zizyphus lotus L. is a medicinal plant which is found in many geographic areas, including the Mediterranean region, e.g. Tunisia. The different parts of this plant are frequently used in traditional medicine in order to treat several diseases such as skin infections, urinary troubles, insomnia and anxiety, digestive disorders, obesity, urinary troubles, diabetes, etc. (Bakhtaoui et al., 2014). Recent studies stipulate onto the anti-inflammatory, analgesic and anti-ulcerogenic activities of aqueous extracts of some parts of this plant on rodents, as well as on the immunomodulatory properties on human T-cell activation, and on IL-2 mRNA expression (Benammar et al., 2014).

In order to investigate more thoroughly onto the properties of *Zizyphus lotus* L., a study dealing with the qualitative evaluation of different methanolic extracts of this plant (originating from roots, stems, leaves and seeds), as well as of their antioxidant properties was carried on.

Materials and methods

All reagents were purchased from Merck and Sigma–Aldrich and were of analytical grade. Different parts (leaves, roots, stems and seeds) of *Zizyphus lotus* L. were collected from north-western Tunisia (Bousalem region) at different times of the year (March, September and October), in 2014. The vegetal materials were grounded into powder and were packed in polythene bags for further use.

The methanol extracts of different parts of the plant were prepared in a similar manner. An amount of ten grams (10 g) of ground samples (leaves, stems, root and seeds respectively) were extracted with 100 mL methanol in a Soxhlet extractor. Several extraction processes were carried on. The obtained extracts were filtered and then concentrated to dryness under vacuum. The residues were stored in a refrigerator at 4 °C until the time of extract use.

The qualitative evaluation of the different extracts of *Zizyphus lotus* was carried on by means of phytochemical screening (for alkaloids, saponnins, terpenes, tannins, quinones, and flavonoids) and thin layer chromatography, using a series of specific standards (catechin, rutine, quercitine, gallic and tannic acids). Determinations were carried out in accordance with procedures described in literature. In order to quantify the total phenol content, the Folin-Ciocalteu reagent was used. The antiradical activities of the methanolic extracts of *Zizyphus lotus* was estimated using the stable free radical 2,2-diphenyl-1-pycrylhydrazyl (DPPH), according to the method of Brand-Williams et al. (1995). Rutine and butyl hydroxyl toluene were used as positive controls.

Results and discussion

The phytochemical analysis of the methanol extracts of the different parts (leaves, stems, root and seeds respectively) of *Zizyphus lotus* revealed that all of these contain (in different amounts) saponnins, terpens, tannins and flavonoids. The roots, stems and seeds also contain sterols. Excepting the leaves, coumarins and alkaloids were detected only in traces. The obtained results showed that the fruit seeds should be considered a source of fatty acids, be-

* simu.georgeta@umft.ro

ing by far richer in saturated and poly saturated fatty acids than any other parts of the plant. In the case of the roots of *Zizyphus lotus*, the phytochemical results revealed a rich content in flavonoids, tannins and saponnins, compounds known for their anti-inflammatory and analgesic activity. The same active compounds (flavonoids, saponins, tannins and alkaloids) detected in the *Zizyphus lotus* leaves, could explain the analgesic effects of this plant and its use in folk medicine. The analysis of the thin layer chromatographic plates revealed the presence of polyphenols, catechin, rutine, quercitin, tannic and gallic acids in the different parts of *Zizyphus lotus*.

Regarding the antioxidant activity of the methanolic extracts originating from the different parts of *Zizyphus lotus*, it was found that the obtained efficient concentration values (IC_{50}) ranged from 4.713 to 6.704 µg/mL, being significantly lower as compared to the positive controls: butyl hydroxyl toluene (21.441 µg/mL) and rutine (13.762 µg/mL). So, one could notice that the studied extracts show an important antioxidant activity. Among all, the seeds methanol extract showed an antioxidant activity five times greater than butyl hydroxyl toluene's one, and three times greater that the rutine's one.

Conclusion

The phytochemical screening of the methanolic extracts originating from different parts of *Zizyphus lotus* L. revealed the presence of some active compounds such as saponnins, terpenes, tannins, quinones and flavonoids. This content in active compounds confirms the importance of *Zizyphus lotus* L. in folk medicine, which could be considered as a natural alternative to some synthetic products.

The methanol extracts of different parts of *Zizyphus lotus* L. showed an important antioxidant activity. According to the obtained efficient concentration values, one could conclude that the seed's methanolic extract has the highest antioxidant activity as compared to the leaves, stems and roots extracts, as well as to the positive controls (rutine and butyl hydroxyl toluene) used in this study.

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Macedonian pharmaceutical bulletin, **62** (suppl) 503 - 504 (2016)
ISSN 1409 - 8695
UDC: 582.475-113.55:615.281(497.7)
Short communication

Antimicrobial activity of Macedonian black pine

Marija Karapandzova[1]*, Ivana Cvetkovikj[1], Gjoshe Stefkov[1], Elena Trajkovska-Dokik[2], Ana Kaftandzieva[2], Svetlana Kulevanova[1]

[1]*University "SS. Cyril and Methodius", Institute of Pharmacognosy, Faculty of Pharmacy, Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*University "SS. Cyril and Methodius", Institute of Microbiology and Parasitology, Faculty of Medicine, 50 Divizija No.6, 1000 Skopje, R. Macedonia*

Introduction

Pines are among the most important and widespread forest trees as they occur in a different range of environmental and climate conditions (Naydenov et al., 2006). *Pinus nigra* Arnold or black pine is a moderately variable species which grows widely throughout Mediterranean and is attributed to Balkan Peninsula as well as Republic of Macedonia where this conifer could be found on several localities (Karadzica, Kozuf, Shara Mountain etc.).

Essential oils isolated from different pine species are widely used as fragrances in cosmetic industry, as flavoring additives for food and beverages and as scenting agents in a variety of household products and intermediates in the synthesis of perfume chemicals (Sezik et al., 2010). Since antiquity, they have been known to possess biological activity, most notably antibacterial, antifungal and antioxidant properties therefore are used in the ethno medical practice and in the folk medicine throughout the world. Moreover, pine oils are used for medical purposes in aromatherapy as carminative, rubefacient, emmenagogue and even as abortifacient agents. Additionally, the systematic and potential usefulness of essential oil studies has become increasingly important because of their growing interest in use in both the food and pharmaceutical industries (Kilic and Kocak, 2014). In this way, the chemistry and the biological activity of the essential oils isolated from different pine species have been intensively studied, particularly needles essential oils.

However, despite the abundant literature on this topic, there are no evident data about the chemistry and biological activity of Macedonian black pine, thus the main objective of the present study was to investigate the chemical composition of the essential oils isolated from needles and to access their antimicrobial activity against certain types of microorganisms in order to define possible application and therapeutic uses of these essential oils as antimicrobial agents.

Materials and methods

Plant material (terminal branches) was collected from two different localities in R. Macedonia (Kozuf and Nidze) and was dried at room temperature, for two weeks. Just before isolation, the needles were separated from the branches and were properly minced.

Essential oil isolation was made by steam distillation in all-glass Clevenger apparatus (Ph.Eur. 8, 2.8.12.). For that purpose, 20 g of minced needles were distilled for 4 hours. For purification purpose, anhydrous sodium sulfate was added to the isolated essential oil to remove residual water.

For GC/FID/MS analysis, the essential oil was dissolved in xylene to obtain 1 μl/ml oil solution.

Essential oil samples were analyzed on Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector according to previously published method by Karapandzova et al. (Karapandzova, 2014).

Antimicrobial activity of essential oils was studied against 14 different microorganisms, including 13 bacterial isolates representing both Gram-positive (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococ-*

* marijakarapandzova@yahoo.com

cus pyogenes and *Enterococcus*) and Gram-negative bacteria (*Acinetobacter* spp., *Escherichia coli* ATCC 25927, *Salmonella enteritidis, Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* and *Proteus mirabilis*) and one strain of *Candida albicans* ATCC 10231. Antimicrobial screening of all tested essential oils was made by disc diffusion and broth dilution method previously described by Karapandzova et al. (2014).

Results and discussion

The total needle essential oil content of Macedonian black pine calculated on anhydrous dried mass yielded from 8.03 to 8.34 ml/kg.

Data analysis of the chemical composition of examined essential oil samples revealed six different classes of components: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenes and other non-terpene components. The most abundant fraction in the essential oil isolated from needles of *Pinus nigra* collected from Kozuf were monoterpene hydrocarbons (39.96%), while sesquiterpenes were declared as dominant (46.45%) in the oil samples isolated from *Pinus nigra* from Nidze. This variability in chemical composition of Macedonian black pines is probably due to the influence of environmental conditions on the biosynthesis of terpene compounds.

Total of eighty nine components were identified in the investigated essential oil samples which represented 87.63% of total oil. Predominant components were monoterpenes: α-pinene (29.23-30.51%), β-pinene (1.44-5.30%) and limonene + β-phellandrene (1.34-1.85%) and sesquiterpenes: *trans*-(E)-caryophyllene (8.56-11.38%), γ-murrolene (0.98-2.11%) and germacrene D (17.32-21.21%).

Previously established differences in the essential oil composition presumably affect their antimicrobial activity. In this regard, essential oil isolated from needles of black pine collected from Nidze has showed antimicrobial activity towards *Staphylococcus epidermidis* and *Streptococcus agalactiae*. Additionally, essential oil isolated from black pine needles from Kozuf revealed good antimicrobial activity against to another three microorganisms as they have showed strong antimicrobial effects toward *Streptococcus pneumonia, Staphylococcus aureus* and *Candida albicans*. Minimal inhibitory concentration (MIC) values for tested essential oil samples ranged from 15.25 to 62.25 µl/ml depending on the microorganism.

According to literature data, essential oils isolated from needles of Macedonian black pine have showed the greatest similarity to the chemical composition of oils obtained from needles of black pines originating from Croatia and Greece with the exception of two components, 13-epimanool oxide (2.25%) which was identified in the oil of Croatian black pine (Idzojtic et al., 2005) and 3-methyl-2-phenyl ester of butyric acid (2.10%) found in essential oils isolated from black pine from Greece (Roussis et al., 1994). Data concerning the antimicrobial activity of *Pinus nigra* essential oils indicated that there are no available published studies regarding this species. In terms of antimicrobial activity of the essential oils isolated from other pines, it is evident that it's in a direct correlation with the chemical composition and largely depends on the dominant components present in the oil.

Conclusion

Macedonian black pines contain essential oil with predominant components α- and β-pinene and have showed good antimicrobial activity against selected Gram (+) bacteria as well as *Candida albicans*. Nevertheless, further studies will be aimed in order to obtain more data about their antimicrobial potential as well as data about their safety usage until their final recommendation as antimicrobial agents.

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Macedonian pharmaceutical bulletin, **62** (suppl) 505 - 506 (2016)
ISSN 1409 - 8695
UDC: 582.477-113.55(497.7)
Short communication

The essential oil composition of Macedonian *Juniperus communis* L. (Cupressaceae)

Ivana Cvetkovikj[1]*, Marija Karapandzova[1], Floresa Sela[1], Gjose Stefkov[1], Maja Simonoska Crcarevska[2], Marija Glavas Dodov[2], Svetlana Kulevanova[1]

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia*
[2] *Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Juniperus communis L. (Cupressaceae) (juniper) is coniferous evergreen tree or shrub, with needle-like leaves and berry-like cones, usually incorrectly consider as berry fruit. Actually, the plant grows as male and female plant and produce male and female cones, but only female cones are used as medicinal source for isolation of juniper oil (*Juniperi aetherolem*), valuable natural product for many purposes. The medicinal use of the juniper essential oil comprises: diuretic, antiseptic, digestive, stomachic and antireumathic activity, while commercial purposes include uses in food industry, in production of alcohol beverages, as well as, cosmetics and perfume production. The oil possesses specific chemical composition where as predominant components are considered the monoterpene hydrocarbons (α-pinene, β-pinene, β-myrcene, sabinene and limonene), followed by the fraction of oxygen-containing monoterepenes (EMA/HMPC/12401/2010), where terpinene-4-ol is considered to be responsible for the diuretic effect of the juniper oil (Chatzapoulou and Kastsiotis, 1993; Orav et al., 2010). The oil composition is highly variable, and depends on genetic and environmental factors (source, geographical origin, maturity of the berries, age of the plant, environmental factors) (Jo and Kim, 2005; Tasic et al., 1993). Furthermore, the differences in the essential oil chemical composition usually trigger variations in its activity.

Over the years, the juniper berries and the juniper berry essential oil are natural drugs that are commercially exploited and exported from Republic of Macedonia (RM). Regarding this, the aim of the present work was determination of the chemical composition and possible chemical variability of the isolated juniper oils from wild growing plants in RM.

Materials and methods

Samples of juniper berries were collected in late autumn in 2010, 2011 and 2012 from six regions in RM (North-West Mountain - Mtn.: Bistra Mtn., and Shara Mtn.; Central Mtn.: Karadzica, Jakupica, and Dautica; Porechie: Kichevo, Makedonski Brod, and Samokov; Pelagonija region: Bitola, Pelister, Prilep, and Resen; Black Drim region: Debar, Karaorman, and Velestovo, and Maleshevija: Berovo). The collected plant material was air dried, stored at cool, and dark place, until analysis.

The essential oils were isolated by steam-distillation in the Clevenger-type apparatus according to the method described in the Ph.Eur.8.0 (2014). The oils were dried over anhydrous sodium sulfate and stored in vials in refrigerator until analysis.

Essential oil samples were analyzed with Agilent 7890A Gas Chromatography system equipped with flame ionization detector (FID), and Agilent 5975C Mass Quadripole detector. For this purpose, an HP-5ms (30 m x 0.25 mm, film thickness 0.25 μm) capillary column was used. Operating conditions and identification of the components were previously published (Sela et al., 2011).

Microsoft Excel® (Microsoft Corp. Redmond, WA, USA) was used for obtained data tabulation, while multivariate statistical analysis software SIMCA 14 (Unimetrics

* ivanacvetkovikj@gmail.com, ivanacvetkovikj@ff.ukim.edu.mk

AB, Sweden) was used for depth analysis. Values of p less than 0.05 were considered significant.

Results and discussion

With GC/FID/MS, 93 components were determinate in the essential oil isolated from juniper berries from all geographic regions in RM. The juniper oil was composed mainly from monoterpene hydrocarbons: α-pinene (15.59-43.68%), β-myrcene (2.89-26.50%), sabinene (1.65-16.34%), and β-pinene (0.00-6.34%). The oxygen-containing monoterpene, terpinen-4-ol was presented from 0.02-4.35%. In addition, some sesquiterpene compounds were also important constituents of the oil and were present in larger percentages (germacrene D 1.25-12.89%; *trans*-E-caryophyllene 1.61-4.05% and α-humulene 1.61-4.60%). The analysed essential oils showed differences in the chemical composition. According literature data, differences and variability in the composition of juniper oil originated from different regions in Europe and America were already reported. α-Pinene was declared as main constituent present from 27.00% in the Greek samples, over 28.60-38.20% in samples from Montenegro, and up to 46.63% in the Iranian samples (Rezvani, 2010). Other important components such as sabinene, germacrene D, myrcene, β-pinene and limonene were found in different amounts.

Having in mind that chemical composition in various samples of the essential oil mostly differs due to the influence of temperature, and/or geographic region (Jo and Kim, 2005), in depth statistical analyses using principal component analysis (PCA), followed by Hierarchical Cluster Analysis (HCA) (calculated with Ward`s criterion and sorted by size), partial least square - discriminant analysis (PLS-DA) and partial least square (PLS) analysis were carried out.

PCA applied on data related to all 93 identified constituents of the oil resulted with mathematical model with six principal components that explained 99.5% of the total variations, with no identified outliers in the model. Dendrogram created by subsequent HCA pointed that analyzed samples could be clustered in two groups.

The samples in the first cluster were correlated with α-pinene and β-myrcene, while the second cluster was linked to germacrene D, β-elemene, *trans*-E-caryophyllene, α-humulene, γ-elemene, β-cadinene, γ-cadinene. PLS-DA preceded PLS analysis with a rationale for discriminatory variables identification. Nine components (germacrene D, α-pinene, β-myrcene, sabinene, α-humulene, β-elemene, *trans*-E-caryophyllene, γ-elemene and γ-cadinene) showed statistical significance and hence were identified as discriminatory variables.

Conducted PLS analysis pointed to the relation of discriminatory variables with the year and geographical region of harvesting, as well as the mean maximal, mean average and mean minimal temperature in the month of collection (October). The PLS analysis suggested a model with four components that explained 94% (R^2Y) of the variables with prediction (Q^2) of 90.2%. Statistical analysis of the results enabled determination of significant variables and establishment of correlation between the oil chemical compositions and/or the harvesting year, geographical region and mean temperature values.

Conclusion

The GC/MS analyses of the chemical composition of the essential oils isolated from the berries of Macedonian juniper revealed presence of 93 components in total. HCA resulted with dendrogram where the samples were grouped in two clusters, one correlated with two monoterpene hydrocarbons, and second with the sesquiterpene hydrocarbons fraction. With PLS-DA, nine discriminatory variables were identified with statistical significance. Generally, the chemical composition of the juniper oil from RM is affected by the temperature and/or the year of harvesting.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 507 - 508 (2016)
ISSN 1409 - 8695
UDC: 528.929.4:547.56(497)
Short communication

Polyphenolic profile of wild growing populations of *Salvia fruticosa* Mill. from Balkan Peninsula

Ivana Cvetkovikj[1]*, Gjoshe Stefkov[1], Marija Karapandzova[1], Jasmina Petrevska-Stanoeva[2], Marina Stefova[2], Svetlana Kulevanova[1]

[1] *Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia*
[2] *Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius Univeristy, Arhimedova 5, 1000 Skopje, R. Macedonia*

Introduction

Salvia species, commonly known as sage, have been used since ancient times for treatment of many different ailments, aches, epilepsy, colds, bronchitis, tuberculosis, hemorrhage, and menstrual disorders. Although there are around 1000 species of *Salvia*, only the pharmacopoeial herbs (*Salvia officinalis* L. and *Salvia fruticosa* Miller) have significant commercial importance (Dincer et al., 2013).

Essential oil, polyphenols and terpenes are considered as main chemical compounds responsible for the pharmacological activity of these species (Kintzios, 2000; Ramos et al., 2010).

S. fruticosa (Greek sage) is an endemic species of the Eastern Mediterranean basin and is the most widespread sage species in Greece (Kintzios, 2000; Ramos et al., 2010). The leaves of this herb have been used for treatment of various skin, blood, and infectious ailments (Ali-Shtayeh et al., 2000) while the essential oil is effective against different microorganisms. Greek sage posses hypoglycemic effect and can be used against inflammations, hepatitis, and tuberculosis (Pitarokili et al., 2003).

However, the most studies have focused on the essential oil, while there are few reports regarding the phenolic composition (Kintzios, 2000).

Therefore, the aim of the present study was to examine the polyphenolic profile of 15 different wild growing populations of *S. fruticosa* from Greece and Albania.

Materials and methods

Plant material (*Salvia fruticosa* Mill., Lamiaceae), was collected from 15 different indigenous populations from nine different locations from Greece (Kavoussi - 2, Rhizoscaro - 2, and Vrysses - 2) and Albania (Porto Palermo-Qeparo - 4, Llogora -1 , Dhermi - 1, Borsh - 1, Ilias Vuno - 1, and Palase - 1). The leaves were air dried, packed in paper bags and kept in a dark and cold place until analysis. Plant identity was verified and voucher specimens were deposited at the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, R. Macedonia.

The 70% methanolic extracts were prepared in ultrasonic bath by the method described by Cvetkovikj et al. (2013).

Qualitative analysis was carried out using an HPLC system from the Agilent 1100 series with photo-diode array and mass detector (G2445A Ion-Trap Mass Spectrometer) equipped with an electrospray ionization (ESI) (Agilent Technologies, Waldbronn, Germany), controlled by LCMSD software (Agilent, v.4.1). MS data were acquired in the negative ionization mode. The eluents were: formic acid (1%, V/V) (A) and acetonitrile (B) and the separations were achieved using the method established by Cvetkovikj et al. (2013), on a Zorbax Eclipse XDB RP C-18 column (150 mm × 4.6 mm, 5 μm, Agilent, Germany), protected with a guard column (4 mm × 4.6 mm, RP-18, 5 μm, Agilent, Germany). The phenolic compounds were identified using their UV and mass spectral data (deprotonated molecular ions and their corresponding fragments and losses).

* ivanacvetkovikj@gmail.com;
ivanacvetkovikj@ff.ukim.edu.mk

Results and discussion

With HPLC–DAD–ESI-MS^n, qualitative characterization of 15 different extracts from Greek sage was performed and a total of 37 different compounds were detected. The detected compounds were classified in five groups: 12 derivates of hydroxycinnamic acid were the predominant group, followed by the group of 12 flavone glycosides of luteolin or apigenin, six phenolic diterpenes, five flavones and only one flavanone glycosides of hispidulin.

Rosmarinic acid together with the flavone, nepetin (6-hydroxyluteolin 6-methyl ether), and the phenolic diterpene (rosmanol isomer) were detected in all 15 examined *S. fruticosa* samples. Thirteen compounds (luteolin diglucoronide, luteolin 7-*O*-glucuronide, luteolin 7-*O*-glucoside, hydroxyluteolin 7-gucuronide, apigenin 7-*O*-rutinoside, hispidulin7-*O*-rutinoside, apigenin 7-*O*-glucoside, apigenin glucoronide, salvianolic acid K, rosmarinic acid, rosmanol isomer, carnosol isomer and eupatorin) were found in amounts higher than 5.00% in at least one population and were considered as principal components of the methanolic extracts. Rosmarinic acid was predominant constituent (32.72-59.88%), followed by luteolin 7-*O*-glucuronide (6.18-19.35%), salvianolic acid K (5.73-21.62%) and rosmanol isomer (1.48-12.56%).

Statistical analysis of variance (ANOVA) of the 13 polyphenolic compounds detected in the extracts from *S. fruticosa* from nine different localities from two Balkan countries (Greece and Albania) revealed that there wasn't a statistically significant difference in the polyphenolic composition of the *S. fruticosa* populations, regardless its origin.

The obtained results for the polyphenolic characterization of the methanolic extracts were in accordance with the available literature. Ecarschou et al. (2002) identified rosmarinic acid as major constituent in the ethanolic extracts while hispidulin, savigenin, cirsimaritin and the diterpenes casrnosic acid, carnosol and 12-methoxycarnosic acid were predominant compounds of the ethyl acetate extracts that showed antifungal activity (Exarchou et al., 2015). In ethyl-acetate and n-butanolic extracts from *S. fruticosa* from Crete, Greece, rosmarinic acid was not detected but derivates of ferulic acid and flavone glycosides as well as derivates of luteolin were identified and were considered as dominant components (Atwi et al., 2016).

Conclusion

With LC/DAD/ESI-MS^n analysis of 15 specimens of *S. fruticosa* from Greece and Albania, 37 polyphenolic compounds were detected, where as predominant group were considered the hydroxycinnamic acid derivates, with rosmarinic acid present with more than 30% in all analysed populations. The flavone glycosides of luteolin and apigenin were second dominant group. Qualitative analysis showed no statistically significant difference in the polyphenolic composition indicating that the polyphenolic profile of *S. fruticosa* is more species allied rather than locality related.

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Macedonian pharmaceutical bulletin, **62** (suppl) 509 - 510 (2016)
ISSN 1409 - 8695
UDC: 615.322:616.31
Short communication

The possibilities of application of medicinal plant materials in stomatology

Elvira Kovac-Besovic[1]*, Salih Saracevic[1], Adnan Besovic[2], Kemal Duric[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina*
[2]*Medimpex d.o.o. Sarajevo, Igmanska 38, 71320 Vogošća, Bosnia and Herzegovina*

Introduction

The value of medicinal plants to the mankind is very well proven. It is estimated that 70 to 80% of the people worldwide rely chiefly on traditional health care system and largely on herbal medicines (Farnsworth and Soejarto, 1991; Shanley and Luz, 2003; Shengji, 2002).

Natural medicinal raw material of plant origin has application in therapy of the all pharmacological indication. More and more, they are taking integral part of the official pharmacy and because of their use in official therapy, they rising to a higher level continually.

However, the application of the medicinal plant raw material in dentistry is much smaller.

Although, stomatology can be classified as medical or health community, still it has own specifications. For this reason, it can be induced the possibility for using plant material in treatment of caries, dental plaque, gingivitis, periodontal disease. The application is possible for different oral inflammations as they represent anti-inflammatory, antimicotic and antiviral agent.

Furthermore, it is important the possibility for application, different plant material and their products in preparation for oral hygiene. Permanent progress of stomatology as scientific area results with introduction of a new technology and new synthetic drugs but these impose also the use of potential natural medical raw material.

The carriers of pharmacological effects in plant material are different chemical substances: tannins, flavonoids, saponins, anthraquinones and their products: essential oil, mucus, balsams etc. (Samuelsson, 2004).

Accordingly, the aim of this study was assessment of the plant material and the extracted chemical substances capacity in stomatology practice.

Material and methods

In this study 25 different medical plants were presented and additionally was estimated their potential application in stomatology due to their content of chemical defined and pharmacological active substances.

Results and discussion

For the purpose of the study, 25 different medical plants were discussed: *Ononidis radix* is used for rinsing the mouth with toothache due to the triterpenes and flavonoids; *Plantaginis radix,* used against toothache (iridoids and aucubine); *Tormentillae rhizome*, for toothache and for oral inflammations, while ground herbal powder is effective for teeth due to tannins; *Rubi fruticosi folium,* to strengthen the right (flavon glycosides, phenolic acids, galotannines); *Solidaginis virgaureae herba* is used for inflammation of the oral mucosa as it contains flavonoids (rutin, quercetin); *Benzoe tinkinensis,* as addition to preparation for mouthwash (benzoic acid); *Symphyti radix* - mouthwash (tannines, alkaloids, alantoin); *Caryophylli flos* for toothache for inflammation of the oral mucosa, to reduce local pain and in dentistry (due to the presence of the essential oil components: eugenol, aceteugenol, β-caryophyllene); *Verbasci flos* as mouthwash, while the root is used for toothache (iridoid glycosides: aucubin, saponins, flavonoids); *Zingiberis rhizome* is used as toothpaste and for mouthwash and gargle (gingerol, essential oil components: zingiberen, citral); *Caricae*

* elvira-kb@hotmail.com

fructus for ulcers treatment; *Aloe capensis* to treat diseases of the oral cavity (anthracene derivatives); *Anisi fructus* removes the bad odor from the mouth due to anethole and anisaldehyde; *Frangulae cortex* can be used against the purulent processes teeth (anthraquinones); *Millefolii herba* for toothache (essential oil, azulene); *Althaeae radix* as mouthwash; *Capsici fructus,* for garglingin inflammation of the mouth and pharynx (capsaicinoids, essential oil, carotenoids); *Myrrha* for halitosis and also has the potential to strengthen the teeth (essential oil: eugenol, m-cresol, alcohols); *Melissae folium* against deposits in the mouth and as toothache (essential oil: citronellal, citronellol, nerol, geraniol, citral); *Menthae piperitae folium* is used as toothpaste (essential oil: menthol, menthone, menthofuran); *Chamomillae flos* as toothpaste (essential oil: chamazulene, bisabolol, farnesene, flavonoids); *Iridis rhizome* as tooth powder, (essential oil); *Basilici herba* as toothpaste (essential oil: 1,8-cineole, linalol, methyl chavicol); *Salviae folium* as gargling agent and for disinfection of the mouth (essential oil: thujones, 1,8-cineole, borneol) and *Aetheroleum Melaleucae* for inflammatory processes due to the presence of 1,8-cineole (Heinrich, 2004).

Plant products have long been used in dentistry as part of various dental materials right from impression materials to eugenol, which forms an integral part of the dental clinic. The use of herbs in dental practice is not limited to only material sciences. A single herb shows a variety of effects like anti-inflammatory, antibacterial, antifungal activity and many more. Hence the incorporation of these herbs in dental practice will prove to be a valuable adjunct in dental treatment.

Conclusion

The use of natural medicinal raw materials is constantly increasing. The use in dentistry is not monitored enough. However, the above example of uses of medicinal plant material gives a broad possibility. Phytotherapeutic treatment in dentistry may include all of the most common toothache to the incorporation of the implant.

This paper is only an introduction for future clinical and laboratory investigation as a momentum for return to herbal medication in the treatment of different infective diseases in stomatology.

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Macedonian pharmaceutical bulletin, **62** (suppl) 511 - 512 (2016)
ISSN 1409 - 8695
UDC: 665.57:543.544
Short communication

Investigation of chemical substances of essential oils in commercial perfumes by method of thin layer chromatography

Elvira Kovac-Besovic*, Azra Besovic, Haris Niksic

Department of Pharmacognosy, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

Introduction

Essential oils are natural products or complex mixtures of hundreds of chemical compounds isolated from aromatic plants. Most of these compounds can be grouped into four major groups: aliphatic compounds, terpenes and terpene derivatives, benzene derivatives and miscellaneous compounds (Oyen and Dung, 1999). They represent the base in the perfume production. However, in the analysis of certain types of perfume, it can be examined from which plant species are used essential oils for their product (Kovac-Besovic, 2001).

Essential oil and plant materials containing such oils are natural products of great economic importance. Their main use is in perfumery, cosmetics, flavoring food and drink, for scenting incense and cleaning product (Sarac, 2009). All the world's leading brands of the highest quality perfumes use not one, but several different essential oils. With their combination, are achieved a number of so-called "fragrance notes" by which they are famous and required. In pharmaceutics, essential oils are used to give drugs an agreeable smell or taste. A few essential oils or pure compounds isolated such oils are used directly as medicines due to their antimicrobial, antifungal or anti-inflammatory activity (Samuelsson, 2004).

Thin layer chromatography (TLC) within a short period of time has become most important technique for the identification, characterization and determination of chemical compounds as well as complex mixtures (Nigam et al., 1965). With this technique can be analyzed the chemical substances of natural and synthetic origin (Kovac-Besovic, 2001).

Therefore the aim of the present study was to determine and confirm the essential oil components used for perfume production with thin layer chromatography.

Materials and methods

In this study were analysed five different commercial perfumes taken randomly, marked as sample 1, 2, 3, 4 and 5. The contents written of the label (declaration) of each of the perfume gives the presence of chemical substances that are integral components of different essential oils derived from various plant materials.

The label given for perfume No. 1 declared the following components: linalool, geraniol, isoeugenol, limonen, citral, citronelal.

The label given for perfume No. 2 declared the following components: linalool, geraniol, citral, citronelal.

The label given for perfume No. 3 declared the following components: linalool, geraniol, eugenol, limonen, citral, citronelal.

The label given for perfume No. 4 declared the following components: linalool, geraniol, isoeugenol, limonen, citral, citronelal, farnesol.

The label given for perfume No. 5 declared the following components: linalool, geraniol, limonen, citral, citronelal.

Components of the essential oils and extracts of plant material used as base for perfume production and chromatographic analysis were: Aetheroleum Rosae (geraniol, citronelol), Aetheroleum Lavanulae (linalool), Aetheroleum Citri (citral), Aetheroleum Melissae (citral and citronelal), Aetheroleum Caryophylli (eugenol), Aetheroleum Aurantii floris (farnesol).

One mL of different essential oils was diluted with toluene and 5 µL were used for TLC.

Commercial perfumes were prepared in toluene in ratio 1:30 (Wagner and Bladt, 1996).

As TLC adsorbent, silica gel GF_{254} was used, while toluene-ethyl acetate in ratio 93:7 was used as mobile phase for separation of the components from the essential

* elvira-kb@hotmail.com

oils as well as the perfumes. The detected components were visualized under UV lamb on 254 nm and 366 nm, while p-anisaldehyde-sulfuric acid was used as spray reagent.

The mobile phase is suitable for the analysis and comparison of all important essential oils, but the plates obtained with chloroform (Melissae folium) and dichlormethane (Caryophylli aetheroleum, Lavandulae aetheroleum), can be used as solvents systems for the drugs and their essential oils.

Results and discussion

Preliminary experiments were done on five randomly provided perfumes to determine and confirm the nature of constituents present (declared on the label), using a mixture of toluene-ethyl acetate (93:7) as developing solvent. Additionally, essential oils (Aetheroleum Rosae, Aetheroleum Lavandulae, Aetheroleum Citri, Aetheroleum Melissae, Aetheroleum Caryophylli, Aetheroleum Chamomillae) and plant material that naturally contain the target components were used. Performing TLC, visualized with p-anisaldehyde sulfuric acid and seen under UV lamp at 254 nm and 366 nm, in all investigated samples were detected: linalool, geraniol, citral and citronellal. In four samples was detected lemon, in two samples farnesol and isoeugenol, eugenol in one sample, while in three samples was identified coumarin.

Conclusion

The examples described above show that TLC has the ability to separate mixtures of substances of similar structure as are the perfumes and essential oils. Additionally it is important to mention that using TLC different perfumes were evaluated for their content declared on the labels provided from the manufacturer. Finally this was done with cheap and easy to perform technique.

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Macedonian pharmaceutical bulletin, **62** (suppl) 513 - 514 (2016)
ISSN 1409 - 8695
UDC: 582.929.4-13.55(497.115)
Short communication

Chemical composition of the essential oils of some *Thymus* spp. (Lamiaceae) from Kosovo

Verka Nedanova*, Nebija Flurim, Marija Karapandzova, Ivana Cvetkovikj, Gjoshe Stefkov, Svetlana Kulevanova

Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia.

Introduction

Among the aromatic plants belonging to the Lamiaceae family, numerous of species and varieties of wild-growing plants of the genus *Thymus* are very important. *Thymus* species are well known as medical plants that contain tannins and flavonoids and are considered as aromatic due to their essential oil content. Several studies emphasized the existence of marked chemical differences among essential oils extracted from different species or varieties. This chemical diversity is generally a function of three factors: genetical and physiological factors as well as the environmental conditions, that can affected the biological activity of the oils (Mustafa et al., 2012).

Production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests as essential oils have been shown to possess antimicrobial and antifungal properties. Nowadays, essential oils and theirs components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use (Mohammadi et al., 2013).

Beside this, the representatives of genus *Thymus* from Kosovo were poorly investigated in the past thus the aim of this study was essential oil chemical analysis of several wild-growing *Thymus* species collected from different locations in Kosovo.

Material and methods

Total of 20 samples of eight wild-growing *Thymus* species were analyzed. The samples of *Thymus tosevii* (Ošljak and Zapluže), *Thymus jankae* (Zapluže), Thymus *longicaulis* (Belobrod), *Thymus pulegioides* (Vata), *Thymus moesiacus* (Brezovica and Prevalla), and *Thymus tosevii* ssp. *substriatus* (Gllobоçica) were collected from localities in South Kosovo. The samples of *Thymus balcanus* were collected on Ljumbard Mtn., West Kosovo while the sample of *Thymus pannonicus* from Gërmia, East Kosovo.

Plant material was harvested in full flowering stage during summer, 2013. The material was put on the paper sheets in a shade and left to air dry. Essential oils were isolated by steam distillation in a Clevenger-type apparatus for 3 hours.

The essential oil composition was analyzed by GC/FID/MS on Agilent 7890A Gas Chromatography system equipped with flame ionization detector (FID) and Agilent 5975C Mass Quadripole detector. Operating conditions were as follows: oven temperature 60 °C (5 min), 1 °C/min to 80 °C (2 min) and 5 °C/min to 280 °C (5 min); flow rate of 1 mL/min (He); injector T=260 °C; FID T= 270 °C; 1 µL injection volume at split ratio 1:1.

The MS was operated in scan mode. Identification of the components present in essential oils was made by comparing mass spectra of components in essential oils with those from NIST, Wiley and Adams mass spectra libraries, by AMDIS (Automated Mass Spectral Deconvolution and Identification System) and by comparing literature and estimated Kovat′s (retention) indices that were determined using mixture of homologous series of normal alkanes from C_9 to C_{25} in hexane, under the same above mentioned conditions. The percentage ratio of essential oils components was computed by the normalization method of the GC/FID peak areas and average values were taken into further consideration.

* verce.nedanova@yahoo.com

Results and discussion

With gas chromatography, 140 compounds were identified in essential oil samples of *T. balcanus* (South Kosovo), representing 76.23-98.62% of the oil. Sesquiterpens were found as predominant components, with E-caryophyllene (26.96%) as the most abundant compound. One oil sample has showed extremely deferent composition with monoterpene linalool (59.51%) as major constituent. Compared with literature, similarities were found with the essential oil isolated from *T. balcanus* from R. Macedonia, which contains E-caryophyllene (22.42%), β-pinene (12.48%) and α-pinene (5.64%) as main constituents (Kulevanova et al., 1998).

On the other hand, 78 compounds were identified in the essential oil samples of *T. jankae* (South Kosovo), representing 97.62% of the total oil. Monoterpenes were identified as main compounds (83.27%) with linalool acetate (19.89%), carvacrol (13.84%) and linalool (10.33%) as predominat constituents. Large amounts of linalool (28.1-35.5%) were also found in essential oils of the Macedonian *T. jankae* (Baba mountain) (Kulevanova et al., 1998).

Oxygenated monoterpens (75.01%) were dominated fraction in the essential oil of *T. longicaulis*. As main compounds were detected alcohols linalool (37.69%) and geraniol (17.73%) followed by other monoterpenes such as: β-myrcene (5.87%), terpinen-4-ol (4.79%) and *cis*-sabinene hydrate (4.18%).

The chemical profile of two samples of *T. moesiacus* (Brezovica and Prvale) was characterised with geraniol (13.82% and 13.12%, respectively) and linalool (6.86% and 10.61%, respectively). The phenolic compound thymol was determined in higher percent (13.97-19.02%) in the oil sample from Brezovica whereas carvacrol (22.33%) was dominated in the sample from Prevale. These four compounds represented 52.32-53.47% of total oils.

Sesquiterpens were predominant in the essential oil of *T. pannonicus* (72.25%) with germacrene D (17.98%) as main constituent. Larger amount of germacrene D (12.12%) was found in the oil samples of *T. pannonicus* from Romania, but the most abundant component in this oil was α-terpinyl acetate (48.83%) (Boz et al., 2011).

47 compounds were identified in one essential oil sample from *T. pulegioides* from Vata (South Kosovo), representing 99.29% of the oil. The oxygenated monoterpenes thymol (33.48%) and geraniol (23.36%) were the main constituents. Large amounts of γ-terpinene (10.49%) were also found. These three constituents represented 67.33% of the total oil.

The quantitative variability of linalool (2.23-30.79%) was also noticed in three samples of *T. tosevii* followed by different percentages of thymol (1.23-5.41%) and carvacrol (2.29-40.34%), as well as linalool acetate (0.48-22.33%). Variability in the essential oil composition of *T. tosevii* was previously reported by Kulevanova et al. (1996) for the samples from Macedonia. The content of some important constituents of the oil varied in broad ranges such as 7.88-20.92%, 0.24-21.79%, 0.58-30.08% and 0.05-26.87% for linalool, geraniol, thymol and carvacrol, respectively (Kulevanova et al., 1996).

The alcohol linalool (30.78%) was determine as main constituent in the oil of *Thymus tosevii* ssp. *substriatus* followed by high quantities of linalool acetate (21.77%) and thymol (13.21%).

Conclusion

Chemical composition of essential oils of eight *Thymus species* from Kosovo had showed great variability. As the essential oils of *Thymus* are valuable because of some monoterpene phenols and alcohols (thymol, linalool, geraniol), the most of the species (*T. pulegioides, T. tosevii, T. tosevii* ssp. *substriatus, T. moesiacus, T. jankae* and *T. longicaulis*) from Kosovo represent biological source of essential oil that could be use for exploitation and further utilization. On the other hand *T. balcanus* and *T. pannonicus* can be exploited for isolation of the sesquiterpene compounds, germacrene D and E-caryophyllene, which were predominant constituents in the oils of these species.

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Macedonian pharmaceutical bulletin, **62** (suppl) 515 - 516 (2016)
ISSN 1409 - 8695
UDC: 613.83:633.888
633.888
Short communication

Routes of cannabis administration: a brief review

Gordana Geshtakovska*, Gjoshe Stefkov

Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Nowadays, the use of cannabis and its legalization for medical use has become a worldwide trend, due to his significant pharmacological effects. Due to the legal constraints on the possession and use of C. sativa, relatively little research on the medicinal qualities of this plant has been conducted. Ben Amar (2006) has recently shown that, the therapeutic applications of cannabis and its derivatives have been studied by various world bodies, including the Scientific Committee of the House of Lords in Great Britain, the Institute of Medicine in the United States and the Senate Special Committee on Illegal Drugs in Canada. Since there is a lack of valuable and reliable information about the routes of cannabis administration and dosage, in this review, we have done overview of six different ways of medical cannabis administration, their risks and benefits.

Smoking

Smoking cannabis produces the most immediate relief and permits the most refined control of the dosage. When cannabis is smoked, the active ingredients are deposited directly into the blood stream, after being absorbed through the mucus membrane of the lung. Whereas the central nervous system and physiological effects occur within minutes by the smoking route or by vaporization, these effects proceed on a time scale of hours in the case of oral ingestion.

Determining correct dosage is easier with smoked cannabis, since the effects are usually felt within 30-60 sec. and develop fully within 5-15 min. The effects may last from 30 min. to 3 h.

Using natural-based chemical free rolling papers, such as those derived from hemp or rice is recommended for health reasons and to avoid using tree products. Although medical marijuana is nontoxic, smoking it can be hazardous over the long term, because toxic compounds are created in the combustion process. The World Health Organization (WHO) suggests that a typical marijuana cigarette (joint) contains between 0.5 g and 1.0 g of cannabis (average 0.75 g). Commercially available strains of cannabis can contain up to 30% THC. An average joint of a 10% THC cannabis strain would contain 75 mg of THC (0.75 g x 10% THC content).

Vaporizing

Vaporizers can be used as a smokeless alternative. Cannabis vaporizers are designed to let users inhale active cannabinoids while avoiding smoke. Vaporizers work by heating the cannabis just below the point of combustion, the point at which smoke is produced. When the cannabis is properly heated, THC and other cannabinoids are emitted in the form of a vapor. This markedly reduces the amount of irritating particulate materials that get inhaled, resulting in much less inflammation and damage to the lining of the lungs. Many patients who find smoked cannabis highly irritating, report effective relief inhaling through vaporizers. Dose is through self titration (gradually adjusting the dose of medicine, until the desired effect is achieved).

Using vaporizers, typically between 50 mg and 500 mg of dried marijuana is used at once. Using a 10% THC strain would mean that between 5 mg and 50 mg of THC would be contained in this dose.

Sublingual delivery

The sublingual (under the tongue) or oro-mucosal (in the oral cavity) delivery method of an oil or tincture pro-

* gorde.gestakovska@gmail.com

vides another rapid onset of action as the medication is readily absorbed into the blood system. Tinctures are usually prepared in a base of alcohol, oil or glycerol. Many concentrated tinctures are taken by dropper under the tongue, and within a few minutes the patient will feel the effects. Effects of tinctures are usually felt within 5 min. up to 1 h and last about 4 h. It is recommended to start with about 3 drops of tincture, and wait an hour before increasing the dosage incrementally and as necessary. Other tinctures may be in a spray container and sprayed in the mouth to be absorbed in the oral cavity and thence into the bloodstream. The sublingual spray is a compromise between the inhaled and oral routes: compared to the oral administration, it reduces the first-pass metabolism, thus increasing the bioavailability of the drug and allowing a greater dose-titration.

Oral ingestion

Oral administration results in a slower onset of action, lower peak blood levels of cannabinoids and a longer duration of pharmacodynamic effects, compared to smoking. Taking cannabis by mouth in pill form or swallowed as a liquid, have both benefits and drawbacks. Since the cannabinoids are fat-soluble, their absorption through the gut is slower and less predictable, being dependent upon the individual's metabolism as well as the contents of the stomach. The onset of action may take as long as 30 min. to an hour, making it more difficult to determine an effective dose, especially for the novice patient. In addition, when taken orally, the medication gets metabolized through the liver before getting into the bloodstream. The liver converts the THC to another chemical, called 11-hydroxy-THC which is more psychoactive than THC, and so the effects will be different than if inhaled or taken sublingually. The advantage of the oral route is that it will last much longer, so a patient does not have to medicate as frequently. This can be helpful for glaucoma patients who are trying to maintain a lower intraocular pressure (Mathre, 2015).

Topical application

Cannabis can be applied externally as a topical ointment, lotion, or poultice, and may be used in the treatment of skin inflammations, arthritis or muscle pains. The goal is for the medication to be absorbed at the specific location being treated. Although it is unclear how well the cannabinoids are absorbed through the skin, the more soluble terpenoids and flavonoids also have anti-inflammatory properties that can be effective.

Rectal administration

Rectal administration of marijuana has many advantages. In addition to be a viable option for patients who can't ingest or inhale cannabis, cannabis suppositories are the best choice. Cannabis suppositories are made either cannabis-infused coconut oil or cocoa butter infused with FECO oil (full infused cannabis oil). Avoiding the gastrointestinal tract, prevents metabolism by the stomach and liver, which breaks down the many different cannabinoids into their constituent parts (including THC), and allows the active constituents to reach the blood in much higher concentrations.

50% of THC is usually transformed into its more psychoactive form, 11-Hydroxy-delta-9-tetrahydrocannabinol, when taken orally. Rectal administration avoids these effects, whilst allowing a higher proportion of THC to reach the bloodstream. While Δ9-THC itself is not absorbed through the rectal route, the pro-drug Δ9-THC-hemisuccinate is absorbed; this fact, combined with decreased first-pass metabolism through the rectal route, results in a higher bioavailability of Δ9-THC by the rectal route (52-61%) than by the oral route. In humans, rectal doses of 2.5-5.0 mg of the hemisuccinate ester of Δ9-THC were associated with peak plasma levels of Δ9-THC ranging between 1.1 and 4.1 ng/mL within 2-8 h, and peak plasma levels of carboxy-Δ9-THC ranging between 6.1-42.0 ng/mL within 1-8 h after administration (Abramovici, 2013).

Conclusion

It is important to remember that in addition to differences in the amount of cannabis used with the various routes of administration, each on is different with respect to the percentage of THC/ other cannabinoids that it delivers to the system and the timing of that delivery. For each pathology, it remains to be determined what type of cannabinoid and what route of administration are the most suitable to maximize the beneficial effects of each preparation and minimize the incidence of undesirable reactions. To maximize the benefits (efficacy) and reduce the undesirable effects (toxicity), new formulations for administering and delivering cannabinoids are currently under investigation.

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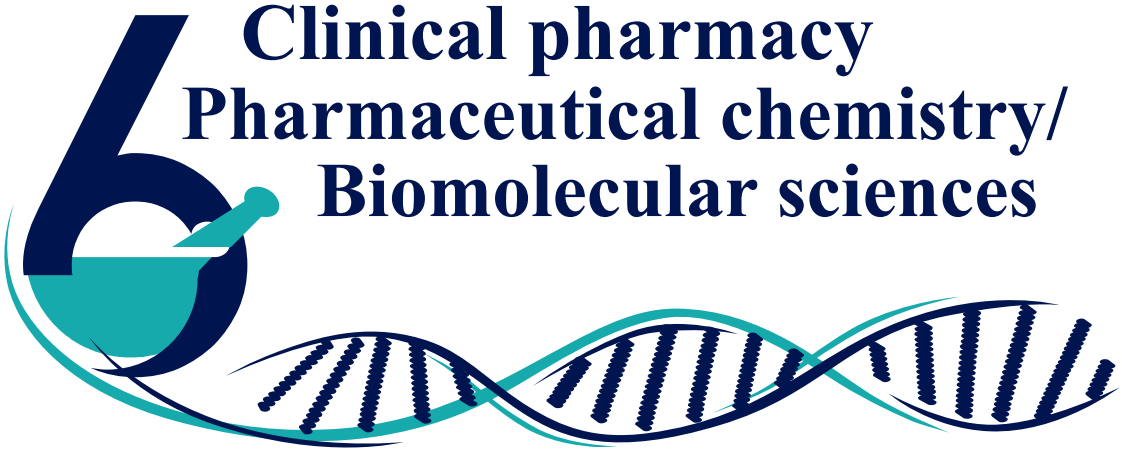

Clinical pharmacy
Pharmaceutical chemistry/
Biomolecular sciences

Macedonian pharmaceutical bulletin, **62** (suppl) 519 - 520 (2016)
ISSN 1409 - 8695
UDC: 615.15:614.254
Short communication

Pharmacotherapeutic interventions and consults - Daily practice of a clinical pharmacist and academician

Dorothea Rudorf

Massachusetts College of Pharmacy/Health Sciences, 179 Longwood Ave., Boston MA 02115, USA

Clinical pharmacy services –impact on health care

Current clinical pharmacists play a critical role in therapeutic management and medication safety by applying their pharmacotherapeutic expertise to ensure rational and effective medication use and optimal patient outcomes (ACCP, 2008). Close collaboration with physicians, nurses and other healthcare providers in interdisciplinary teams is vital.

Many studies have evaluated the role of a clinical pharmacist and the impact of clinical pharmacy services in different patient groups in both outpatient environments (community pharmacies and ambulatory clinics) and inpatient facilities (teaching hospitals and nursing homes), (Anderson and Schumock, 2009; Gallagher et al., 2014; Rotta et al., 2015) and in various specialty settings (e.g. emergency department) (Davis et al., 2016; Lada and Delgado, 2007).

Clinical pharmacy interventions – clinical and economic benefits

One of the main activities of clinical pharmacists are clinical interventions and they are described in several ways (Alderman and Farmer, 2001; Kjeldsen et al., 2014; Westerlund and Marklund, 2009). Most literature reviews conclude that clinical pharmacy interventions contribute to improve patient outcomes and cost savings focusing on specific clinical benefits (e.g. reduced adverse drug effects and medication errors, enhanced medication adherence, appropriate use of medications and prescriptions) and economic benefits (e.g. cost-effectiveness) (Anderson and Schumock, 2009; Gallagher et al., 2014; Touchette et al., 2014). However, studies differ regarding their methodology and outcomes measures, and the description of pharmacist interventions and their implementation are inconsistent (De Rijdt, 2008; Rotta et al., 2015).

Pharmacy students - clinical contributions

Academia shapes the culture of clinical pharmacy by training future professionals, e.g. in experiential practice rotations (Rathbun et al., 2012). Previous studies examining pharmacy students' interventions in a variety of practice settings have documented their clinical and economic benefits; however, there are differences regarding study scope, design, and level of detail (Divall et al., 2010; Mersfelder and Bouthillier, 2012; Shepler, 2014; Shogbon and Lundquist, 2014; Woolley et al., 2013). Although studies quantify and categorize student interventions, few provide specific details or discuss trends and the impact of clinical pharmacy preceptors on pharmacy students' clinical contributions (Divall et al., 2010; Mersfelder and Bouthillier, 2012; Woolley et al., 2013).

Clinical preceptor - impact on pharmacy student interventions

This presentation focuses on the daily practice of a clinical pharmacy preceptor and academician in a large teaching institution in educating future pharmacists to provide clinical pharmacy services, specifically through identifying and communicating pharmacotherapeutic interventions and consults and documenting their outcomes.

It includes the results of a recent study assessing the quantity, characteristics, and acceptance rate of 1172 interventions performed by 30 pharmacy students at an inpatient institution under the guidance of this preceptor (Talbot et al., 2015).

* dorothea.rudolf@mephs.edu

Study methods

During their mandatory 6-week inpatient advanced practice experiential rotations, pharmacy students accompanied medical teams on daily rounds, seeing patients on one of three general medicine floors. Under the direct supervision of the same preceptor, the students suggested drug therapy-related interventions, modifications of pharmacotherapy to enhance patient care and safety. Students documented their interventions in a detailed log, including date, brief summary of the clinical situation, description of suggested intervention with evidence-based justification, and medical team's acceptance or rejection. Each intervention was reviewed for appropriateness by the preceptor. Intervention data was categorized by acceptance/rejection, stratified into specific intervention types, and qualified by therapeutic classes and specific medications involved. Descriptive statistics were used in the analysis.

Study results

The mean number of interventions/student/rotation was 39 (range: 8-83). Overall, the medical team accepted 84% of suggested interventions; 4% were partially accepted. Most common intervention types were: adjusting dose/frequency/duration to match indication (17%), initiating indicated medication (12%), renally adjusting dose/frequency/duration (10%), changing administration schedules to prevent interactions (6%), and discontinuing non-indicated medications (6%). Therapeutic classes most frequently involved were: antibiotics (24%), gastrointestinal agents (22%), cardiovascular drugs (13%), supplements (10%), and CNS medications (9%). Overall, 214 unique medications were associated with interventions, most commonly omeprazole (12%), vancomycin (7%), warfarin (3%), ciprofloxacin (3%), and cefepime (2.3%). Students' weekly intervention rate increased as rotations progressed.

Study conclusion

It was concluded that the high number of clinical interventions cataloged by this study emphasizes the significant impact that Pharmacy students can have on educating providers and enhancing patient care. The increasing weekly intervention suggestion rate is likely related to students developing familiarity with the medical team, gaining awareness of therapeutic problems, and building confidence in initiating and substantiating interventions. The high acceptance rate may be indicative of the medical teams' past positive experiences with the students' valuable clinical pharmacy contributions. The quality of these beneficial interventions was likely augmented by consistent preceptorship.

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Macedonian pharmaceutical bulletin, **62** (suppl) 521 - 522 (2016)
ISSN 1409 - 8695
UDC: 615(497.4)
Short communication

Status of clinical pharmacy in Slovenia

Aleš Mrhar

Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

Introduction and objective

Clinical pharmacy in Slovenia includes all services performed by pharmacists practising in clinics and hospitals, community pharmacies, community health centres, nursing homes and other settings where medicines are prescribed and used. Its main mission is to move the focus of attention from the drug to the single patient or population receiving drugs.

Clinical pharmacists in Slovenia are following the overall goal of clinical pharmacy activities, i.e., to promote the correct and appropriate use of medicinal products and devices. These activities aim to:

• maximize the clinical effect of medicines, i.e., using the most effective treatment for each individual patient,

• minimize the risk of treatment-induced adverse events, i.e., monitoring the therapy course and the patient's compliance with therapy, and

• minimize the expenditures for drug treatments, i.e., trying to provide the best treatment alternative accompanied by the cost-effective use of drugs for the greatest number of patients.

The aim of this contribution is to present recent achievements and perspectives of clinical pharmacy in Slovenia in terms of professional, educational and research activities.

Methods

Clinical pharmacists in Slovenia have carried into effect all well established methodological approches to implement clinical pharmacy activities at primary, secondary and tertiary level of the healthcare system. The term "clinical" describes the type of activities which are related to the health of the patient(s) in general and as such does not necessarily imply an activity implemented only in a hospital setting but also in the community care.

Results

Professional activities at primary level

There are two types of services that are devoloping currently in community care: first, offering medication review in community pharmacies and second, establishing the practice of pharmacist-consultant on ambulatory (outpatient) basis in community health centres and in nursing homes. Medication review, performed in community pharmacies, evaluates patient's medicines with the aim of managing the risk and optimizing the outcome of medicine therapy by detecting, solving and preventing drug-related problems. It helps patients use their medicine more effectively by improving knowledge, concordance and use of medicines. It is offered as Medication use review which includes medication history and patient information, and Advanced medication review which is based on medication history, patient information and clinical information. Clinical information is obtained from patient's medical records and direct contact between dispensing pharmacist and attending physician. Medication use review is performed by pharmacist with competences, while Advanced medication review should be carried out only by specialist clinical or community pharmacist with appropriate competences.

The practice of pharmacist-consultant, performed on ambulatory (outpatient) basis has been introduced at the end of 2012 as a project run by Health Insurance Institute of Slovenia. The main purpose of the project is to bring together general practitioners and pharmacists to work closely in order to ensure proper use of drugs in clinical practice. Multistep procedure is started by general practitioner who identifies either patients with potential drug adverse reactions or patients with potential drug-drug inter-

* ales.mrhar@ffa.uni-lj.si

actions in the case of polypharmacotherapy. These patients and their medical records are referred to the pharmacist who works on the basis of Advanced medication review. The pharmacist's report is sent back to the general practitioner who optimizes the pharmacotherapy in accordance with the patients. The acceptance rate of pharmacist's proposals is approximately 70%. As the 3 year project clearly showed significant decline of drug-related problems, Ministry of Health of Slovenia has decided in 2016 to introduce the practice of pharmacist-consultant in the regular health program financed by the Health Insurance Institute of Slovenia.

Professional activities at secondary and tertiary level

A full scale model of clinical pharmacy activities in Slovenian hospitals has been introduced in 2007. They are performed in the wards at the patients' bedside and in the hospital management system. Clinical pharmacists participate in medical rounds, perform Advanced medication reviews, implement medication reconciliation process, perform therapeutic drug monitoring activities and prepare drug formularies. Additionally, they participate in hospital committees for drugs, antibiotics and hospital infections, take an active role in creating pharmacotherapeutic guidelines and start setting up clinical pharmacy departments.

Three cases of good clinical pharmacy practice in Slovenian hospitals are presented. Clinical pharmacists in General hospital Murska Sobota participate at healthcare meetings and ward rounds, they run medication reconciliation at hospital admissions and discharges and perform Advanced medication reviews in appointed patients. Clinical pharmacists at the University clinic Golnic are integrated in the treatment of patients at higher risks for adverse drug reactions (those with decreased renal function, prescribed with strong inhibitors or inducers of drug enzymes and drug transporters, prescribed with strong opioids, prescribed with drugs where TDM is performed and prescribed medicines with a feeding tube), oncology patients, patients with tuberculosis and patients with hereditary angioedema. Additionally, clinical pharmacists successfully implemented on a full scale a medication reconciliation model for all oncological patients admitted to the clinic and introduced TDM service for theopylline (Rugelj et al., 2015). Moreover, recently, a randomized, double-blind, controlled trial on clinical pharmacist's intervention reducing clinically relevant drug-drug interactions in patients with heart failure, was completed at the University clinic Golnik and published (Roblek et al., 2016). And finnaly, clinical pharmacists at the University Medical Centre Maribor successfully introduced on a full scale TDM for vankomycin, gentamicin, methyldigoxin and valproic acid. All patients receiving above-mentioned narrow therapeutic index drugs are entitled to TDM service.

Education activities

Slovene Chamber of Pharmacies runs specialistic courses on clinical pharmacy and community pharmacy. Currently, in Slovenia there are 60 specialists all together, by 2020 additional 90 specialists with competences will be available to perform clinical pharmacy activities in Slovenian healthcare system.

Faculty of Pharmacy, University of Ljubljana runs PhD course on clinical pharmacy. By 2020 we expect 20 graduates able to design and conduct research activities in the area of clinical pharmacy.

Additionally, clinical pharmacists collaborate in under and postgraduate pharmacy courses and courses for physicians and nurses,and finnally, clinical pharmacists organize pharmacokinetic courses in the hospital wards for physicians and nurses.

Research activities

Clinical pharmacists perform clinical studies and run outcomes research in terms of examine closely clinical, humanistic and economic outcomes, and publish original and review research articles in peer-reviewed journals.

Conclusion

Clinical pharmacy is implemented successfuly in majority of Slovenian clinics, hospitals, pharmacies and community health centres, it is incorporated in international cooperation on many levels, and finnally, it is on a good way to grow up as an essential part of the Slovenian healthcare system. It is our aim to create a strong team of academic and professional clinical pharmacists with specializations and doctorates in clinical pharmacy, able to run qualificative routine clinical practice and advanced clinical research, both in favour of the patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 523 - 524 (2016)
ISSN 1409 - 8695
UDC: 615.213.015
Short communication

Population pharmacokinetic modeling of therapeutic drug monitoring data from patients with epilepsy

Daniela Milosheska*, Tomaž Vovk, Iztok Grabnar

Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana Slovenia

Introduction

Therapeutic drug monitoring (TDM) is a clinical specialty that aims at improving patient care by dose individualization of drugs for which clinical experience or clinical trials have shown an improved outcome in the general or special populations. TDM can be especially beneficial for dose adjustments for drugs with dose-dependent pharmacokinetics, as an aid in the diagnosis of clinical toxicity, for evaluation of drug-drug interactions and drug compliance (Milosheska et al., 2015).

Clinical pharmacokinetics in practice is applied to TDM for drugs that are characterized by a narrow therapeutic range and a large variability in pharmacokinetics. Moreover, population modeling is a powerful tool to quantitatively evaluate the influence of various clinical, biochemical, demographic factors and other sources of variability on the dose-concentration relationship. TDM in clinical practice is initiated for a number of antiepileptic drugs (AEDs) particularly due to the episodic nature of the condition and difficulties to assess the clinical efficacy of AEDs. Initially TDM was employed for the old generation of AEDs such as carbamazepine, valproic acid, phenytoin, and primidone, which are characterized by complex and variable pharmacokinetics. On the other hand, benefit of TDM for the new generation of AEDs including lamotrigine, oxcarbazepine, topiramate, stiripentol, tiagabine, levetiracetam, and zonisamide, is often questioned as they have more favourable pharmacokinetics, wider therapeutic range and better tolerability in terms of interaction and adverse effects profile (Patsalos et al., 2008). Therefore, substantial research is needed to better document the value of TDM of the new AEDs in clinical practice. The aim of the present report was to provide evidence of the usage of population pharmacokinetic models as extensions of the therapeutic drug monitoring in patients with epilepsy treated with lamotrigine (LTG) or oxcarbazepine (OXC).

Materials and methods

Two prospective clinical studies were conducted on patients with confirmed epilepsy on stable OXC or LTG therapy at the University Medical Centre Ljubljana, Slovenia. Both studies were approved by the National Medical Ethics Committee of the Republic of Slovenia and were carried out according to the Declaration of Helsinki. At least two blood samples per patient were drawn, approximating trough and peak steady-state concentrations. Plasma concentrations of OXC and its main metabolite were determined by liquid chromatography tandem mass spectrometry method, developed and validated for the study purposes. For LTG plasma concentration measurements an adapted HPLC method with UV detection was used. Patients enrolled in LTG study were genotyped for metabolizing enzymes (*UGT1A4* and *UGT2B7*) by real-time PCR genotyping using TaqMan Allelic Discrimination Assays on the ABI 7500 instrument (Applied Biosystems, Foster City, California, USA) and for transporters (*ABCB1* and *SLC22A1*) using a fluorescence-based competitive allele-specific (KASPar) assays according to the manufacturer's instructions (KBiosciences, Herts, UK). The population pharmacokinetic analysis was performed employing a nonlinear mixed effects modeling approach using NONMEM software (ver. 7.3, Icon Development Solutions, Ellicott City, MD, USA.) (Bauer, 2011).

Results and discussion

A total of 100 pharmacokinetic profiles from adult patients on stable LTG therapy were available for pharmacokinetic analysis. The structural model used was a one-

* daniela.milosheska@gmail.com

compartment model with first order absorption and elimination. With a base model, absorption rate (inter-individual variability) was estimated at 1.96 h^{-1} (72.8%), oral clearance at 2.32 L/h (41.4%) and distribution volume at 77.6 L (30.2%). Residual variability was most adequately described using a proportional error model and was estimated at 19.8%. LTG clearance was associated with UGT2B7 genetic polymorphism, patient's weight, renal function, smoking and co-treatment with enzyme inducing or inhibiting drugs. This study showed that there is a significant variability in LTG pharmacokinetics and therefore TDM can be useful in various clinical settings for therapy individualization.

A total of 84 plasma concentration measurements of OXC and its active metabolite 10-monohydroxyoxcarbazepine (MHD) from 18 pediatric patients (age 6 months-3years) with epilepsy on stable OXC therapy were included into the study. The structural model was comprised of two-compartment model for the parent drug and one compartment model for the disposition of the active metabolite. The estimated parameters were absorption rate constant (Ka), volume of the central and peripheral compartment of the parent drug ($V1_{OXC}$ and $V2_{OXC}$), elimination and distribution clearance of the parent drug (CL_{OXC} and Q_{OXC}), and clearance and distribution volume of the metabolite (CL_{MHD} and $V1_{MHD}$). Estimated parameters (inter-individual variability) of the base model were Ka 0.581 h^{-1} (90.9%); CL_{OXC} 33.7 L/h (41.4%); $V1_{OXC}$ 21.3 L (152%); $V2_{OXC}$ 3299 L; Q_{OXC} 26.4 L; CL_{MHD} 0.128 L/h (26.4%); $V1_{MHD}$ 3.13 L. Model that include allometric scaling of the clearance and distribution volume parameters and maturation function on OXC and MHD clearance was adopted as the final model. Developed model provided new insights into the developmental aspects of OXC and MHD disposition in pediatric patients. Future studies are needed to confirm those findings and to evaluate the developed pharmacokinetic model as a tool for therapy optimization.

Conclusion

Performed clinical studies demonstrated that there is a considerable variability in LTG and OXC pharmacokinetics and the application of therapeutic drug monitoring can be useful for therapy optimization in patients with epilepsy. Identification and quantification of the sources of variability among patients is essential for individualization of drug therapy, and it can be accomplished by employing population pharmacokinetic approach. Therefore, pharmacokinetic modeling can serve as valuable extension of therapeutic drug monitoring and can be used for therapy optimization and individualization in routine clinical practice significantly contributing to more rational pharmacotherapy of antiepileptic drugs.

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Macedonian pharmaceutical bulletin, **62** (suppl) 525 - 526 (2016)
ISSN 1409 - 8695
UDC: 615.015:577.2(497.7)
Short communication

The role of drug metabolizing enzymes in personalized therapy

Aleksandra Kapedanovska-Nestorovska[1]*, Zorica Naumovska[1], Krume Jakovski[2], Zoran Sterjev[1], Aleksandar Dimovski[1], Ljubica Suturkova[1]

[1] *Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje, Republic of Macedonia*
[2]*Faculty of Medicine, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje, Republic of Macedonia*

Introduction

Individual differences in drug response, both beneficial and adverse, has long been recognized as complex and common problem in clinical practice (Ramamoorthy et al., 2015). Pharmacogenetics (PGx) aims at identifying genes and genetic variants that affect the clinical outcome of therapy, i.e. determination of inherited genetic differences and genetic mechanisms that condition or disposition efficacy and toxicity of drugs (Jones 2013). PGx prediction of the therapeutic outcome in each patient individually, is a challenge and it's usage in clinical practice is still far from reality. The extensive research efforts undertaken over the past decade have identified several genetic markers that are strongly associated with outcomes of interest. Appraising the drug related function of CYP450 (CYP2D6, CYP2C9, CYP2C19, CYP3A5 and AKR1D1) genetic variants is the core for efficient modeling of population specific, cost-effective, PGx platform for individualization of drug therapy (Cabaleiro et al., 2015; Holmes et al., 2011; Jiang et al., 2015; Kapedanovska Nestorovska et al., 2014; López-Rodríguez et al., 2008; Patel et al., 2014; Rejon-Parrilla et al., 2014) .

The objective of this study is to determine the differential expression of polymorphic CYP450 genes and the correlation of mRNA levels in the liver and peripheral blood, to evaluate the clinical validity of the CYP450 phenotype in prediction of pharmacokinetic properties and therapeutic outcome of substrate drugs (CYP2D6 for risperidone, CYP2C19 for clopidogrel and CYP2C9 for ibuprofen) and to assess cost-effectiveness of PGx platform for individualized treatment with risperidone and clopidogrel in our country.

Materials and methods

The studies included a total of 482 subjects, of which 230 healthy volunteers and 252 patients. The presence of single nucleotide polymorphisms is determined by using the Real-Time Polymerase Chain Reaction while the relative mRNA level of the CYP2D6, CYP2C19, CYP2C9, CYP3A5 and AKR1D1 genes, in the liver and peripheral blood, was determined by the method of qRT-PCR. Plasma and urinary concentrations of risperidone and 9-OH risperidon as well as of ibuprofen in healthy volunteers and patients with psychiatric disorders are determined using a validated HPLC-MS / MS and HPLC. Decision tree model, including only direct drug related costs was used to evaluate the economic viability of the PGx application in individualization of Rispiridone and Clopidrogel therapy in our country.

Results and discussion

According to the results, the relative CYP450 mRNA levels and the degree of their mutual correlation in liver and peripheral blood indicate tissue specific, sexually dimorphic, cis /trans regulated gene expression and activity. The CYP3A5 gene expression in blood can be used as a biomarker to study the physiological and pathological variations of CYP2D6, CYP2C9, CYP2C19 and AKR1D1 enzymes. Association of CYP450 allele variants with drug pharmacokinetics and treatment outcome, (CYP2D6 for risperidone, CYP2C9 for ibuprofen and CYP2C19 for clopidogrel), confirm the genetic basis of variability in drug response in patients from R. Macedonia. Risperidone metabolic ratio is a useful therapeutic biomarker to recommend CYP2D6 genetic testing to guide the present or future treatment of patients.The PGx defined Risperidone dose strategy (assuming 99% test accuracy) was associ-

* alka@ff.ukim.edu.mk

ated with €7.6 /month/patient increase in total treatment cost and health gain of 0,11 QALY, yielding an ICER of €69.32/ QALY, compared to the traditional approach. Reduced PGx test accuracy (95% and 50%) augmented the ICER (€610.73/QALY and €2300.22/QALY, respectively), due to the €30.53/ month increase in the treatment for each incorrect genotyped patient. Total accumulated cost per patient for the PGx guided clopidogrel therapy was €99.049 versus € 107.62 for the traditional treatment strategy while the mean drug-associated cost was e €21.09 and €9.68, respectively. The cost associated with and due to side events hospitalization was 1.5-fold less in PGx compared to the traditional treatment. Economic assessment of genetic screening testing for mutations that affect the level of expression and functional activity of CYP2D6 and CYP2C19 genes justifies the application of PGx individualized treatment with risperidone and clopidogrel in our country.

Conclusion

The established association between the examined genetic variations, drug pharmacokinetics, clinically significant patient outcomes as well as the obtained pharmacoeconomic evidence, highlighte the opportunities of implementing PGx guided drug treatment in R. Macedonia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 527 - 528 (2016)
ISSN 1409 - 8695
UDC: 615.214.015.4
Short communication

Influence of efflux transporter protein P-glycoprotein (*ABCB1/MDR1*) on therapeutic outcome

Zorica Naumovska[1]*, Aleksandra Kapedanovska-Nestorovska[1], Ana Filipce[2], Zoran Sterjev[1], Aleksandar Dimovski[1], Ljubica Suturkova[1]

[1]*Faculty of Pharmacy, Ss. Cyril & Methodius University, MajkaTereza 47, 1000 Skopje, R. Macedonia*
[2]*Faculty of Medicine, Ss. Cyril & Methodius University, 50 Divizija 6, 1000 Skopje, R. Macedonia*

Introduction

Synonymous polymorphic variants of ABCB1gene C1236T (rs1128503), C3435T (rs1045642) and non-synonymous variant G2677T/A (rs2032582) thet results in cahange of the aminoacid 893Ala with Ser/Thr are commonly associated with P-glycoprotein expression and function. P-gp has been linked with predisposition to psychiatric disorders such as schizophrenia, bipolar disorders and depression. The P-gp expression and functional activity plays important role in pharmacotherapy resistance and unpredictable therapeutic response to antipsychotics (Bruhn et al., 2014). Establishing the association between concentrations of risperidone and its active metabolite 9-OH risperidone in the body (plasma and urine) with the genetic profile of the metabolic and transporter proteins will enable better therapeutic approach and achievement of desired therapeutic outcome (Zhu et al., 2007).

Objective

The aim of this research is to determine the association of polymorphic variability of *ABCB1* gene with the relative mRNA expression of the transport protein in the body, to determine the relationship of P-glycoprotein with the predisposition to schizophrenia and bipolar disorder, as well as to evaluate the inadequate response and the occurrence of side effects in patients treated with risperidone.

Materials and methods

The total of 222 subjects, of which 124 healthy subjects (107 healthy volunteers and 45 postmortem individuals) and 54 patients (bipolar disorder and schizophrenia) from Republic of Macedonia were included in the study. The presence of single nucleotide polymorphisms for the *ABCB1* for three polymorphisms C1236T [rs1128503], G2677A/T [rs2032582] and C3435T [rs1045642] were analyzed by Real-Time PCR while the relative expression of ABCB1 gene in different tissues (blood, brain, liver, kidney, duodenum, jejunum, ileum colon) from the same individuals. Plasma and urinary concentrations of risperidone and 9-OH risperidon in healthy volunteers and patients with psychiatric disorders are determined using a validated HPLC-MS / MS and HPLC.

Results and discussion

Our result confirmed the influence of ABCB1gene polimorhosms, gender and age on P-gp mRNA relative expression. Determination of relative expression of ABCB1 gene in different tissues (blood, brain, liver, kidney, duodenum, jejunum, ileum colon) from the same individuals allows interpretation of the tissue distribution of the transport protein in the body. The homogeneity of the data enabled building of a predictive model for determining the relative expression of mRNA of the transporter protein in different tissues with an acceptable error (4-11%). The model is based on the obtained results for the influence of C1236T, G2677T/A and C3435T polymorphisms and haplotypes, gender, age relative expression of *ABCB1* in blood and their mutual interactions on the relative mRNA expres-

* zose@ff.ukim.edu.mk

sion of P-glycoprotein (Marzolini et al., 2004).

Due to the active role of P-glycoprotein in the transport through the blood brain barrier (BBB), *ABCB1* has a possible role in the predisposition for occurrence of central nervous system diseases. The results of this study have confirmed the role of the *ABCB1* gene in predisposition to psychiatric disorders and increased risk of developing bipolar disorder in carriers of the heterozygotes and mutant homozygotes for polymorphic variations in 1236 and 2677 in comparison to the normal genotype carriers (Abbott, 2013).

HPLC-UV method for determination of the concentrations of risperidone and 9-OH risperidone in plasma and urine, that can be used in clinical practice for therapeutic drug monitoring (TDM) was optimized and validated (Jovanovic et al., 2010).

Pharmacogenetic analyzes confirmed the influence of polymorphic variability of the transporter to the pharmacokinetic properties of risperidone in healthy individuals from the study of bioequivalence and patient population. It gives an opportunity for clinical application of this analysis in order to avoid side effects and to improve the therapeutic activity.

Our findings suggest that the allele frequencies in Macedonians are similar to those reported for Caucasians of European descendant and have shown statistically significant differences in comparison with the Asians and African population. We have identified eight different haplotypes in our population but, three haplotypes CGC, TTT and CGT represent almost 90% of ethnical Macedonians. This study may contribute to population specific data on *ABCB1* gene and has to be taken into account in order to for future establishment of the association and functional impact of *ABCB1* polymorphisms with various drug responses and disease predispositions in R. Macedonia (Naumovska et al., 2014).

This study suggested influence of ABCB1 genetic variation on P-gp activity or expression and predisposition to mood disorders, but not to schizophrenia. One of the futures prospective of our work might be the evaluation of the influences of polymorphic variants in the promoter region on ABCB1 expression and estimation of its influence of predisposition for certain psychiatric disorders. One of the most important limiting factors in the study was the restricted number of the patients, but obtained results are in agreement with most of the findings from other relevant studies.

Conclusion

Obtained results confirm the role of pharmacogenetics as a modern tool for rational pharmacotherapy, which is economically justified.

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Macedonian pharmaceutical bulletin, **62** (suppl) 529 - 530 (2016)
ISSN 1409 - 8695
UDC: 616.419-006.448-08
Short communication

What do we learned from new treatment of multiple myeloma?

Sonja Genadieva Stavrik

University Hermatology Clinic, Medical Faculty, 1000 Skopje, Macedonia

Myeloma multiplex still remains incurable disease. In the recent years there is a huge improvement in treatment of patients with multiple myeloma, particularly in younger patients with less than 65 years, mainly based on the introduction of novel agents, such as bortezomib, thalidomide and lenalidomide. Doubling survival in myeloma patients as compared to the 1990s when only chemotherapy was used is based on introducing of autologous stem cell transplantation and novel agents such as bortezomib, thalidomide and lenalidomide. So the main question is what do we learn nowadays from these improvement?

First of all today, we are facing the redefinition the therapeutic goal: "cure"or "disease control", that means to convert the disease in a chronic condition which enables the patient to survive more than 20 years with a good quality of life. Conventional chemotherapy did not fulfill these expectations and in the era of conservative approach with conservative chemotherapy younger patients lost 18 years of life, and most of them suffered the emotional burden of several relapses until disease become refractory. But the mainstay of these is based in the tests that are currently used to define complete remission. It become clear that previous evaluation have not enough strength to predict disease outcome. Low relative sensitivity is landmark of the criteria that have been recently used to define treatment response such as less than 5% plasma cells in the bone marrow, the disappearance of the M-protein by immunofixation, and the complete disappearance of any extra-osseus plasmocytoma (Palumbo et al., 2011). Today we have more sensitive tools which are required and enable us to improve the assessment of treatment efficacy in the patients with multiple myeloma.

Today, physicians are able to offer wider variety of treatment options for both young and elderly patients with multiple myeloma. Therapeutic options should be tailored and personalized according to patient's characteristics by balancing efficacy and toxicity of each drug. Effective treatment should be concentrated at the early phase of disease, when clones are more drug sensitive, long – lasting remission are more frequent, and serious adverse events are less prominent. This approach significantly improves quality of life and may ultimately prolong overall survival (Genadieva Stavric et al., 2014; Moreau et al., 2015).

Initial therapy for multiple myeloma depends to a certain extent on patients characteristics such as: eligibility for autologous stem cell transplantation per se; age and comorbidities. The role of induction therapy is to induce remission, but patient's characteristics have a significant role in the initial treatment approach. Goals of treatment are: to eradicate the tumor clone, including cancer stem cell, to search for an appropriate balance between efficacy and toxicity with three different but complementary aims: quality of life, survival prolongation and eventually the dream of cure. This can be achieved if we use appropriate tools to evaluate treatment efficacy (Rajkumar et al., 2014; Ria et al., 2014). Achieving the lowest level of minimal residual disease can be an important goal of therapy, a step in the path to cure (Bianchi et al., 2015; Paiva et al., 2015).

When autologous stem cell transplantation is a therapeutic option for a newly diagnosed young, fit patient, the patient must be treated with agents that do not compromise hematopoetic stem/progenitor cell collection, so after a few cycles of induction therapy progenitor stem cells should be collected. Nowadays initial treatment options for a young newly diagnosed patient with multiple myeloma should be based by a risk adopted approach conforming to the maxim of optimizing the therapeutic index by balancing efficacy with potential side effect (Paiva et al., 2015; Rajkumar et al., 2014; Ria et al., 2014).

If an hematologist would search for cured patients with multiple myeloma, he would stop in the registry of transplanted patients who underwent allogeneic transplantation. Unfortunately there is only a small number of these pa-

* sgenstav@yahoo.com

tients. The main reasons for that are: high transplant related mortality, donor limitations, advanced age. In the field of autologous transplantation there is operationally "cure" for only 3-10% of multiple myeloma patients which will remain in complete remission after high-dose chemotherapy and autologous stem cell transplant. The main advantages of autologous transplantation are: achieving complete remission in 15-30%, the possibility of long treatment-free period with excellent quality of life; prolongation of survival by one year. There is still a role of autologous stem cell transplantation in the era of novel agents. With autologous transplantation after induction therapy there is a possibility for further decrease of the tumor mass. Incorporating the novel agents in the induction protocols improved the complete remission rates and this is the first step towards higher complete remission rate after transplantation. Today, complete remission is considered as "condition sine qua non" for an improved survival (Paiva et al., 2015). The efficacy of treatment is mainly related to the achievement of a durable response. We already know that mechanism of action of high-dose melphalan and novel agent are different, so they are acting complementary. There are at least five active classes of treatment: alkylators, corticosteroids, proteasome inhibitors, immunomodulatory drugs, and antracyclines. All these drugs have significant activity against multiple myeloma when are used alone, but having in mind that they may have complementary mechanism of action, their activity is increased further when they are combined between each-other. So, today we are facing numerous doublet, triplet and quadruplet combinations which have been tested through the use of these drugs.

Therapeutic options should be tailored and personalized according to patient's characteristics by balancing efficacy and toxicity of each drug which is especially important for elderly patients. In the mode of sequencing treatment for elderly patients with multiple myeloma, our goal is to achieve and maintain maximal response while limiting treatment-related toxicities as much as possible. Second-generation novel agents, such as carfilzomib, pomalidomide, elotuzumab, bendamustine are currently being evaluated as an option to improve treatment outcome in myeloma patients (Bianchi et al., 2015).

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Macedonian pharmaceutical bulletin, **62** (suppl) 531 - 532 (2016)
ISSN 1409 - 8695
UDC: 615.212
Short communication

Improved analgesics: BU08028 a novel, bifunctional NOP/MOP ligand

Gerta Cami-Kobeci[1]*, Mei-Chuan Ko[2],Lawrence Toll[3] and Stephen M. Husbands[1]

[1]*Department of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, UK*
[2]*Wake Forest University, Winston-Salem, North Carolina, USA*
[3]*Torrey Pines Institute for Molecular Studies, Port St. Lucie, Fl, USA*

Introduction

Although mu opioid (MOP) analgesics, such as morphine, are the preferred analgesics, they possess well characterised, unwanted effects such as respiratory depression and abuse potential.

When a NOP (nociceptin receptor) agonist is co-administered with a MOP agonist, the combination produces a synergistic effect, resulting in enhanced analgesia (Ko and Husbands, 2013). This suggests the possibility of obtaining strong analgesia with only low efficacy partial agonism at both MOP and NOP receptors. Buprenorphine possesses partial MOP agonist activity and, importantly, low efficacy, modest potency NOP partial agonism. However, one study has detected no NOP receptor involvement in buprenorphine-induced physiological responses in primates (Cremeans et al., 2012) and so an improved analgesic might have a buprenorphine-like profile but with enhanced NOP activity. Structure-activity relationship studies suggested that the region of space occupied by the *tert*-butyl group in buprenorphine was key to good NOP receptor activity (Cami-Kobeci et al., 2011a, 2011b; Khroyan et al., 2011).

To this end we have synthesized novel buprenorphine-like analogs with increased NOP affinity and efficacy.

Materials and methods

Access to analogues of buprenorphine in which the *t*-butyl group is replaced by alternative bulky groups was by the standard procedure of Grignard addition to ketone.

Binding affinities of the new compounds, plus various standards, were determined in CHO cells transfected with human receptor cDNA, as previously described (Spagnolo et al., 2008); the displaced selective radioligands were [^{3}H]DAMGO, [^{3}H]Cl-DPDPE, [^{3}H]U69593 and [^{3}H]N/OFQ for binding to MOP, delta opioid (DOP), kappa opioid (KOP) and NOP receptors respectively.

The ligands were assessed for functional activity in the [^{35}S]GTPγS-binding assay in human receptor transfected CHO cells (Spagnolo et al., 2008).

BU08028 has been evaluated in vivo for attenuation of acute pain, using the tail flick assay and these results will be presented. To characterize the pharmacological profile of BU08028 as an analgesic in rhesus monkeys, acute thermal nociception and capsaicin-induced thermal allodynia assays were conducted.

Results and discussion

Of the analogues of buprenorphine with variation in the C20 substituents BU08028 having *t*-pentylgroup,has a Ki of less than 10 nM at each receptor in the opioid receptor family, making it the first universally high affinity opioid ligand. More importantly, even though the structure is very similar to that of buprenorphine, its affinity at NOP receptor is almost 10 fold higher than that of buprenorphine itself.

In vitro functional activity, as determined by [35S] GTPγS binding, indicated that BU08028 has virtually no efficacy at delta and kappa receptors. At mu receptors, the functional activity is very similar to buprenorphine, with only 21% stimulation relative to DAMGO. At NOP receptors, the EC50 is 43.5 nM, approximately 6 times more potent than buprenorphine, and has significantly higher ef-

* snezanacupara@yahoo.com

ficacy showing but it is far more efficacious, having 44% stimulationrelative to N/OFQ, ascompared to buprenorphine which has stimulation in the range of 15%.In the tail flick assay BU08028produced a dose-dependent increase in antinociception through MOP receptor activation.

Systemic administration of BU08028 (0.001-0.01 mg/kg) in rhesus monkeys, dose-dependently produced antinociception against acute thermal nociception and capsaicin-induced thermal allodynia. Compared to buprenorphine (0.01-0.1 mg/kg), the antinociceptive effect of BU08028 was more potent and much longer-lasting (i.e., more than 24 hours). BU08028-induced antinociception was attenuated equally by both mu opioid (MOP) receptor antagonist naltrexone (0.03 mg/kg) and NOP antagonist J-113397 (0.1 mg/kg), indicating that BU08028 is a bifunctional MOP/NOP agonist in primates. When administered alone in doses up to 0.01 mg/kg, BU08028 did not induce itch scratching, a standard side-effect of opioids such as buprenorphine.

More importantly, BU08028 at antinociceptive doses did not compromise physiological functions including respiration and cardiovascular activities measured by the radio-telemetric probes. Compared to MOP agonists, buprenorphine and remifentanil, BU08028 did not produce reinforcing effects in monkeys under the progressive-ratio schedule of drug self-administration.

Conclusion

We have discovered BU08028, and other compounds, having binding affinity at MOP receptors similar to that of buprenorphine and, as desired, higher affinity and considerably higher efficacy than buprenorphine at NOP receptors. In measures of hyperalgesia, both in rodents (rats) and non-human primates (rhesus monkey) BU08028was a potent, long-acting anti-hyperalgesic with both MOP and NOP components to its activityin the absence of common side effects associated with MOP agonists in primates.This study strongly supports the therapeutic potential of ligands with mixed MOP/NOP actions as innovative analgesics in humans.BU08028 therefore represents a potential analgesic agent, with low side effect profile.

This work was supported by NIDA grants DA020469 (SMH), DOD/Army W81XWH-13-2-0045, US-PHS grants DA-035359 and DA-032568 (M-ChKo) and DA023281 (LToll).

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Macedonian pharmaceutical bulletin, **62** (suppl) 533 - 534
(2016)
ISSN 1409 - 8695
UDC: 615.22(474.3)
Short communication

Innovative drug discovery projects in the Latvian Institute of Organic Synthesis: from meldonium to new cardioprotective drug methyl-GBB

Maija Dambrova[1,2]*, Edgars Liepinsh[1]

[1]*Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, LV1006, Riga, Latvia*
[2]*Riga Stradins University, Dzirciema Str. 16, LV1007, Riga, Latvia*

Introduction

The Latvian Institute of Organic Synthesis is a target driven research institution that combines the findings from novel academic research practices with applied research in collaboration with pharmaceutical industry. The core competences of the Latvian Institute of Organic Synthesis are medicinal chemistry and innovative drug discovery with outstanding expertise in molecular modelling, organic synthesis, medicinal chemistry and preclinical drug development for the treatment of infections, cancer, cardiovascular diseases, and neuronal disorders. During the last decade several new drug candidate molecules have been discovered in the Latvian Institute of Organic Synthesis.

From meldonium to new cardioprotective drug methyl-GBB

Meldonium (mildronate; 3-(2,2,2-trimethylhydrazinium) propionate) is a clinically used cardiometabolic drug. The mechanism of action of the drug includes lowering the L-carnitine and its metabolite contents in body tissues through inhibition of enzyme γ-butyrobetaine hydroxylase and organic cation/carnitine transporter type 2 protein (Dambrova et al, 2002; Dambrova et al., 2016).

After investigation of molecular mechanisms of meldonium, we started screening program which resulted in the discovery of a novel compound, 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB) (Tars et al., 2014). Methyl-GBB treatment decreased the acylcarnitine contents and lead to cardioprotective effects by limiting long-chain fatty acid oxidation and facilitating glucose metabolism. *In vivo* pretreatment with methyl-GBB attenuated the infarct size and significantly improved 24 h survival of rats (Liepinsh et al., 2015). In apolipoprotein E knockout mice, methyl-GBB treatment reduced the size of atherosclerotic plaques (Vilskersts et al., 2015). In the experimental models of diabetes methyl-GBB administration improved insulin sensitivity and reduced blood glucose levels (Liepinsh et al, 2016). These results provide evidence that the pharmacological decreasing of the acylcarnitine content may represent a novel treatment strategy for cardiovascular diseases.

Conclusion

The Latvian Institute of Organic Synthesis hosts the innovative synergy of academic achievements in organic chemistry and pharmacology with competent applied research in medicinal chemistry to drive smart drug discovery projects.

Acknowledgements

Supported by the Latvian State Research Program BIOMEDICINE. Dr. E. Liepinsh received funding from the 7th Framework Programme project InnovaBalt.

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Macedonian pharmaceutical bulletin, **62** (suppl) 535 - 536 (2016)
ISSN 1409 - 8695
UDC: 615.277
Short communication

DNA topoisomerase inhibitory activity and 3D-QSAR analysis of benzazoles

Esin Akı-Yalcin*, Ismail Yalcin

Pharmaceutical Chemistry Dept., Faculty of Pharmacy, Ankara University, Ankara, Turkey

Introduction

DNA topoisomerases (Topo) are enzymes that isomerise the tertiary structure of DNA without changing its primary structure. The high degree of conservation of these enzymes among prokaryotes and eukaryotes indicates an essential role in cell biology. Because its structure is a double helix, DNA is under tortional stress that results in multiplex twisting of the molecule. To be processed for replication or gene expression, the supercoiled DNA must become accessible to nucleic acid polymerases or components of the transcription machinery. This change requires relaxation and untangling of the intertwined DNA strands, which are the typical tasks of Topo (Hande, 2003).

In humans, two classes of Topo are well characterized, type I and type II. Topo type II (Topo II) are useful as drug target, since they have an indispensable function in cell biology and they lack biological redundancy. Inhibitors of these enzymes have become central parts of both primary and adjuvant chemotherapy regimens in neoplastic diseases, and they probably will remain so for the foreseeable future.

Classical Topo II inhibiting agents such as epipodophyllotoxins or anthracyclines interfere with the breakage-reunion reaction of Topo II by stabilizing this cleavable complex. The stabilization of the cleavable complex and not the inhibition of the Topo II activity is supposed to play the decisive role in the cytotoxic effect of the classical Topo II interacting agents. Accordingly, resistance against classical Topo II-inhibiting agents can result from any process that leads to an altered binding of Topo II to drugs or DNA and a reduced formation of cleavable complexes. Indeed, it was demonstrated that decreased Topo II catalytic activity can mediate drug resistance to cancer cells (Beck et al., 2001).

Because of Topo II is the target for some of the most active anticancer drugs such as etoposide, teniposide, and doxorubicin used in the treatment of human malignancies, detailed investigations of bi- and ter-benzimidazole derivatives revealed that these compounds constitute a new class of Topo I and II inhibitors. Work on such compounds indicates that a fused ring system in the structure is critical for the activity.

In the past years, we synthesized several derivatives of benzazoles, such as benzoxazoles, benzimidazoles, benzothiazoles, and oxazolo(4,5-b)pyridines as isosteric fused heterocyclic compounds to investigate their eukaryotic DNA Topo II inhibitory activity (Pinar et al. 2004) and realized their three dimentional quantitative structure activity relationships (3D-QSAR) analysis by using comparative molecular similarity indices analysis (COMSIA) method (Tekiner-Gulbas et al., 2006).

A training set of 37 compounds of benzazoles, which are possessing benzoxazole, benzimidazole, benzothiazole, and oxazolo(4,5-b)pyridine fused heterocylic nucleus at their structure, were tested for their eukaryotic DNA Topo II inhibitor activity in cell-free system by using relaxation assay. The relaxation assay utilises supercoiled plasmid as substrate and has been used by many investigators to study the catalytic activity of Topo I and II types. Inhibitory activities were presented as micromolar concentrations of the compounds that cause 50% inhibition per unit of enzyme (IC50), under the assay conditions. From the plots obtained with three different concentrations of the drugs, IC50 values were obtained and the results are the averages of two to three estimations. If no inhibition was obtained at 100 μM, the drug was assumed to have no inhibitory activity on eukaryotic DNA Topo II (Pinar et al., 2004).

For the 3D-QSAR studies performed by using CoMSIA methods running the SYBYL program package with

* esinaki@ankara.edu.tr

the IRIX 6.5 operating system of a training set of benzoxazole, benzimidazole, and oxazolo(4,5-b)pyridine derivatives, which were their eukaryotic Topo II inhibitory activities observed in cell-free system by using relaxation assay, to assume the predictions for their structure activity relationships (Tekiner-Gulbas et al., 2006).

From among the training set of 37 compounds, 28 benzazole derivatives were found to be able to inhibit the eukaryotic DNA Topo II in cell-free system at an initial concentration of 100 μg/ml. These 28 compounds were further tested at a lower range of concentrations to define their inhibitory activity and etoposide was used as the standard drug in order to compare their activity. Of these 28 compounds, 12 derivatives had IC50 values between 11.4 and 46.8 μM range and they were considered as positive Topo II inhibitors. Among these compounds, 2-phenoxymethylbenzothiazole, 6-nitro- 2-(2-methoxyphenyl)benzoxazole, 5-methylcarboxylate-2-phenylthiomethylbenzimidazole, and 6-methyl-2-(2-nitrophenyl)-benzoxazole were found as more active than the reference drug etoposide. Moreover, 5-nitro-2-(4-ethoxyphenyl)benzoxazole, 5-(4-fluorophenyl-carboxyamide)-2-phenylbenzoxazole), 5-methyl-2-phenylthiomethylbenzimidazole, and 5-nitro-2-phenoxymethylbenzimidazole had Topo II inhibitory activities comparable to etoposide.

The results indicate that either having sterically bulky substituents such as phenylacetamide or phenoxyacetamide groups at position 5 or holding a non-aromatic moiety as cyclohexyl or cyclopentyl rings and/or a pyridine ring at position 2 of the fused heterocyclic nucleus causes a severely reduced or lack of activity. On the other hand, different fused heterocyclic nuclei in the structures of the most potent Topo II inhibitors are indicating bioisosteric properties for the enzyme inhibitory activity (Pinar et al., 2004).

For the 3D-QSAR studies, the best performed CoMSIA model was obtained from the combination of two fields (i.e., steric and hydrophobic). The LOO cross-validated PLS analysis of the best model gave rise to a cross-validated value (q2) of 0.562, suggesting that the model is a useful tool for predicting Topo II inhibitory activity. The correlation coefficient between the calculated and experimental activities non crossvalidated value (r2) of 0.968 with standard error 0.073 indicates that the fitness of analyzed results is 96.8% compared to experimental results. The respective relative contributions of steric and hydrophobic fields are 35% and 65%, indicating that hydrophobic field is more predominant. The established model was validated using a test set of compounds, which were not included in the development of the model (Tekiner-Gulbas et al., 2006).

There are two significant contours representing the favored steric area to increase the inhibition against the Topo II enzyme. If a bulky substituent, such as methoxy group, is attached on ortho position of 2-phenyl-5-nitro-benzoxazole, it will occupy into favorable for steric contour and will enhance the activity. According to the 5-methylcarboxylate-2-phenylthiomethylbenzimidazole, both meta and para positions of the phenyl group, which are attached to the 2nd position of benzimidazole ring system, fit into the favorable for steric contour and improve the activity.

In CoMSIA study, hydrophobic similarity index fields are also constructed and an area found, which is placed on phenyl ring of the most active compound 5-methylcarboxylate-2-phenylthiomethylbenzimidazole, means favorable for hydrophobic. The phenyl group of another the most active compound 2-phenyl-5-nitro-benzoxazole also fits into the same favorable area. On the other side, nitro group of 2-phenyl-5-nitro-benzoxazole and carbonyl group of ester moiety of 5-methylcarboxylate-2-phenylthiomethylbenzimidazole play a very important role for increasing Topo II inhibitory activity. We could say that hydrophilic area is more significant than hydrophobic area to enhance the activity. Because all phenyl rings attached at the 2nd position of benzazole ring system fit into one of the favorable hydrophobic contours.

In conclusion, the results point out that benzimidazole, benzoxazole, benzothiazole, and/or oxazolopyridine derivatives also exhibit significant Topo II inhibitory activity and may provide advanced opportunities to design and develop new chemotherapeutic agents.Furthermore, the observed COMSIA contour plots provide many useful insights into relationships between structural features and inhibitory activity for these benzazole derivatives.

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Macedonian pharmaceutical bulletin, **62** (suppl) 537 - 538 (2016)
ISSN 1409 - 8695
UDC: 615.849.2.014.2:615.373
Short communication

Development and standardization of Rituximab-conjugates for labeling with Lutetium-177 and Yttrium-90

Emilija Janevik-Ivanovska[1]*, Darinka Gjorgieva Ackova[1], Katarina Smilkov[1], Icko Gjorgoski[2], Trajce Stafilov[3], Petre Makreski [3], Zorica Arsova-Sarafinovska[4], Lajos Baloch[5], Angela Carollo[6], Alberto Signore[7], Adriano Duatti[8]

[1] *Faculty of Medical Sciences, Department of Pharmacy, Goce Delcev University, Krste Misirkov bb, Stip, Republic of Macedonia*
[2] *Faculty of Natural Sciences and Mathematics, Institute of Biology, University "Ss. Cyril and Methodius" , Arhimedova 3, Skopje, Republic of Macedonia*
[3] *Faculty of Natural Sciences and Mathematics, Institute of Chemistry, University "Ss. Cyril and Methodius", Arhimedova 3, Skopje, Republic of Macedonia*
[4] *Institute of Human Health, 50 Divizija 6, Skopje, Republic of Macedonia*
[5] *Department of Nuclear Medicine and Translational Biological Sciences , National Public Health Center, National Research Directorate for Radiobiology and Radiohygiene, Budapest, Hungary*
[6] *European Institute of Oncology, Division of Nuclear Medicine, via Ripamonti, 435, Milano, Italy*
[7] *Nuclear Medicine Unit, Department of Medical-Surgical Sciences and of Translationa Medicine, "Sapienza" University of Rome, Via di Grottarossa 1035, Italy*
[8] *University of Ferrara, Department of Chemical and Pharmaceutical Sciences, Via L. Borsari, 46 Ferrara, Italy*

Introduction

Our work was focused on the investigation for a ready to use prepared freeze dried rituximab immunoconjugates as potential radiopharmaceuticals for labeling with Lu-177 and Y-90 in order to increase the stability and higher efficiency and lower toxicity. We tested three bifunctional chelating agents (BFCA's), *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA and 1B4M-DTPA conjugated to the same antibody using previously established protocol for conjugation (Gjorgieva Ackova, 2014, 2015; Smilkov, 2014).

The main goal was to investigate chemical characterization of the immunoconjugates, labeled with "cold" non-radioactive isotopes of Lutetium and Yttrium in the same conditions as with radioactive Lutetium 177 and to show the chemical behavior and toxicological properties.

Material and method

The conjugation of antibody with three different bifunctional cleating agents was performed using using previously established protocol for conjugation. The concentrations were adjusted to 1 mg/mL and the solutions were then lyophilized.

The purified immunoconjugates were formulated in absence of any cryoprotectant at the concentration of 10 mg/mL, and subsequently lyophilized according to selected protocols.

The process of freeze drying was completed using Labconco Free Zone Stoppering Tray Dryer, (USA), using protocol described by Park in 2013, modified to our experience.

Concentration of the antibody/immunoconjugate was determinate before and after freeze drying and reconstitution using UV spectrophotometer (Jenway UV/VIS spectrophotometer 6715), and semi-micro UV polypropylene tubes with 0.1M PBS pH=8.0, at 280 nm in triplicate.

After freeze drying both characterization of the conjugates and determination of the average number of BFCA attached to each antibody molecule is performed by MALDI-TOF mass spectrometry and integrity of the antibody was evaluated using SDS-PAGE electrophoresis, on 12% bis-tris acrylamide gel.

The spectroscopic characterization of all three freeze dried immunoconjugates, in terms of monitoring the secondary protein structure (and its preservation), was achieved

* emilija.janevik@ugd.edu.mk

by FT-IR and Raman spectroscopy.

The freeze dying immunoconjugates after reconstitution were labeling with Lu-177 (555 MBq/mg in 0,5 M NH_4OAc) and radiochemical purity was determined by instant thin-layer chromatography on silica plates with a mobile phase of ammonium acetate : methanol (1 : 1) using Cyclone Plus Phosphore Imager (Perkin Elmer).

The obtained radioimmunoconjugates were characterized by SE-HPLC, using a Zorbax Bio Series GF-250 column and the elution process was monitored on UV detector at 280 nm and radiodetector (Wojdowska, 2014).

Toxicological studies were performed in Wistar rats after injection of rituximab labeled with cold Lutetium and Yttrium. Biodistribution studies were performed in 5-6 week old nude mice grafted with Raji cells ($2x10^6$ cells in 0.5 mL medium solution) after injection of radioactive Lu-177-Rituximab.

Results and discussion

The 3 day protocol of freeze drying without the presence of mannitol, showed the greatest similarity in the elution profiles of the immunoconjugate prior lyophilization (Gholipour, 2014).

After freeze drying, the pellets obtained corresponded to the composition and the time until complete reconstitution after addition of saline showed no significant difference in the time of complete dissolution of the lyophilisates, i.e. all tested samples were completely reconstituted in 2 min.

The average number of BFCA attached to each antibody molecule performed by MALDI-TOF mass spectrometry shows presence of two main peaks corresponding to MW of 146491 Da (unconjugated antibody) and 149873 Da (conjugated antibody) which corresponds to an average of 6.1 groups of *p*-SCN-Bn-DOTA (M = 551.61 g·mol-1), two peaks also, corresponding to MW of 146477 Da (unconjugated antibody) and 151246 Da (conjugated antibody) corresponds to an average of 8.8 groups of *p*-SCN-Bn-DTPA (M = 540.54 g·mol-1) attached to a molecule of rituximab and two peaks corresponding to MW of 146848 Da (unconjugated antibody) and 151506 Da (conjugated antibody) corresponding to average of 8.3 groups 1B4M-DTPA (M = 555.58 g·mol-1) attached to a molecule of rituximab.

All immunoconjugates (both before and after lyophilization) were separated in two distinct Mw species which migrated in two bands (upper at ~50 kDa and lower at ~25 kDa) confirming the migration behavior typical for IgG antibodies which are composed of twoidentical subunits each composed by two polypeptide chains: two heavy and two light chains, linked via 4 disulfide bonds. The obtained fragments correspond to molecular masses of rituximab heavy and light chain given at the literature (Bil, 2007;)

In the experimental IR (in the region 2000-500 cm^{-1}) and Raman spectra (2000–400 cm^{-1} region) we observed retaining of native structure of the antibody and no obvious aggregation.

The radiochemical purity and determination of radioimmunoconjugates by SE-HPLC, obtained after radiolabeling the with Lu-177 was higher than 5%. These conjugates were stable for 48h in 0.9% NaCl, however, progressive aggregation was observed.

Animal studies showed no toxicity and SPECT images in mice showed good localization of the tumor, as confirmed by ex-vivo organ counting.

Conclusion

After evaluation of all the obtained results obtained we can conclude:

- Three immunoconjugates were synthesized, using *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA and 1B4M-DTPA using the a selected ratio, 1:20
- Protocol for lyophilization was established, yielding lyophilisates with favorable physicochemical properties.
- The non-radioactive labeling with Y and Lu showed preserved secondary structure in all three types of immunoconjugate, confirming their stability in conditions of freeze-drying and labeling
- During labeling with Lu-177 all three types of radioimmunoconjugates showed high radiochemical purity, over 95%, which was confirmed both in ITLC and SE-HPLC.

The selection of the most appropriate immunoconjugate kit suitable for labeling with Lu-177 or with Y-90 can be made after stability study of the formulation and completition of cell culture studies.

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Macedonian pharmaceutical bulletin, **62** (suppl) 539 - 540 (2016)
ISSN 1409 - 8695
UDC: 616.833-097:579.835.12
Short communication

Guillain Barré syndrome (GBS): new insights in the molecular mimicry between C. jejuni and human peripheral nerve (HPN) proteins

Aida Loshaj - Shala[1,2]*, Luca Regazzoni[3], Armond Daci[2], Marica Orioli[3], Katerina Brezovska[1], Ana Poceva Panovska[1], Giangiacomo Beretta[3], Ljubica Suturkova[1]

[1]*Faculty of Pharmacy, University Ss. Cyril and Methodius, Skopje, Blv. "Mother Theresa" 47, 1000 Skopje, Republic of Macedonia*
[2]*Department of Pharmacy, Faculty of Medicine, University Hasan Pristina", Mother Theresa 10 000, Prishtina, Kosovo*
[3]*Department of Pharmaceutical Sciences DISFARM, University of Milan, University of Milan Via Mangiagalli 25, 20133 Milan, Italy*

Introduction

The Guillain-Barre Syndrome (GBS), is an acute inflammatory disorder inducing arms and legs muscle weakness (tetraplegia), as well as the loss of deep tendon reflexes (areflexia).

The development of GBS is mainly associated with an antecedent infection caused by Campylobacter jejuni (C. jejuni). These infections are not uniquely associated with any clinical subtype, but severe axonal degeneration is more common following C. jejuni (Hughes et al., 1999). Experimental evidence implicates concomitant production of specific serum antibodies against several types of gangliosides that was thought to play an important role in the development of the disease (Willison and Yuki, 2002). On the other hand, proteins belonging to the family of heat shock proteins (HSP) are also believed to be etiological factors in many autoimmune diseases whose pathophysiologies are thought to stem from immune responses against HSP (Yonekura et al., 2004).

Hence, to shed further light on the mechanisms that trigger the immune responses in post infectious autoimmune diseases such as GBS, the aim of this study was to investigate the profiles and immunoreactivity of proteins from human peripheral nerve (HPN) tissue, the main pathological target of the disease, and from C. Jejuni (O:19), as the most often causative agent for GBS.

Materials and methods

In the first part of the study, proteins were isolated from HPN and C. Jejuni (serotype O:19, ATCC 43446). Human peripheral nerve was obtained at autopsy within 8 hr after death from patients who died from non-neurological disease (Department of Forensic Medicine, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Macedonia). Afterward proteins were extracted, separated by 1D SDS-PAGE using a Protean IIxi electrophoresis cell (Protean IIxi, BioRad), and their potential cross reactivity identified by Western blotting. The electrophoretic bands containing the proteins of interest were isolated, subjected to trypsin digestion, the resulting peptides submitted to nHPLC-nESI-MS and MS/MS (Dionex UltiMate3000 nanoflow LC system connected to an LTQ-Orbitrap XL) analysis and the results elaborated using SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Fisher Scientific, Rodano, MI, Italy) for proteins identification and evaluation of their similarities.

Results and discussion

Western blot analysis of the immunoreactivity of isolated proteins to sera from patients with GBS, revealed signals for at least 8 immunoreactive bands for HPN and 8 for C. jejuni O:19 – GBS. Bands corresponding to proteins associated to the strongest immunogenic response (60-70

* aidashala@gmail.com

kDa) and bands that revealed positive but weaker signal (45 kDa), were subjected to proteomic analysis.

For C. jejuni, no proteins were identifiable with sufficient confidence level in the band corresponding to Mw~45 kDa, while at Mw~60-70 kDa, 11 proteins were identified. Among them, 2 out of 3 and 7 out of 11 proteins respectively, were proteins previously identified in microorganisms from the genre Campylobacter, while the remaining proteins were in the overall from other microorganisms. All these proteins were from the family of chaperone/co-chaperone proteins (GroEL, DnaK and HtpG).

By contrast, the main proteins identified in the corresponding 45 kDa HPN band were enzymes and structural proteins. In HPN, proteins identified in the mass range around 70 kDa (the SDS-PAGE band associated to the strongest immunogenic response) were human serum albumin (HSA) neurofilament light peptide and different forms of cytoskeletal keratin. Three proteins from the HSP 70 family (heat shock 70 kDa protein 1A/1B mw=70KDa, heat shock cognate 71 kDa protein mw=70.9 KDa, and heat shock-related 70 kDa protein 2 mw=70 KDa) were found, as well as HSP 60 (mw=60 KDa). Cross verification of these proteins in UniProt bacterial proteins database rendered as reliable matching proteins (similarity score above 45%) those belonging to bacterial chaperone DnaK proteins.

In a recent study, de Jong and colleague reported the identification of seven epitopes present at different positions along the in constitutive self HSP 70 (De Jong et al., 2014).

Alignment of these peptides with the corresponding sequences in the above selected chaperones from different Campylobacter species (Mw~70 kDa) and human HSP 70, showed that two of those epitopes showed ~100% of sequence homology.

In analogy to what done for HSP 70, the presence of the HSP 60 consensus sequence L256xxLxxNxLxxxxxxx-AVKAPGFGDxRKxx reported by Elfaitouri et al. (2013) was verified after alignment of mitochondrial 60 kDa heat shock protein identified in HPN with GroEL chaperone and with 60 kDa chaperonin (both proteins share around 50% of similarity with HSP 60). In the cases of GroEL chaperone there was total coincidence of all key amino acids, while for 60 kDa chaperonin only lys264 was replaced by an isoleucine residue.

Therefore, the microbial chaperone proteins DnaK (~70 kDa) and the different forms of HSP 70 in HPN identified in this study, as well as bacterial GroEL an human HSP 60, reciprocally share high primary sequence homology and conservation of their known epitopes.

C. jejuni chaperone proteins (~70 kDa) can be suggested as possible antigens determining the induction of autoimmune disorders such as GBS or, at least, to play a relevant role in these diseases, possibly through a molecular mimicry mechanism (Elfaitouri et al., 2013).

In particular, in our case, it can be speculated that the HSP neuroprotective effect (after activation of the heat shock response in consequence of infection) may be inhibited due to the interference of specific anti-HSP antibodies, eliciting the neurodegenerative effect of anti-GM1 autoantibodies on the peripheral nervous system.

Conclusion

To the best of our knowledge, this is the first study investigating the molecular identity of cross reacting, immunogenic proteins involved in GBS. The high resolution and mass accuracy provided by the Orbitrap analyzer, allowed to obtain an extensive, although presumably incomplete, identification of candidate proteins such as human and bacterial HSP. The results of this study confirmed that C. jejuni DnaK (~70 kDa) and human peripheral nerve HSP 70, and bacterial GroEL and human HSP 60 share high sequence homology, in good accordance with the massive amount of work showing that HSP are highly conserved in evolution, leading to magnificent similarities in structure and composition between mammalian HSPs and their homologues in microorganisms. Taken altogether, these results strongly suggest a potential involvement of chaperone molecules in the development of the autoimmune response associated to GBS.

Hence, further studies that could help identify new molecular targets in inflammatory and autoimmune conditions such as GBS should be focused on the better understanding of the multifaceted contribution of HSPs in immune response and inflammation, thus to allow the development of new diagnostic, therapeutical and pharmaceutical tools for the treatment of these harmful diseases.

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Macedonian pharmaceutical bulletin, **62** (suppl) 541 - 542 (2016)
ISSN 1409 - 8695
UDC: 616.348/.35-006.6-085.277
Short communication

Impact of *KRAS* mutations on capecitabine adjuvant monotherapy in CRC patients

Nadica Matevska-Geshkovska[1]*, Marija Staninova[1], Ivana Trajkovska[1], Aleksandar Eftimov[1], Milco Panovski[2], Natalija Petrushevska-Angelovska[3], Biljana Grozdanovska[3], Aleksandar Dimovski[1]

[1]*Center for Biomolecular Pharmaceutical Analyses, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*
[2]*University Clinic for Abdominal Surgery, University "Ss. Cyril and Methodius", Vodnjanska 17, 1000 Skopje, Macedonia*
[3]*University Clinic for Radiotherapy and Oncology, University "Ss. Cyril and Methodius", Vodnjanska 17, 1000 Skopje, Macedonia*

Introduction

Colorectal cancer (CRC) is a common, heterogeneous disease that arises through the aggregate effects of multiple genetic mutations and epigenetic alterations involving genes that regulate cell growth and differentiation (Croce, 2008). In addition, CRC tumors are often comprised of cytogenetically different clones that arise from the initial transformed cell through different secondary or tertiary genetic alterations. This significant molecular heterogeneity of CRC leads to differences in the clinical presentation and response to the therapy, and therefore affects the clinical management of the patients.

The vast majority of the CRC cases (~75%) is described as sporadic CRC and has no apparent predisposing etiology. However, in recent years, a couple of particular morphological subtypes of sporadic CRC have been recognized (Jass, 2007). All of these subtypes present rather different clinical features and molecular signatures and potentially have different underlying molecular origins. Sporadic microsatellite stable (MSS) tumors usually arise through the classical adenoma-carcinoma pathway and demonstrate a high level of chromosomal instability (CIN) and mutations in genes as *APC, KRAS, TP53, TGFBR2, PI3KCA* etc. Mutations in *KRAS* (predominantly point mutations in codon 12 and 13) lead to a constitutively active GTP-bound protein and subsequent downstream activation of the MAPK signaling cascade rising to unregulated proliferation and differentiation of the cancer cells. Moreover, somatic changes in *KRAS* are thought to be an early event in colon carcinogenesis and appear concordant between primary tumor and metastatic sites (Artale et al., 2008). Their role as negative predictive biomarkers for anti-EGFR targeted therapy (cetuximab, panitumumab) is well established (Javle and Hsueh 2009). However, the data from the studies exploring the potential role of the *KRAS* mutations as prognostic biomarkers or as predictive biomarkers for standard 5-fluorouracil based adjuvant therapy are less consistent, which in part can be due to the significant heterogeneity of the assay methodology and the datasets that are analyzed. Having that in mind, we conducted a prospective study in order to evaluate the possible prognostic effect of the *KRAS* mutations in patients treated with capecitabine adjuvant monotherapy and to better understand the relationship between *KRAS*-mutation status and survival after CRC diagnosis.

Materials and methods

Study population

A total of 97 patients with histologically proven stage III or high-risk stage II colon cancer were included in the study. All recruited patients underwent a surgical resection of the tumors and were submitted to capecitabine adjuvant therapy according to standard protocol. After the completion of the therapy, follow-up visits were scheduled for a total of 5 years.

MSI analysis

Microsatellite instability in tumor samples was analyzed with multiplex fluorescent polymerase chain reaction

* nmatevska@ff.ukim.edu.mk

followed by capillary electrophoresis on 3500 Automated Genetic Analyzer (Life Technologies, USA). MSI status was determined using 9 STR markers: BAT25, BAT26, D2S123, D5S107, D5S346, D17S250, D18S58, D18S61 and D18S535 derived from the panel of microsatellite loci defined by the NCI (Boland et al., 1998). The tumor samples were classified as MSI if instability was present at more of 30% of the loci screened.

KRAS mutation analysis

KRAS mutations were analyzed in tumor samples using custom designed assay. Exon 2 of the *KRAS* gene was amplified using the following primers: AAGGCCTGCTGAAAATGACTG and AGAATGGTCCTGCACCAGTAA. Bi-directional sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit. Sequencing products were purified using BigDye XTerminator® Purification Kit (Life Technologies, USA) and analyzed by a capillary electrophoresis on a 3500 Genetic Analyzer (Life Technologies, USA).

Statistical analysis

Differences in the demographic and clinical characteristics of the patients were tested using unpaired Student t-test (continuous variables) or Fisher's exact test (categorical variables). The primary end-point of the study was disease-free survival (DFS), defined as the time from end of the adjuvant therapy to the first recurrence diagnosis. Survival curves were generated according to the Kaplan and Meier method and survival distributions were compared with the use of the log-rank test. Hazard ratios and 95 percent confidence intervals were computed with the use of Cox proportional-hazards regression model.

Results and discussion

A mutation in exon 2 of the *KRAS* gene was observed in a total of 26 (26.8%) patients, of which 21 (21.6%) in codon 12 and 5 (5.2%) in codon 13. The most common mutation was Gly12Asp which accounted for 38.5% of all *KRAS* mutations. There was no statistically significant association between the *KRAS* mutations and the clinical features of patients, although there was a trend towards higher incidence of *KRAS* mutations in patients with stage III CRC (p=0.07). The survival analyses showed an association of the *KRAS* mutations with a shorter DFS; the five-year survival of patients carriers of *KRAS* mutation was 42.3% versus 73.2% in patients with wild-type *KRAS* (p=0.004). The multivariate analysis adjusted for the gender, stage and tumor localization, showed that the presence of *KRAS* mutation is an independent predictor for poor prognosis in patients treated with adjuvant capecitabine monotherapy (HR 2.48; 95% CI 1,23-5,01; p=0.011). This leads to a conclusion that only wild-type *KRAS* CRC patients could benefit from adjuvant capecitabine monotherapy. The association of *KRAS* mutations with poor DFS was even more evident in patients with microsatellite stable tumors. The 5-year survival of patients with MSS tumors harboring *KRAS* mutation in codon 12 or 13 was 31.8% versus patients with wild-type *KRAS*, where the survival is 71.9% (p=0.001). The hazard ratio of 2.64 (CI 1.22 – 5.70; p=0.014) indicate an increase in the risk of relapse in patients with MSS tumors compared to unselected group of patients. In models stratified according to clinicopathologic characteristics, no difference in DFS was observed in patients with stage II and III, i.e. the presence of *KRAS* mutation was associated with poor outcome of treatment with capecitabine regardless of stage. However, different patterns in survival were observed according to gender of the patients, where the presence of the *KRAS* mutation remained a strong negative predictive factor for DFS only in male patients (HR 4.87; 95% CI 1.94 – 12.20; p< 0,001), where the average five-year survival of carriers of *KRAS* mutation was 29 months compared to 83.5 months in patients with wild-type *KRAS* (p<0.001). In contrast, in the female subgroup of patients, the presence of *KRAS* mutations had no significant effect on survival (58 vs. 79.7 months, p=0.93). Since there is no significant difference in the frequency of *KRAS* mutations according to the gender (p=0.71), these differences in survival are probably not due a statistical error, and represent the possible protective effect of the estrogen hormones.

Conclusion

The mutations in the *KRAS* gene are a strong negative predictive factor for survival after adjuvant capecitabine monotherapy, especially in male patients. Routine *KRAS* testing can give significant contribution towards decision making regarding the treatment strategies in CRC by identifying patients with a highly aggressive disease and poor survival irrespective of the capecitabine adjuvant monotherapy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 543 - 544 (2016)
ISSN 1409 - 8695
UDC: 615.33.015.4
Short communication

Binding site description of 2-substituted benzothiazoles as potential RND efflux pump inhibitors

Ismail Yalcin[1]*, Serap Yilmaz[1], Kayhan Bolelli[1], Esin Aki-Yalcin[1], Ufuk Over-Hasdemir[2]

[1]*Pharmaceutical Chemistry Dept., Faculty of Pharmacy, Ankara University, Ankara, Turkey*
[2]*Medical Microbiology Dept., Faculty of Medicine, Marmara University, Istanbul, Turkey*

Introduction

The resistance-nodulation-cell division family (RND) efflux pumps exemplify a unique phenomenon with drug resistance in different gram negative bacterial strains as a single mechanism causing resistance against several different classes of antibiotics. In Escherichia coli AG102 and Acinetobacter baumannii SBMox2 strains the well characterized RND efflux pumps are AcrAB-TolC and the AdeABC respectively. Most of the antibiotics were found to be substrates for these pumps by increasing the expression of the efflux pump genes, leading to multidrug resistance (MDR) and the treatment failure and death caused by these gram negative bacterial infections or underlying diseases are common (Sun et al., 2014). Consequently, the need of searching new therapeutic solutions that suppress the activity of efflux pumps and restore the sensitivity of commonly used antibiotic is essential.

RND efflux pumps, which are only found in Gram-negative bacteria, have a tripartite composition. RND type efflux pumps contain an inner membrane transporter protein (RND pump), an outer membrane protein (OMP) channel, and a periplasmic membrane fusion protein (MFP). They are allowed direct extrusion of various antibiotics from the cytosol or periplasmic space to the outside of the bacterial cell, and have been found to be associated extensively with clinically significant antibiotic resistance (Sun et al., 2014).

Recent studies reported that RND type efflux pumps, which are named AcrAB-TolC in E. coli and AdeABC in A. baumannii, comprise a transporter protein (RND pump) AcrB in E. coli or AdeB in A. baumannii acting as a proton/drug antiporter, an outer membrane channel protein TolC in E. coli or AdeC in A. baumannii, and a periplasmic membrane fusion protein AcrA, which serves as a linker between TolC and AcrB in E. coli or AdeB, which serves as a linker between AdelC and AdeB in A. baumannii (Sun et al., 2014).

The emergence of MDR strains of Gram-negative bacteria pathogens is a problem of ever increasing significance (Sun et al. 2014). Interestingly, these RND efflux pumps decrease the antibacterial activity of dissimilar antibiotic structures, which can be considered a MDR mechanism. Because of bacteria become insensitive to different classes of antibiotic therapy, new therapeutic approaches must be looked for, searching for new molecules to block efflux, to restore drug susceptibility to resistant clinical strains.

The goal of this study is (i) to define the potential RND efflux pump inhibitor (EPI) activity of our previously synthesized BSN coded 2-substituted benzothiazoles by observing the reversal antibacterial activity of antibiotics particularly to chloramphenicol (CHL) and/or ciprofloxacin (CIP) in the AdeABC efflux pump overexpressor Acinetobacter baumannii SbMox2 and/or AcrAB-TolC efflux pump overexpressor E. coli AG102 clinical isolates, and (ii) to examine the structure activity relationships by describing the binding site features of these tested compounds and to analyze the active site protein-ligand interactions of RND efflux pump AdeABC in A. baumannii by generating pharmacophore hypothesis.

Materials and methods

A well-known standard microdilution assay was used to determine the minimum inhibitory concentration (MIC) of our previously synthesized BSN coded 2-substituted

* yalcin@ankara.edu.tr

benzothiazoles derivatives (Yilmaz et al. 2013), CHL and CIP and MICs of CHL and CIP were determined in the presence and absence of the BSN coded compounds.

A common feature pharmacophore hypothesis, HipHop method, was generated by using the Accelrys Discovery Studio 2.1 software to explain the specification of the structure activity relationships of pharmacophoric sites of the tested BSN coded 2-substituted benzothiazoles in the targeted AdeABC efflux pump. This tool builds pharmacophore hypotheses (overlaying common features) for which the fit of individual molecules to a hypothesis could be correlated with activity of the molecule.

A set of potential AdeABC efflux pump inhibitors of BSN coded 2-substituted benzothiazoles, which exhibited 16-folds or greater reduction in the MIC value of CHL after used in combination in A. baumannii SbMox-2, was selected as the EPI active training set to use in the HipHop pharmacophore generation method. Among the tested BSN coded compounds, the most active molecules, BSN4, BSN6, and BSN23, were used to derive common feature-based alignments and considered as "reference compounds" specifying a principal value of 2 and a maximum omitting features value of 0.

Results and discussion

For the antibacterial activity test against A. baumannii SbMox-2 and/or E. coli AG102 clinical isolates, BSN coded 2-substituted benzothiazoles were first tested alone to observe their intrinsic antibacterial affinity. However, when they were tested alone they did not exhibit any significant intrinsic antibacterial activity. But, when they were tested in combinations with CHL or CIP against the AdeABC overexpressor A. baumannii SbMox-2 mutant, a reversal in the antibacterial activity of 22, 20 fold double dilution better MIC values were observed respectively for CHL and CIP. Moreover, the combinations of the tested compounds with CHL or CIP against the AcrAB-TolC overexpressor E. coli AG102 strain were exhibited a reversal antibacterial activity of 6, 10 fold double dilution better MIC values respectively for CHL and CIP. Among the tested BSN coded benzothiazoles, BSN4, BSN6, and BSN23 reversal the antibacterial activity of CHL revealing a MIC values of 0.125 µg/ml against the AdeABC overexpressor A. baumannii SbMox-2 strain.

The generated 3D-common feature pharmacophore hypothesis containing two Hydrogen Bond Acceptors (HBA) and three Hydrophobic Aromatics (HpAr) was anticipated as the common-feature functions to explain the pharmacophoric site specifications of the EPI activity of BSN coded 2-substituted benzothiazole compounds. The generated pharmacophore model reveals that the two HBA and three HpAr features are found significant for binding to the active site of the target protein. Three HpAr features demonstrate the appropriate active shape of the molecule, displaying the required place of bulky aromatic moieties. Two HBA atoms or groups at the given positions are necessary in the molecule to bind to the target protein.

Conclusion

The generated pharmacophore model revealed that when the tested compounds substituted by a benzyl group instead of phenyl ring attached to the benzothiazole nucleus then, they could not be able to show any match with the hydrogen bond acceptor feature of nitrogen atom in the thiazole ring at the fused ring system. Therefore, these compounds showed lower fit value and were not able to match with all the mapped pharmacophore common-features in the anticipated model. This observation explains why 2-phenylbenzotiazole structure is more favourable than 2-benzylbenzotiazole for increasing potency in this set of compounds.

In conclusion, the generated 3D-common feature pharmacophore hypothesis reveals that the conformational properties of the compounds are significant for the AdeABC efflux pump inhibitor activity against the multi-drug resistant A. baumannii SbMox-2 strain and compounds possessing 2-[4-(4-substituted-2-phenyl-acetamido)phenyl]benzothiazole and/or 2-[4-(4-substituted-3-phenylpropionamido)phenyl]benzothiazole structures are important for improving the AdeABC efflux pump inhibitor potency.

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Macedonian pharmaceutical bulletin, **62** (suppl) 545 - 546 (2016)
ISSN 1409 - 8695
UDC: 615.218.032.21:616.211-002-056.3
616.211-002-056.3-085.218.032.21
Short communication

Ectoine nasal spray in treatment of allergic rhinitis

Vladimir Šaranović

Srpsko Lekarsko Društvo, ORL sekcija
KBC Zvezdara , ENT Clinic, Preševska 31, Belgrade, Serbia

Allergic rhinitis definition and treatment

Allergic rhinitis is clinically defined as an inflammation of the nose with characteristic symptoms such as rhinorrhoea, nasal obstruction, sneezing, and/or itching of the nose. The symptomatic disorder of the nasal mucosa and tissue is associated with an IgE-mediated immune response to allergens and is characterized by two phases: an immediate response after allergen exposure (early phase) and a late phase occurring up to 12 hours later, which predominantly causes nasal congestion (Calderon Moises et al., 2010). If a concurrent respiratory infection is present, a patient's probability of developing bronchial asthma as comorbidity increases. Likewise, the risk of developing further allergies with more severe symptoms rises over the time of the disease (http://www.bitop.de).

The optimal treatment of allergic rhinitis depends on several individual factors. A stepwise therapeutic approach, however, is generally recommended. Current guidelines favour second-generation oral or topical H1 antihistamines for treating allergic rhinitis (Angier et al., 2010). Moreover, intranasal glucocorticosteroids and intranasal decongestants are highly recommended as effective treatments for nasal blockage (Bousquet et al., 2008).

Azelastine is a new-generation antihistamine applied topically as nasal spray or eye drops. It is used as treatment of allergic rhinitis, hay fever, and allergic conjunctivitis. Although azelastine is regarded as effective possible first-line treatment for allergic rhinitis, common side effects, such as bitter taste of the drug and local irritation reactions and rare side effects such as fatigue or headache, can occur.

Ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxyclic acid) is a compatible solute which is naturally produced by bacteria, conferring resistance to external stress factors such as extreme temperatures, high salt concentrations, and ultraviolet radiation. It acts via a mechanism called "preferential exclusion" and "preferential hydration (DEGAM, 2008). Ectoine is expelled from proteins or lipid membranes, resulting in the modulation of the solvent characteristic of surrounding water. Thus, ectoine is able to form a protective and stabilizing hydrate capsule around the protein and therefore helps to protect biomolecules and proteins from irreversible structural modifications by inhibiting dehydration. This indirect effect leads to a more compact and more stable folding of proteins and increases the stability of lipid membranes by increasing their fluidity (Dirschka, 2008). The effect derives from the mechanism of halophilic bacteria which stabilises the osmotic balance in the microorganic cell, where extremolytes such as ectoine are accumulated in the cytosol to equal out the varying salt concentration in the outer area (Lentzen and Schwarz, 2012). Stabilization of membranes such as those lining the airways or eyes might reduce the potential water loss of such membranes and protect them against invading allergens, thereby limiting the inflammatory cascade induced by stress mediators at the membrane level, as has been shown for lung epithelia and skin cells (Lentzen and Schwarz, 2012). In vitro experiments have further shown that ectoine inhibits apoptosis, triggered by nanoparticles (Smiatek et al., 2012), and likewise blocks the activity of ceramides, which are regarded as central molecules in the sphingolipid metabolism as well as in the induction of apoptosis (Sydlik et al. (a), 2009). Currently, ectoine is used in dermatological products for successfully treating skin diseases such as atopic dermatitis (Sydlik et al. (b), 2013). Still widely unknown is the use of ectoine in nasal sprays or eye drops. In such medical devices, ectoine may strengthen the hydroprotection of the nasal membrane and may alleviate the infection of the inflamed tissue (Vestweber, 2009).

* jelena.ognjenovic@esensa.co.rs

Toxicological studies and results of human studies reflect the excellent safety profile of products containing ectoine, therewith making them promising candidates for the treatment of allergic rhinoconjunctivitis (Vestweber, 2009).

Review from clinical studies

In order to investigate the efficacy of treatment with ectoine, data from published as well as unpublished clinical studies were reviewed.

The current noninterventional, open-label study investigated treatment of allergic rhinitis comparing the intranasal glucocorticoid beclomethasone with that of ectoine containing nasal spray. Within the study, different mode of action, on the one hand the glucocorticoid, was compared to a physical, membrane stabilizing molecule. Importantly, it was shown that nasal symptom scores of both treatment groups improved significantly over the study period of 14 days. Although advantages of the beclomethasone spray in comparison with the ectoine spray were shown, results of the ectoine group showed its potential clinical efficacy. Glucocorticoids bind to specific glucocorticoid receptors which are present on almost all cells of the body. Following binding, transcription of a number of inflammatory cytokines and chemokines can be modulated, which in turn results in decreased recruitment and activation of inflammatory cells. In allergic rhinitis, this results in a quick improvement of inflammatory symptoms which was confirmed in the results of the beclomethasone group. Oppositely, ectoine acts physically via a mechanism called "preferential exclusion." In the presence of ectoine, membranes and lipids are protected indirectly. As ectoine is expelled from the surface of proteins and lipids, those are protected by a water shell, thereby increasing the fluidity of membranes and resulting in the preferential formation of the native conformation of proteins. This might stabilize mucous membranes such as lining epithelia of the nose, thereby protecting those from invading allergens and reducing allergen-induced inflammations as shown in different model systems and as reported in congress report. It is understood that many allergens which cause allergic rhinitis symptoms have protease activities which act by impairing epithelial barrier function. This in turn results in increased penetration of allergens into nasal mucosa. The barrier stabilizing properties of ectoine may counteract this scenario by improving the epithelial barrier and stabilizing membranes. In allergic rhinitis, this might protect the nasal mucosae from invading allergens, resulting in improvement of symptoms.

A recent placebo-controlled study in an environmental challenge chamber showed that 3 hours after application of the ectoine nasal spray and eye drops the symptoms were decreased by ~20%.

Conclusion

Taken together, this meta-analysis demonstrated that the application of ectoine-based nasal spray and eye drops improves symptoms of allergic rhinitis and rhinoconjunctivitis. This easy-to-apply, well-tolerated, naturally-based nasal and ocular treatment, which has no unpleasant taste and virtually no side effects, effectively reduces allergic rhinitis symptoms and represents an exciting alternative for rhinoconjunctivitis sufferers.

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Macedonian pharmaceutical bulletin, **62** (suppl) 547 - 548 (2016)
ISSN 1409 - 8695
UDC: 616.34-002-085.37.07
Short communication

Monitoring of azathioprine active metabolite concentration in patients with inflammatory bowel disease in R. Macedonia

Kristina Pavlovska[1]*, Maja Slaninka,[1] Miceska Emilija Atanasovska,[1] Marija Petrushevska,[1] Kalina Gjorgjievska[1], Dragica Zendelovska[1], Igor Kikerkov[1], Jasmina Tonik Ribarska[2], Petranka Mishevska[3], Ljudmila Efremovska[4]

[1]*Department of Preclinical and Clinical Pharmacology and Toxicology, Medical faculty, University "Ss Cyril and Methodius", 50ta Devizija 6, 1000 Skopje, Macedonia*
[2]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*
[3]*Gastroenterohepatology clinic, Vodnjanska 17, 1000 Skopje, Macedonia*
[4]*Instutute of Physiology, Medical faculty, University "Ss Cyril and Methodius", 50ta Devizija 6, 1000 Skopje, Macedonia*

Introduction

In clinical practice, the most often investigated enzyme and routinely determined in patients treated with azathioprine (AZA) is thiopurine methultransferase enzyme (TPMT). Inherited variations in the TPMT enzyme activity are especially important in determination of individual differences between patients regarding the therapeutic response as well as the toxic effects of AZA treatment (Al Hadithy et al., 2005; Ansari et al., 2002). Additionally, inherited variations in TPMT enzyme activity have an impact on production of active 6-thioguanine nucleotide metabolite (6-TGN) after administration of AZA or 6-mercaptopurine (Wright et al., 2004). Regarding this, higher concentration of 6-TGN metabolite (235-450 pmol/8x108 Er) means better outcome of the disease activity, and therapeutic response as well (Cuffari et al., 2011; Dubinsky et al., 2000).The aims of this study were to assess the relationship between TPMT enzyme activity and concentration of 6-TGN metabolite and to determine the impact of concentration of 6-TGN metabolite on the disease activity in patients with inflammatory bowel disease (IBD), both Crohn's disease and ulcerative colitis, treated with AZA.

Materials and methods

Thirty-nine (39) patients with IBD from the Gastroenterohepatology clinic, Skopje were included in this study. All of them had used AZA in a period of more than 3 months. Determination of TPMT enzyme activity was performed using the ELISA method (Bradford and Shih, 2011). 3-5mL of blood volume was taken in a tube with EDTA or heparin as an anticoagulant. In a period of 30 minutes after the blood draw, samples were centrifuged (1000g, 15 min.). Plasma was taken out and freezed on -20 or -80°C until analysed. The technique of determination of the TPMT enzyme activity using ELISA method was performed in accordance with the reference manual of the manufacturer of the ELISA kit, "HumanThiopurineMethyltransferase (TPMT) ELISA Kit, My BioSource, USA. In order to establish a control group, blood samples were taken from healthy volunteers as well. Eritrocytes were than isolated from the blood sample and concentration of the 6-TGN was determined using the HPLC method. The bioanalytical method was validated according to the EMA Guideline on validation of bioanalytical method (EMA, 2011). The disease activity was assessed using the well known, Crohn's disease activity index (CDAI) (Gasche et al., 2000) and the Ulcerative colitis activity index (UCAI) (Walmsey et al., 1998). According to the concentration of 6-TGN metabolite, patients were divided in three groups: subtherapeutic group (<200 pmol/8x108 Er); ther-

* kristinap81@yahoo.it

apeutic (200-400 pmol/8x108 Er) and overdosed (>400 pmol/8x108 Er) (Smith et al., 2013). Results assessment of 6-TGN metabolite concentration in patients was established for the first time in Macedonia in a collaboration between two institutions, Department of Preclinical and Clinical Pharmacology and Toxicology, Medical Faculty, Skopje and the Faculty of Pharmacy, Skopje.

Results and discussion

The average value of TPMT enzyme activity in all patients was 18,49 U/mL Er ± 8.27 U/mL Er (min. 1.9 U/mL Er, max. 35.8 U/mL Er). The average value of 6-TGN metabolite concentration was 437,46 pmol/8x108 Er ± 198.82 pmol/8x108 (min. 64.8 pmol/8x108 , max. 905.5 pmol/8x108).

The results of this study have shown linear indirect moderate correlation between the TPMT enzyme activity and 6-TGN metabolite concentration in patients with IBD treated with AZA (Spearman Rank Order Corellation: R=-0,3632). As the TPMT enzyme activity was increasing, 6-TGN concentration was decreasing (p>0,05).

Adittionaly, and increased concentration of 6-TGN correlated with increased probability of remission.

Conclusion

Monitoring of 6-TGN metabolite concentration in combination with determination of the TPMT enzyme activity may predict the therapeutic response in patients with IBD treated with AZA. As well, the result suggests that the method could be useful for detection of patients who do not respond to this therapy in order to identify new alternative treatments.

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Macedonian pharmaceutical bulletin, **62** (suppl) 549 - 550 (2016)
ISSN 1409 - 8695
UDC: 615.282
Short communication

The relationship between plasma protein binding and molecular properties of selected antifungal agents

Jadranka Odović[1]*, Jovana Trbojević[2], Jasna Trbojević-Stanković[3], Ratomir Jelić[4], Biljana Stojimirović[3]

[1]*Department of Analytical Chemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia;*
[2] *Ivančić i sinovi d.o.o., Palmotićeva 13, 11000 Belgrade, Serbia*
[3]*School of Medicine, University of Belgrade, Belgrade, Serbia*
[4]*Faculty of Medicinal Science, University of Kragujevac, Kragujevac, Serbia*

Introduction

Fungal infections are widely spread in the global population today. Antifungal drugs are therapeutic agents extensively used to treat these infections. They can be classified in two groups: first, the natural antifungal antibiotics and second, synthetic drugs. According to the way of administration antifungal drugs can be topical and systemic agents. For the systemic, drug's properties such as absorption, distribution, plasma protein binding, metabolism or route of elimination considerably influence their therapeutic success (Lemke and Williams, 2013). The drug's in vivo efficiency is significantly influenced by its plasma protein binding (PPB). In vivo the drug molecules can be bound to proteins and lipids in plasma, to proteins and lipids in tissues, or can be free (unbound) and diffuse among the aqueous environment of the blood and tissues. Depending on a specific affinity for plasma protein, a portion of the bound and unbound drug may differ. In most cases, only free drug molecules interact with the therapeutic target, a receptor, to produce effective therapy results and free drug's fraction is the one that can be metabolized and excreted. One of the advantages of drug modeling is the ability to find an optimal drug PPB range (Lemke and Williams, 2013).

A number of drug's molecular physicochemical properties notably influence its ADME properties. Lipophilicity, molecular weight, molecular volume, polar surface area and solubility play important roles in drug absorption, penetration into tissues, distribution as well as the degree of plasma protein binding and route of elimination. In our previous papers we studied the relationship between Angiotensin-converting enzyme inhibitor's lipophilicity and protein binding data (Odovic and Trbojevic-Stankovic, 2012). Furthermore, the aim of this study was to investigate the relationship between computed molecular properties of seven selected systemic antifungal drugs and their plasma protein binding (PPB) data available in literature.

Materials and methods

Seven selected systemic antifungal drugs, amphotericin B, fluconazole, itraconazole, ketoconazole, posaconazole, terbinafine and voriconazole were investigated in this study.

The PPB degree data for selected antifungal agents was obtained from relevant literature (Lemke and Williams, 2013; www.drugbank.ca). The values of antifungal drugs' molecular descriptors, aqueous solubility (logS), electronic descriptor - polar surface area (PSA), constitutional parameter - molecular weight (Mw), geometric descriptor - volume value (Vol) and seven different lipophilicity parameters, logP values (AlogPs, AClogP, milogP, AlogP, MlogP, XlogP2 and XlogP3) were calculated using two different software packages (www.molinspiration.com; www.vcclab.org). The selection of most suitable logP value was evaluated on the basis its best agreement with values of PPB obtained from literature.

Microsoft Excel 2003 and Origin 7.0 PRO (Origin Lab Corporation, USA) were used for statistical analysis.

* jodovic@pharmacy.bg.ac.rs

Results and discussion

According to the data obtained from literature, antifungal drugs mostly have relatively similar and high values of plasma protein binding. These values range from 90% for amphotericin B to 99.8% for itraconazole. Ketoconazole, posaconazole and terbinafine have also high and almost equal values of PPB (99%, 98%, 99%, respectively) while fluconazole and voriconazole represent exceptions with their lower values of plasma protein binding (12% and 58% , respectively) (www.drugbank.ca).

The five molecular descriptors, logP, PSA, Mw, Vol and logS, for all studied systemic antifungal drugs were calculated using software packages, Virtual Computational Chemistry Laboratory and Molinspiration Depiction Software (www.molinspiration.com; www.vcclab.org).

The methods used for logP calculation can be divided into property-based and substructure-based methods and there are two groups of substructure-based methods: fragmental and atom-based. The differences between calculation methods led to distinctions between absolute logP values. It is generally accepted that molecules with high lipophilicity show higher values of plasma protein binding in comparison to the less lipophilic ones with similar properties. Considering all this, in the first stage of the study the relationships between numbered calculated lipophilicity descriptors, logP values, and plasma protein binding data obtained from relevant literature for selected antifungal drugs were investigated using simple linear regression. The best correlation was obtained for the relationship between antifungal drugs' plasma protein binding data and AClogP lipophilicity descriptor. Consequently, lipophilicity descriptor AClogP was chosen for further correlations. Following, the correlations between plasma protein binding data and other calculated molecular descriptors, PSA, Mw, Vol, logS were investigated providing relatively poor correlation ($R2 < 0.35$).

In continuation, the relationships between antifungal drugs' plasma protein binding data and two different molecular descriptors were investigated using multiple linear regression analysis (MLR). The relationship between antifungal drugs' protein binding data and their lipophilicity with application of additional molecular descriptors, Mw, Vol, PSA, logS as additional independent variables provided significantly higher correlations (with R2 varying from 0.81 to 0.85). The obtained relations are presented with following Equations (1)-(4).

PPB predicted(%) = 12.699(±3.213)AClogP + 0.046(±0.027)Mw + 17.105(±16.654) (1)

With n = 7; R2 = 0.851; S.D. = 15.714; F = 11.451;

PPBpredicted(%) = 12.406(±3.306)AClogP + 0.049(±0.030)Vol + 19.185(±16.114) (2)

With n = 7; R2 = 0.847; S.D. = 15.922; F = 11.101.

PPBpredicted(%) = 14.921(±3.477)AClogP + 0.097(±0.072)PSA + 25.055(±15.557) (3)

With n = 7; R2 = 0.823; S.D. = 17.136; F = 9.312;

PPBpredicted(%) = 15.144(±3.618)AClogP – 2.207(±1.806)logS + 28.532(±14.628) (4)

With n = 7; R2 = 0.814; S.D. = 17.566; F = 8.764.

As can be seen the best correlation was obtained using MLR analysis with application of two molecular descriptors, AClogP and Mw or AClogP and Vol data, as independent variables ((Equation (1) and (2)). However, all established correlations can be considered as good with R2 higher than 0.80.

Conclusion

The discovery of new pharmacologically active substances and modeling of drugs with antifungal activity led to necessity of predicting drugs properties and their ADME data. The correlations that were found between antifungal drugs' plasma protein binding data and their calculated molecular descriptors, confirmed that lipophilicity accompanied with other molecular properties, such as molecular weight, volume value, polar surface area and aqueous solubility are essential for drugs plasma protein binding. The presented computational technique could be regarded as additional, in vitro approach appropriate for evaluation of plasma protein binding of investigated drugs especially important in design of new synthesized antifungal drugs, since only free drug molecules can interact with the receptor to produce effective therapy results.

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Macedonian pharmaceutical bulletin, **62** (suppl) 551 - 552 (2016)
ISSN 1409 - 8695
UDC: 615.234:616.248
616.248-085.234
Short communication

Zileuton in treatment of patients with bronchial asthma

Naim Morina[1]*, Gëzim Boçari[2], Ali Iljazi[3], Liridona Gashi[4], Naime Morina Shaqiri[5]

[1] *Department of Pharmacy, Faculty of Medicine. University of Prishtina. Clinical Centre, Mother Theresa Str., Prishtina. Kosova*
[2] *Department of Pharmacology, Faculty of Medicine. University of Tirana*
[3] *Kosovo Occupational Health Institute, Clinical Centre N.N., Gjakova, Kosova*
[4] *Institute of Biochemistry Clinical Centre, Mother Theresa Str., Prishtina. Kosova*
[5] *Kosovo Medicines Agency, Mother Theresa Str., Prishtina. Kosova*

Introduction

Research of the leukotrienes originates from the classic pharmacologic studies in 1940 from Kellaway and Trethewie (Kellaway and Trethewie,1940). While studying albumin, they discovered a slow reacting substance, which stimulated smooth muscle. They named it as a slow reacting substance – SRS and based on the pharmacologic activity they managed to conclude that it was a unique substance, found only in immunologically sensitized tissues by an antigen. Decades later, SRS was called anaphylaxis slow reacting substance (SRS-A).

Zileuton is absorbed immediately after oral administration and extensively metabolized by CYP and UDP glucuronosyltransferase. Even in this case, initial medicine is responsible for the therapeutic effect. Zileuton is a medicine with short effect and a half-life of approximately 2.5 hours and also very much bound to the proteins (93%). Pharmacologic effects of cys-LTs' occur not only as a consequence of the activation of cys-LT1 receptor; for example, cys-LTs' which trigger the vascular smooth muscle contraction (Gorenne et al., 1995). and stimulate expression of the P-selectin generated by endothelial cells via receptor LT2 (Pedersen et al., 1994).

Work studied the effect of antileukotriene –Zileuton in the treatment of patients with bronchial asthma and increased bronchial reactivity, comparing it with control group treated with salbutamol (beta2 adrenergic receptor agonist) applied via inhalation.

Materials and methods

Formation of leukotrienes depends on the lypoxygenation of the arachidonic acid by 5-lypoxygenase. Zileuton is an active and powerful inhibitor of the activity of 5-lypoxygenase and as such inhibits generation of its products. Consequently, besides inhibition of cys-LTs', zileuton also inhibits the formation of leukotriene B4 (LTB4), which is a powerful chemotactic of other eicosanoids too, which depend on the synthesis of leukotriene A4 (LTA4). Theoretically, therapeutic effects of 5-lipoxygenase should include all those seen at the antagonist cys-LT1, but also other effects which include inhibition of the LTB4 and other products of 5-lipoxygenase.

Pharmacologic effects of cys-LTs' occur not only as a consequence of the activation of cys-LT1 receptor; for example, cys-LTs' which trigger the vascular smooth muscle contraction (Gorenne et al., 1995) and stimulate expression of the P-selectin generated by endothelial cells via receptor LT2 (Pedersen et al., 1994).

Work studied the effect of antileukotriene –Zileuton in the treatment of patients with bronchial asthma and increased bronchial reactivity, comparing it with control group treated with salbutamol (beta2 adrenergic receptor agonist) applied via inhalation.

$$SRaw = Raw \times ITGV$$

Raw and the SRaw were taken for analyses.

Parameters of the lung function are determined with Body plethysmography. Raw and ITGV were registered and specific resistance (SRaw) was calculated.

In persons with bronchial asthma and increased bronchial reactivity (n=21), Zileuton applied in a dose of 600 mg first day (oral route administration, 4 x 1 tabl.). After measurement of initial values, 1 capsule 600 mg of zileu-

* naim-mor@hotmail.com

ton applied and measured Raw and ITGV after 60, 90 and 120 min. In the end, in terms of control, applied salbutamol (beta2-adrenergic agonist) in the form of aerosol and in a dose of (2 inh. x 0,2 mg); Raw and ITGV values were measured again and SRaw was calculated.

Zileuton, as antagonist of leukotriene receptor (600 mg, tablet) administered orally 2 days in row at home (4 x 600 mg) and after 1 day, respectively 2 days reported at the ambulance and measured initial values, tablet administered orally at the ambulance, and measured Raw and ITGV after 60, 90 and 120 min. At the end, as control, administered salbutamol (beta2-adrenergic agonist) in the form of aerosol and in a dose of (2 inh. x 0,2 mg), and Raw and ITGV values were measured again and SRaw was calculated.

Gained results grouped and analysed. Statistic data processing included determination of the average values (X), standard deviation (SD), standard mistake (SEM), and testing of significance of changes in between groups of patient treated with antileukotriene substances.

Gained results tested with a t-test in order to ascertain significant changes in between examined groups. In order to compare groups, utilized was statistic test ANOVA. This test is used in cases when necessary to evaluate more than two groups. Potential mistakes with t – test avoided with ANOVA used as a method of statistic test.

Results and discussion

Results of this research, in patients with bronchial asthma, indicate that Zileuton administered in a dose of 600 mg first day (oral route administration, 4 x 1 tabl.) has not caused significant decrease of the specific resistance (SRaw) of airways (p value 0.1 > Apha 0.05).

Zileuton, administered 2 days in a row in a dose of 600 mg (4 x 1 tabl. per day), has caused significant decrease of the specific resistance (SRaw) of airways (p value 0.03 < Alpha 0.05). Treatment of the control group with salbutamol (agonist of beta2-adrenergic receptor) is also efficient in removal of the increased bronchomotor tonus, causing significant decrease of the resistance (Raw), respectively of the specific resistance (SRaw), (p value 0.05 = Alpha 0.05).

Antagonists of leukotriene in doses administered 1 and 2 days after administration of Zileuton at home in the same patient, does not significantly cause decrease of the arterial systolic and diastolic pressure (AP) (p value 0.1 > Alpha 0.05).

Clinical trials with antileukotriene medicines were quite heterogeneous in response to the therapy, with patients that can be classified in two groups, those "responding" on the treatment and those "not responding" on it. For patients responding to the treatment with antileukotriene, heart, lungs, and blood treatment clinics have recognized these medicines as alternative to inhaled steroids, in small doses, in order to maintain slight chronic asthma under the control. More studies are needed to define the role of these medicines in moderate and severe asthma. (Barnes and Miller, 2000). Some clinical trials indicated that antagonists of leukotriene have an affinity in reduction of the dose of inhaled steroids necessary to control asthma exacerbations (Jarvis and Markham, 2000).

Side effects of the patients administering zileuton are similar to those of patients administering placebo. In estimated 4 to 5% of the patients administering zileuton there is an increase of liver enzymes responsibly within 2 first months of treatment. Hepatic enzymes should be monitored in patients that have just entered the treatment with zileuton, in order to be protected from a potential toxicity of the liver.

Conclusion

Results of this research indicate that Zileuton, administered in a dose of 600 mg (oral route administration, first day 4 x 1 tabl.) in patients with bronchial asthma has not caused significant decrease of the specific resistance (SRaw) of airways, (p value 0.1 > Alpha 0.05).

Antileukotriene - Zileuton administered 2 days in a row, in a dose of 600 mg (4 x a day 1 tabl.), has caused significant decrease of the specific resistance (SRaw) of airways (p value 0.03 < Alpha 0.05). Treatment of the control group with salbutamol (agonist of beta2-adrenergic receptor) is effective in removal of the increased bronchomotor tonus, by causing significant decrease of the resistance (Raw), namely specific resistance (SRaw), (p value 0.05 = Alpha 0.05).

This suggests that Zileuton is a powerful selective inhibitor of the activity of 5-lypoxygenase and as such inhibits generation of its products. Consequently, besides inhibition of cys-LTs' formation, Zileuton also inhibits the formation of leukotriene B4 (LTB4), which is a powerful chemotactic of other eicosanoids too, which depend on the synthesis of lekotriene A4 (LTA4). According to gained results, the effect of antileukotriene (Zileuton) is not immediate after oral administration, but the powerful effect of the Zileuton seen only after two days of inhibition of cys-LTs', and inhibition of leukotriene B4 (LTB4) and A4 (LTA4) based on the recordings made of the specific resistance of airways (SRaw).

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Macedonian pharmaceutical bulletin, **62** (suppl) 553 - 554 (2016)
ISSN 1409 - 8695
UDC: 615.277.015
Short communication

Docking studies of neurokinin-1 receptor antagonists as an anticancer target

Esin Aki-Yalcin*, Özüm Öztürk, Kayhan Bolelli, Ismail Yalcin

Ankara University, Faculty of Pharmacy, Pharmaceutical Chemistry Dept., 06100 Ankara, Turkey

Introduction

Over the last two decades, research regarding Neurokinin-1 (NK1) receptor has been pursued aggressively to develop drugs that might be useful for a branch of pharmacologic purposes including; anticancer, antiviral and antiemetic and dozens of molecules have been entered into various phases of clinical trials. The endogenous ligand neuropeptide Substance P (SP) selectively binds to NK1 receptor at the plasma membrane (Munoz et al. 2011). SP is an undecapeptide that belongs to the tachykinin peptide family and widely distributed in both the central and the peripheral nervous system of mammals. Activation of NK1 receptor by SP stimulates G-protein mediated signaling pathways that are crucial for regulating cellular excitability and function such as cAMP accumulation, arachidonic acid mobilization and phosphatidylinositol turnover. It has been shown that activation of Akt suppresses apoptosis and stimulation of NK1 receptor by SP induces phosphorylation on Akt or Protein Kinase B (PKB) activity in human glioblastoma cells. After binding to the NK1 receptor in tumor cells, SP induces mitogenesis and inhibits apoptosis. Hence NK1 receptor antagonists can lead to apoptosis and inhibit tumor cell proliferation. Antagonists of these receptors inhibit the development of metastasis by blocking the activation of NK1 receptor by SP. It is shown that NK1 receptors are overexpressed in tumor cells and their antagonists such as aprepitant, L-733,060, and L-732,138 have antitumor activity against several human cancer cell lines such as melanoma, neuroblastoma, glioma, retinoblastoma, pancreatic, larynx, gastric and colon carcinomas (Munoz et al. 2011).

It has been demonstrated that binding sites of peptide antagonists and non-peptide antagonists of NK1receptor are different than each other. SP and peptide NK1 receptor antagonists bind to the extracellular terminal region of the receptor, but non-peptide NK1 receptor antagonists bind to intracellular part of the enzyme between transmembrane helices (Munoz et al. 2011). Ligand binding pocket of an NK1 receptor is a hydrophobic core between the loops of transmembrane TM III-VII. Several residues, such as Gln165 (TM IV), His197 (TM V), His265 (TM VI) and Tyr287 (TM VII) are involved in the binding of many non-peptide antagonists of the NK1 receptor. The other residues that are contributed in non-peptide antagonist binding are Ser169, Glu193, Lys194, Phe264, Phe267, Pro271 and Tyr272 (Almeida et al. 2004).

Over the last decade our group have been designed, synthesized, and working on the new anticancer active compounds. Some of our previously synthesized benzoxazole and benzamide compounds showed significant inhibitory activity for human DNA Topoisomerases and Glutathione S-transferases and also anticancer effects observed on various cell cultures (Pinar et al. 2004).

In this research, we aimed to search the activity of our previously synthesized compound, 2-[4-(4-ethylbenzamido)phenyl]benzothiazole (BSN009), to the new anticancer target NK1 receptor and to identify the binding site features and modes of NK1 receptor and the non-peptide antagonists including our synthesized compound using molecular docking study.

Materials and methods

The cytotoxic activity of tested compounds (BSN009, CP-96345, L-733,060, L-732,138, and aprepitant) were assayed using the MTT colorimetric protocol. MTT is cleaved to formazan by the "succinate-tetrazolium reductase" system (EC 1.3.99.1) which belongs to the mitochondrial respiratory chain and is active only in viable cells. Human

* esinaki@ankara.edu.tr

colon carcinoma cell line HT-29 (ATCC, HTB-38), breast cancer cell line MCF-7 (ATCC, HTB-38), human cervical carcinoma cell line HeLa (ATCC, CCL-2) and mouse embryonic fibroblast cell line NIH3T3 (ATCC, CRL-1658) were used in this study for cytotoxicity experiments.

To analyze the binding site features of the tested compounds the molecular docking studies were performed by using CDocker working with Accelrys Discovery Studio (DS) 3.5 software.

The homology model of the NK1 receptor with CP-96345 was developed by Evers and Klebe. For preparation of protein and ligands the target protein was taken, hydrogens were added and their positions were optimized using all atom CHARMm forcefield and the Powell method available in DS 3.5 protocol. The minimized protein was defined as the receptor using the binding site module. The binding site was defined from the cavity finding method which was modified to accommodate all the important interacting residues in non-peptide antagonist binding site of the NK1 receptor. The protein was held rigid while the ligands were allowed to be flexible during refinement. The docking and scoring methodology was first validated by docking of ligand CP-96345. The docked position of CP-96345 overlaps well with the homology model position,

Results and discussion

Our previously synthesized compound, 2-[4-(4-ethylbenzamido)phenyl]benzothiazole (BSN009) was found as an active compound at a concentration of 50 μM as a result of MTT assay and inhibited colon cancer cell lines growth about by 57.53%. On the other hand, it has also been found that BSN-009 had no toxic effect on the normal cell line.

As a result of the molecular docking studies; BSN-009 was shown similar binding modes with NK1 receptor as known antagonists L-733,060, aprepitant, and L-732,138. The tested 2-substituted benzothiazole, BSN009, has hydrogen bonds with Gln165 (2,39 Angstrom) and His197 (1,83 Angstrom) like other non-peptide antagonists of NK1 receptor Oxygen atom of carbonyl group of BSN009 makes an H bond with His197. The phenyl ring of the benzamide group of BSN009 has a pi-cation interactions with His187. Binding energy values (kcal/mol-1) of BSN009 and aprepitant, which is a well-known NK1 receptor antagonist, is close to each other.

Conclusion

In conclusion, the performed molecular docking study elucidated that Gln165, His197, His265 and Tyr287 are crucial amino acids in the non-peptide binding site of the NK1 receptor. As a result of the molecular docking study and cytotoxic experiments, it can be concluded that BSN009 may be a good anticancer drug candidate as an NK1 receptor antagonist and is worthy to carry on the anticancer in vivo studies. This study also provide a model to design novel and more potent antitumor agents as NK1 receptor antagonists.

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Macedonian pharmaceutical bulletin, **62** (suppl) 555 - 556 (2016)
ISSN 1409 - 8695
UDC: 615.213.015.4
Short communication

Application of isocratic hydrophobic index obatined by RP-TLC of some succinimide derivates in QSA(P)R studies

Jelena Curcic[1]*, Natasa Milosevic[1], Vesna Kojic[2], Natasa Milic[1], Gordana Uscumlic[3], Nebojsa Banjac[4]

[1]*Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 1-3, 21000 Novi Sad, Serbia*
[2]*Oncology Institute of Vojvodina, University of Novi Sad, Put Doktora Goldmana 4, 21204 Sremska Kamenica, Serbia*
[3]*Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia*
[4]*Faculty of Agriculture, Food Technology and Biochemistry, University of Belgrade, Nemanjina 6, 11080, Belgrade – Zemun, Serbia*

Introduction

Drug-like property optimization offers significant opportunities for enhancing drug discovery. Development of a new drug is connected with quantitative structure-activity (properties) relationship, QSA(P)R, research. Lipohilicity is the key physicochemical property of a molecule which depends on its structure and determines its pharmacokinetic behavior and biological activity. Due to its high cost-effectiveness and fair reproducibility, thin-layer chromatography (TLC) is one of important alternatives for quantifying lipophilicity of drug candidates. The aim of this study was to calculate hydrophobicity index C_0 with reversed phase (RP) TLC methods of 14 compounds with assumed antiepileptic activity and to examine whether C_0 values can be applied in QSA(P)R studies.

Materials and methods

RP TLC was used for study of the retention behavior of N-(3- or 4- substituted phenyl)-2,2,-diphenyl-succinimide derivatives. Substance number 1 (s1) is not substituted, s2-s11 have attached in para position: $-CH_3$, $-OCH_3$, -OH, $-NO_2$, -CN, -F, -Cl, -Br, -I, $-COCH_3$, while s12-s14 have in position meta -CN, -OH and -Cl, respectively. Precoated RP-18W/UV254 10×10 cm plates (Macherey-Nagel GmbH and Co., Düren, Germany) were used as stationary phase and binary solutions of water with acetonitrile with a varying volume fraction, φ (0.45-0.65) of organic solvent, were the mobile phase. Each analyzed compound was dissolved in acetone (c=2mg/ml) and 0.2 µl aliquots with a micropipette were spotted on the chromatographic plates. Ascending technique with previous saturation of the chambers was used. After development, the spots were detected at 254 nm with UV lamp and Rf values were measured. For each compound analyzed in this paper, logP values (logarithm of the partition coefficient of the compound) and logD values (log of the distribution coefficient of the compound at pH=7.4) were determined by the usage of different algorithms (www.alogps.com and www.acdlabs.com). Physicochemical parameters as number of rotatable bonds (RB), numbers of hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), polar surface area (PSA) and molar weight (MW) were obtained, as well. Finally, for each compound k_a (constant of absorption in min^{-1}) and logBB (logarithm of blood-brain barrier, BBB permeability) were predicted (www.acdlabs.com). All calculations and statistical analysis were done by use of software package Origin 8.0.

Results and discussion

Retardation factor, R_M was calculated for each compound according to the equation: $R_M=\log(1/R_f-1)$. Furthermore, in the linear correlation of the R_M values versus φ, the chromatographic retention constant R_{M0}, was obtained as extrapolated value to 0% point: $R_M=R_{M0}+S\times\varphi$. The isocratic chromatographic hydrophobicity index, C_0, is calculated as R_{M0}/S and it is defined as point when the amount of

* curcicjelena@yahoo.com

the analyte in the mobile and the stationary phase is equal. Since, C_0 is usually applied for quantifying lipophilicity of the compound; it has been compared to the computer calculated logP values. Highly significant linear correlation with good correlation coefficients has been observed between the C_0 values and in silico logP/logD values (r^2=0.78, r^2=0.94 and r^2=0.77 for MlogP, ClogP and logD, respectvely) in which substance with OH group in position meta (s13) was an outlier. It has been assumed that s13 forms an intramolecular hydrogen bond since OH group as HBD is close to dione group which is HBA. Probably due to this kind of interactions, s13 has reduced affinity to form hydrogen bonds with water and to be eluted with the mobile phase when compared to its isomer, s4 which does not have possibility to form an intramolecular hydrogen bond.

Regarding the physicochemical characteristics and relationship with "druglikness" properties of the compounds investigated, according to Lipinski's rule of 5, poor absorption or permeation are more likely for compounds which have >5 HBD, MW>500, logP>5 and >10 HBA. As previously reported, none of the compounds violates even single rule. Moreover, additional rules for good oral bioavailability were proposed by Veber: ≤10 RB, ≤140Å^2 PSA, or ≤12 HBD+HBA. Analyzed compounds do not violate Veber rules hence good oral absorption can be expected. According to in silico data absorption rate is practically identical for all the examined compounds (k_a between 0.051 to 0.053 min^{-1}). The main assumption is that the effect of the core consisted of three phenyl groups attached to succinimide ring may overwhelm that of the substituent and slight changes in lipophilicity determined by C_0 practically do not affect their absorption rate. Physicochemical properties influence the passive BBB permeation of compounds. Set of BBB rules was compiled by Clark: N+O<6, PSA<60–70Å^2, MW<450, logD=1–3, ClogP–(N+O)>0 in order for the compound to permeate through BBB. All compounds violate one or two rules (high logD and/or PSA), while s5 violate four rules. Nitro group has defined positive and negative poles and it is partially ionized inhibits BBB permeation of s5. Since BBB permeability depends on lipophilicity (Di et al., 2003), logBB was presented as function of C0 and square equation with good statistical quality (r^2=0.658, p<0.001) was obtained.

Conclusion

For the analyzed succinimide derivates, experimentally obtained C_0 values are comparable to the in silico determined logP/logD values with s13 excused from the comparison. Retention parameter, C_0 can be proposed for expressing lipophilicity of the analytes examined in this paper and it can be applied in QSA(P)R studies.

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Macedonian pharmaceutical bulletin, **62** (suppl) 557 - 558 (2016)
ISSN 1409 - 8695
UDC: 616.61-008.64-052
Short communication

Individualization of therapy in patients with renal impairment

Jelena Curcic*, Mladena Lalic Popovic, Svetlana Golocorbin Kon, Natasa Milic, Maja Milanovic, Natasa Milosevic

Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 1-3, 21000 Novi Sad, Serbia

Chronic kidney disease (CKD) is a common, progressive condition that affects over 13% adult population in US only and the number of patients with end-stage renal disease progressively increases every year. The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) classifies CKD into five stages according to estimated glomerular filtration rate. Glomerular filtration rate (GFR) has been usually estimated based on equation that incorporates serum creatinine concentration (SCR) along with demographic data and it is measured in ml/min per 1,73m2: the first stage has GFR >90 ml/min per 1.73 m2 and the second one between 60-89 ml/min/1.73m2, while stages 3, 4 and 5 are being characterized with GFR 30-59, 15-29 or <15 ml/min/1.73m2, respectively.

Based on CKD stage disease individualization of therapy is required especially for drugs which are mainly eliminated through urine and/or have narrow therapeutic index. Other conditions which may required individual dosage regimen are also: application of nefrotoxic drugs, conditions which may lead to development of chronic renal disease as hypertension, diabetes mellitus etc. Furthermore, severe hepatic dysfunction is usually accompanied by some renal impairment (hepato-renal syndrome). Reduced renal excretion has been reported in patients with severe cirrhosis (Child–Pugh class C) for a number of drugs mainly eliminated by renal excretion in unchanged form. Calculated creatinine clearance (CLCR) based on serum creatinine concentration is the most convenient method to estimate GFR as it requires only a single blood sample. As serum creatinine is so highly dependent on age, gender, and body size, a number of formulas and corrections have been developed to estimate the muscle mass and assumed creatinine production (Verbeeck and Musuamba, 2009).

One of the oldest method for GFR estimation in adults is Cockcroft-Gault formula: GFR(ml/min/1.73m2)=[140-age(y)×body weight(kg)]/SCR(mg/dL)×72] multiplied with 0.85 if the patient is women. Estimated creatinine clearance may be calculated also based on Modification of Diet in Renal Disease (MDRD) formulas. MDRD1 formula beside the concentration of creatinine in serum requires additional information as urea concentration in serum, Cu in mg/dL and albumin concentration in serum, alb in g/dL and it is given as: GFR=170×SCR exp(-0.999) × age exp(-0.176) × (0.762 for women) × (1.180 for black) × Cu exp(-0.170) × alb exp(0.318). However, MRDR2 formula requires only serum concentration level i.e.: GFR=186×SCR exp(-1.154)×age exp(-0.203)×(0.742 for women)×(1.212 for black). Moreover, for patients whose GFR is over 60 ml/min/1.73m2 it may be calculated by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation which is considered to give more precise estimation: GFR=a×[SCR(mg/dL))exp(c)/b]×[0.993 exp(age)] where a is for white race 141 for male and 144 for female, and in the black race 163 for male and 166 for female, b is 0.9 for male and 0.7 for female and while c is -0.411 if serum creatinine levels is <0.9 mg/dL and -1.209 if serum creatinine levels is >0.9 mg/dL in male or -0.329 if SCR is <0.7 mg/dL and -1.209 if SCR levels is >0.7 mg/dL in female. If the patient is overweight/obese (BMI >25 kg/m2 according to WHO), Salazar-Corcoran method should be used: GFR is estimated as GFR=[(137-age)×(0.285×body weight(kg)]+12.1×height(m)×height(m)]/[51×SCR(mg/dL)] in male while in female patients the estimation is given as GFR=[(146-age)×(0.287×body weight(kg)]+9.74×height(m)×height(m)]/[60×SCR(mg/dL)].

In children, Schwartz adjusted method is used for estimation of renal impairment degree i.e.: GFR=[k×height(cm)]/SCR(mg/dL) where k=0.33 for pre-term newborns age under 1 year, k=0.45 for full term in-

* curcicjelena@yahoo.com

fants age<1, k=0.55 for children age 1-12 and girls in puberty and 0.7 for boys in puberty (age >12). If the children are obese, Léger (Léger et al., 2002) formula is being applied: GFR=[56.7×body weight(kg)+0.142×height(cm)×height(cm)]/[88.4×SCR(mg/dL)].

GFR can alternatively be calculated based on the serum concentration of cystatin C. Cystatin C, is a small, nonglycosylated basic protein, produced at a constant rate which renal elimination occurs only by glomerular filtration with no tubular secretion, and only minimal extrarenal elimination. The blood concentration of cystatin C depends almost entirely on the GFR and is not substantially affected by diet, nutritional status, or inflammatory or malignant diseases. If cystatin C is being determined by PENIA method GFR can be estimated as: GFR=80.35×cystatin C(mg/L)-4.32, while if it is determined by PETIA method GFR should be calculated as GFR=86.49×cystatine C(mg/L)-1.68(×1.384 for children <14) (Chew et al., 2008).

Once the GFR is determined, individual dosage regimen in patient with chronic kidney disease can be applied based on the following formula: D*=D×Q where D is the maintenance dose for patient with normal renal function, D* is maintenance dose for patient with renal impairment and Q is being calculated as Q= fnonr + fr×(CLCR*/CLCR) i.e. Q=(1-fr) + fr×(CLCR*/CLCR) where CLCR is clearance creatinine in patient with normal renal function and CLCR* is the calculated clearance creatinine for the patient who requires individual dosage regimen, fr is the fraction of drug eliminated by urine and fnonr is the drug fraction eliminated extrarenal. The normal range of GFR, adjusted for body surface area, is 100-130 ml/min/1.73m2 in men and 90-120 ml/min/1.73m2 in women younger than the age of 40 and in pharmacokinetic calculations for CLCR it is usually used 100 ml/min/1.73m2. In children, GFR is 110 ml/min/1.73m2 until 2 years of age in both sexes, and then it progressively decreases. Hence when new dose is being calculated in patient with renal impairment and in comparison with ones who have normal renal function CLCR=110 ml/min/1.73m2 is being used for children under the age of 2 and 100 ml/min/1.73m2 for older (Doogue and Polasek, 2011).

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Macedonian pharmaceutical bulletin, **62** (suppl) 559 - 560 (2016)
ISSN 1409 - 8695
UDC: 616.61
Short communication

Distribution coefficients of novel coumarin derivatives

Lulzime Ballazhi[1]*, Elena Dogazanska[1], Faik Imeri[2], Ahmed Jashari[3], Emil Popovski[4], Goran Stojkovikj[4], Bozhana Mikhova[5], Kristina Mladenovska[1]

[1]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*
[2]*Institute of Physiology, University of Zürich, CH-8057, Zürich, Switzerland*
[3]*Faculty of Natural Sciences & Mathematics, State University of Tetovo, Pashe Deralla bb, 1200 Tetovo, Republic of Macedonia*
[4]*Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Arhimedova Str. No. 3, PO Box 162, 1000 Skopje, Republic of Macedonia*
[5]*Bulgarian Academy of Sciences, Institute of Organic Chemistry with Centre of Phytochemistry, Acad. G. Bonchev 9, 1113 Sofia, Bulgaria*

Introduction

It has been known for many years that coumarins have versatile structure, significant therapeutic potential and are present in many natural therapeutic products (Riveiro et al., 2010). They have multiple biological activities, including anticoagulant (Mousa, 2002), anti-inflammatory (Hadjipavlou-Litina et al., 2007), antimicrobial (Nimavat et al., 2009) and anticancer activities (Belluti et al, 2010). In search for new chemotherapeutics against based on coumarin we have synthesized eight compounds that combine coumarin core and izoxazoles or thiazoles in hydrazinyldiene-chroman-2,4-diones (Jashari et al., 2014; Ballazhi et al., 2014; 2015). Three of the eight compounds, 3-[2-(1,3-thiazol-2-yl)hydrazinylidene]chroman-2,4-dione (**1**), 3-[2-(4-methyl-1,3-thiazol-2-yl)hydrazinylidene] chroman-2,4-dione (**2**) and 3-[2-(4,5-dimethyl-1,3-thiazol-2-yl)hydrazinylidene]chroman-2,4-dione (**3**), have been shown to have most potent antiproliferative effects and induced apoptosis in breast cancer cells (Jashari et al., 2014) and bone and lung metastatic cell lines from breast cancer (Ballazhi et al., 2015). In addition, synergistic effects of tamoxifen (Ballazhi et al., 2014) and doxorubicin (Ballazhi et al., 2016, *submitted for publication*) with these compounds were evaluated and apoptotic effect was confirmed. These compounds were further subjected to physicochemical characterization. In this study their distribution coefficients (logD) in three pH environments were evaluated and compared with the anticipated ones from different software packages.

Materials and methods

4-Hydroxycoumarine (4-HC, reference), n-octanol and the compounds for preparation of phosphate buffer saline (PBS), NaH_2PO4, NaOH and KH_2PO4, were purchased from Merck KgaA (Germany). The tested compounds were synthesized by the procedure previously described (Jashari et al., 2014; Ballazhi et al., 2015). The experimentally obtained distribution coefficients ($logD_{exp}$) between n-octanol and phosphate buffers (pH 7.4, NaH_2PO_4-NaOH, 0.15 M, I = 0.397 M; pH 6.8, NaH_2PO_4-NaOH, 0.15 M, I = 0.397 M; and pH 5.8, Na_2HPO_4-KH_2PO_4-NaOH, 0.15 M, I = 0.6, temp. coef. -0.0028/^oC) were determined by shake-flask method (Ballazhi et al., 2016, *submitted for publication*). The experiments were performed in the systems n-octanol : PBS = 2:1; 1:1; 1:2 (vol), in triplicate. The mixtures of the organic and aqueous phase containing the compounds were shaken on a mechanical shaker for 2 h (shake-water bath, Haake, 25±0.1 ^oC). After equilibration, they were centrifuged for 10 min at 5000 rpm, the n-octanol phase was removed and in both phases the concentrations of the compounds were assayed spectrophotometrically (UV/VIS Perkin-Elmer System, USA) at λ_{max} 290 nm for 4-HC and 410-420 nm for the tested compounds. The $logD_{exp}$ were calculated as the ratio between molar

* lulzime.ballazhi@unite.edu.mk

concentration in n-octanol and aqueous phase. All experiments were made in triplicate. The $logD_{7.4exp}$ values were compared with the calculated ones ($logD_{calc}$) obtained by ChemAxon's software (www.chemicalize.org/), Vega NIC (www.vegaNIC.com), ChemDraw (www.cemistry-software.com), vcclab (www.vcclab.org)-ALOGPS 2.1 and molinspiration (www.molinspiration.com).

Results and discussion

The $logD_{7.4exp}$, $logD_{6.8exp}$ and $logD_{5.8exp}$ values determined for monobasic acid 4-HC were in accordance with the literature data as well as the partition coefficient of the un-dissociated 4-HC in pH 7.4, logP 2.66, which is similar to the value 2.37 determined by van der Giessen and Janssen (1982). When n-octanol/PBS ratio was 1:1, the $logD_{7.4exp}$ ranged between -0.065±0.003 (1) and 0.957±0.038 (3), $logD_{6.8}$ between 0.153±0.001 (1) and 0.996±0.008 (3) and $logD_{5.8}$ between 0.894±0.011 (1) and 1.886±0.018 (3). Small differences and the same tendency were observed between the corresponding $logD_{exp}$ when determined in systems with different n-octanol/PBS ratios. Large discrepancy was noted between $logD_{7.4exp}$ and $logD_{7.4calc}$ values. However, when a simple regression was performed, there was found a strong correlation between the $logD_{7.4exp}$ and $logD_{7.4calc}$ ($0.986 \leq R \leq 0.999$). Depending on the software used, predictions are composed of the molecules' atomic increments, selected atom types to accommodate electron delocalization and addition of contributions of ionic forms. logD of zwitterions is calculated from the logD at the p*I*. Also, the effect of hydrogen bonds on logD is considered if the formation of a six membered ring between suitable donor and acceptor atoms can take place. As the logD values are pH-dependent, the logD calculation relies on the pKa prediction process. Considering data determined in the study, the smallest difference was observed when software package VegaNic was applied ($1.189 \leq \Delta_{(logD7.4calc-logD7.4exp)} \leq 1.527$ for 4-HC; $0.682 \leq \Delta_{(logD7.4calc-logD7.4exp)} \leq 1.096$ for 1; $0.814 \leq \Delta_{(logD7.4calc-logD7.4exp)} \leq 1.393$ for 2; $1.075 \leq \Delta_{(logD7.4calc-logD7.4exp)} \leq 1.488$ for 3), which is based on two well-known molecular descriptors: ALogP (hydrophobicity contribution of 120 atom types) and MlogP (13 structural parameters). The large discrepancy between the predicted and experimentally determined values suggests that the structures of the coumarin derivatives in the aqueous phase and/or in the organic phase are significantly different from the assuming structures. One of the reasons could be the tautomeric behavior of these compounds.

Conclusion

Distribution coefficients of novel coumarin derivatives in different pH mediums were determined. The derivative having thiazole moiety with additional methyl groups attached to the carbons at the positions 4 and 5 (3) showed the highest lipophylicity. Further studies are needed for complete physico-chemical and PK/PD characterization of the synthesized compounds.

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Macedonian pharmaceutical bulletin, **62** (suppl) 561 - 562 (2016)
ISSN 1409 - 8695
UDC: 616-097-085:602.9
Short communication

Mesenchimal stem cells as a new approach in treatment of systematic lupus erythematosus

Magdalena Vrchkovska[1]*, Tatjana Dimitrovska Manojlovik[2] , Marija Vrchkovska[3]

[1]*Pharmacy Organization Medika Karta, Partizanska 110, 7000 Bitola, R. Macedonia*
[2]*DIC / Clinical hospital "D-r Trifun Panovski", Partizanska bb, 7000 Bitola, R. Macedonia*
[3]*Centar for Public Health, Bitola, Partizanska bb, 7000 Bitola, R. Macedonia*

Introduction

Systematic lupus erithematosus (SLE) is multi sistematic autoimmune disease with wide range of clinical symptoms on almost every organ and tissue, with a high level of morbidity and mortality as a result. There is no clear cause for SLE and is hard to be cured (Bertsias et al., 2012).

In recent years, with increasing levels of health care, there are new drugs to treat SLE. Although the survival rate from 1990 till today has increased by 68% (Urowitz and Gladman, 2000) due to the use of glucocorticoids and cytotoxic drugs, excessive use of these drugs leads to many side effects and death cases. Also, significant concerns related to opportunistic infections and secondary malignancy due to immunosuppressive treatments emerge the need for safer and more effective therapy. In recent decades, stem cell transplantation has emerged as a new treatment modality for refractory and severe SLE, mainly hematopoietic stem cell transplantation (HSCT) and mesenchymal stem cell transplantation (MSCT). The study of Liang and Sun (2015) is related to the rationale and current status of the MSCT in the treatment of SLE.

SLE therapy

If the HSC defects are from aquired response, autologus HSCT can be a treatable method after removing the cause of risk, and making reconstruction of the immune system with deleting the auto-reactive lymphocyte clone of hematopoietic origin. No matter of any of this, if genetics is a dominant factor, allogenetic HSCT will be more effective and logically applicative approach (Liang and Sun, 2015).

In the most cases, patients are treated with an allogenetic HSCT for SLE and concominant hematological diseases, and will have complete remission from the first and second conditions. But, beside the encouraging result, there might be some problems with HSCT treatment in SLE (Kushida et al., 2001).

Allogenetic HSCT seems to have a huge curative potential, but also, comes with enormous risk of GVHD (graft versus host disease) that have high occurrence and hold responsibility for worst results of allogenetic transplantation in hematologic disease, with limitation of wider consideration of in autoimmune disease. GVHD are linked with some symptoms, like acute organ toxicity, delayed reconstitution of immune system, increase TRM (transplantation-related mortality), longer remaining of autoreactive lymphocytes, and high rate of opportunistic infections. As a conclusion of above written, autologus and allogenetic HSCT can't be considered as best stem cell therapy (Bertsias et al., 2012).

In recent days, MSCT is new, hopeful stem cell therapy for SLE. Mesenchymal stem cells are multipotent, non-hematopoietic cells that are being considered as a promising new treatment for tissue regeneration. It has demonstrated that mesenchymal stem cells can differentiate into bone and cartilage, and have immunomodulatory effects on T-cells and B-cells. They also can't be detected by the immune system because they lack co-stimulatory molecules.

Combination of anti-proliferative and immunomodulatory characteristics of MSCs and their immunological privilege will make a new approach in treatment of variety of autoimmune inflammatory diseases. By today, MSCs are used in cases with multiple sclerosis, neuromyelitis optica, chronic and acute GVHD.

* magivrckovska@yahoo.com

Combinations of MSC deficiency and SLE began with making some experiments. Transplantation of allogeneic HSCs with bones to get MSCs is a path for prevention of the recurrence of autoimmune diseases in MRL/lpr mice, a murine model of lupus, in cases of easy relapse if HSCs are infused alone. Also, a positive response is found in transplantation with allogeneic HSCs and MSCs namely it increased survival level of MRL/lpr mice, but also with bone marrow transplantation (Ishida et al., 1994).

The named above results stress deficiency in lupus MSC populations and gave a strong impact for greater use of MSCT in lupus treatment, it is hard to see abnormality is from genetic or acquired factors is alike. This problem is very important in HSCT also, because it will determine the best and appropriate source for MSCT, or it would be autologous or allogeneic.

The first study of MSC infusions in humans is the one from 1995, which demonstrates the appropriance of ex vivo expansion and subsequent infusion of autologous MSCs in 15 patient volunteers. From that point, MSCT was considered as a great tool in the usage of engraftment, management graft failure, prevention or treatment of GVHD after HSCT, and treatment of several other autoimmune diseases (Liang et al., 2010).

The most important result of human MSC therapy is arising from clinical trials which have an aim of severe and refractory SLE patient, even with mechanisms with which MSCs conduct their functions of immune modulation and are still not completely understood, but will involve variety of paths. Affiliated Drum Tower Hospital (ADTH) of Nanjing University Medical School is the very first section involved in in MSCT for treatment of autoimmune disease. More than 300 patients with active and persistent SLE, refractory to standard treatment, was under treatment with allogeneic MSCT. The treatment was due from March 2007 through December 2013.

Four patients had a great improvement in creatinine levels in serum. Anti-dsDNA titers decreased after 1 month post-MSCT in all cases. All patients could lower the doses of steroids and CTX, and also there were two cases where patients weren't taking CTX anymore after 6 months results. There was no GVHD and TRM after allogenetic MSCT (Liang et al., 2010).

Results clearly show that MSCs have large therapeutic potential but also have many problems that have to be solved for making way for MSCT to treatment of lupus. One of the problems is getting standard cell production without considering heterogeneity, potential, influence of expansion media on the phenotype, and suitability of the source. Some factors possible change capability of expansion, potency and phenotype, like age of donor and condition of growth, for even the surface makers of MSCs (Liang and Sun, 2015).

Lupus is a mesenchymal stem cell disease, not only hematopoietic, in light of defects in HSCs and also MSCs in SLE patients. At recent days risks of GVHD are limited in great range for the clinical use of allogenetic. However, allogenetic MSCT will be better and more attractive to use then the allogenetic HSCT in treatment of lupus, with preliminary results in efficacy and safety. This will make a new platform in treatment of SLE patients with refractory and severe disease (Liang and Sun, 2015).

Conclusion

The number of patients with SLE in our country is higher to date and unfortunately in Macedonia, doctors use conventional therapy in treatment of SLE. That arise a need for implementation of the latest method in treatment of SLE with aim to improve the quality of life. Also, the number of hospital days will be lower, as well as the need of cure in countries abroad, and costs for medicines. The most important result will be improved quality of patinets` life, where people will have longer life and lower rate of mortality. For that cause, we should promote implementing conditions where this treatment will be in use, with simplifying and making life better for SLE patinets.

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Macedonian pharmaceutical bulletin, **62** (suppl) 563 - 564 (2016)
ISSN 1409 - 8695
UDC: 615.277
Short communication

May bile acids be utilized to enrich oncological armamentarium?

Bojan Stanimirov, Nebojša Pavlović*, Karmen Stankov, Maja Đanić, Vesna Kojić, Svetlana Goločorbin-Kon, Momir Mikov

Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Republic of Serbia

Introduction

Bile acids, a main product of cholesterol catabolism, have traditionally been considered as intestinal emulsifiers of lipophilic xenobiotics. Due to an amphiphathic structure, bile acids have been recognized to facilitate a transport of various substances across biological membranes, influencing their physiological and pharmacological activity (Stojančević et al., 2013). During the past fifteen years bile acids were endowed with a hormone function since these steroid molecule species regulate numerous metabolic processes, determine metabolism and cell fate not only in enterohepatic tissues, but also on a systemic level (Stanimirov et al., 2015). In addition, bile acids and bile acid-activated receptors play a profound role in the process of carcinogenesis; however, this function has not been clearly elucidated jet. All of these peculiar functions are mediated by the interaction of bile acids with several proteins including nuclear and G-protein-coupled receptors as well as cell kinase pathways (Stanimirov et al., 2012). This raises the complexity of bile acid functions on a novel level, emphasizing their role not only as a passive carrier that promotes transport of pharmacological agents across biological membranes, but also as the agents with potential pharmacodynamic function. Doxorubicin is one of the most potent and most commonly used antineoplastic agents for the treatment of both solid and hematological malignancies. Doxorubicin induces the programmed cell death in malignant cell through several pathways, including the DNA strand breaks by topoisomerase 2α inhibition, DNA alkylation, oxidative stress and oxidative damage of nucleic acids, proteins and lipid membranes, ATP depletion etc. However, cumulative, dose-dependent toxicity of this agent may result in hepatotoxic and cardiotoxic events which cast shadow on the quality of life of oncological survivors. Therefore, the enhancement of doxorubicin function along with reducing dose-dependent undesirable effects is one of the main challenges in developing novel doxorubicin formulations. The aim of this study was to determine an effect of secondary bile acid, ursodeoxycholic acid, on the cytotoxic activity and proapoptotic potential of doxorubicin in vitro, in a model of human breast adenocarcinoma.

Materials and methods

Human breast adenocarcinoma MCF-7 cells were treated with increasing concentrations of doxorubicin and ursodeoxycholic acid, and the cytotoxic activity was assessed using colorimetric MTT test. Cells were also simultaneously treated with selected concentration of doxorubicin and the concentration of ursodeoxycholic acid within the range of physiological level. The quantitative analysis of expression of genes involved in apoptosis, Bax and Bcl-2, was assessed using real-time quantitative PCR method. Gene expression was calculated using ΔΔCt method for relative quantification and normalized to β-actin as a housekeeping gene. The statistical analysis was performed using one-way analysis of variance with SPSS statistical software and if the $p \leq 0.05$the results were considered as significant.

Results and discussion

Both doxorubicin and ursodeoxycholic acid expressed dose-dependent cytotoxic activity with half inhibitory

* nebojsa.pavlovic@mf.uns.ac.rs

concentration (IC50) values of 0.64 μM and 320.5 μM, respectively. When the cells were co-treated with 0.25 μM of doxorubicin and non toxic concentration of ursodeoxycholic acid, the cytotoxicity increased up to 39.6% ($p \leq 0.05$), compared to doxorubicin alone which inhibited 33% of cell growth. This result indicates that the addition of ursodeoxycholic acid may significantly increase doxorubicin-induced cytotoxicity in MCF-7 cell line. In the following step we wanted to dissect the effects of ursodeoxycholic acid on doxorubicin-induced apoptosis by determining the expression of apoptosis-related genes Bax and Bcl-2. The expression of a pro-apoptotic gene Bax in cells treated with doxorubicin alone was 3.22 fold higher ($p < 0.01$), and in the group of cells co-treated with doxorubicin and ursodeoxycholic acid was 2.28 fold higher in comparison with control group ($p < 0.01$). The expression of anti-apoptotic gene Bcl-2 in the doxorubicin treated cells was 1.42 fold higher ($p < 0.01$), whereas in co-treated cells was 1.25 fold decreased compared to the control ($p < 0.01$). These results indicate that co-treatment with ursodeoxycholic acid reduced the expression of both apoptosis-related genes compared to cells treated with doxorubicin alone, which is in agreement with well known antioxidant and apoptosis-reducing function of ursodeoxycholic acid (Perez and Briz, 2009). When we assessed the ratio of Bax to Bcl-2, a parameter that determines the susceptibility of cells to the apoptosis, the values of this ratio in doxorubicin treated cells was highly significantly increased in both doxorubicin-treated and ursodeoxycholic acid-co-treated cells, 6.26 ± 0.7 ($p < 0.01$) and 7.89 ± 1.1 ($p < 0.01$), respectively, compared to control group of cells 2.9 ± 0.9. Even though the value of Bax/Bcl-2 ratio in the group of co-treated cells was higher compared to doxorubicin treated cells, the difference was not statistically significant. However, the results were in accordance with the results of MTT test indicating that the co-treatment with ursodeoxycholic acid had driven MCF-7 cells into apoptosis in greater extent than treatment with doxorubicin alone.

Conclusion

The simultaneous treatment with ursodeoxycholic acid in non-toxic concentrations and doxorubicin significantly increased the cytotoxicity towards MCF-7 cell line, by promoting the programmed cell death, apoptosis, at least on the transcriptomic level. Therefore, ursodeoxycholic acid may be exploited as useful agent in developing novel pharmaceutical formulations or antineoplastic strategies relying on doxorubicin protocol.

Acknowledgment

Supported by the Ministry of education, science and technological development, the Republic of Serbia, Grant III41012.

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Macedonian pharmaceutical bulletin, **62** (suppl) 565 - 566 (2016)
ISSN 1409 - 8695
UDC: 616.348
Short communication

Prediction of binding affinities of different bile acids towards multidrug transporters in Lactobacillus acidophilus NCFM - a pharmacoinformatic approach

Maja Đanić[1]*, Nebojša Pavlović[1], Bojan Stanimirov[1], Tijana Stojančević[2], Svetlana Goločorbin Kon[1], Momir Mikov[1]

[1] *Faculty of Medicine, Hajduk Veljkova 3, 21000 Novi Sad, University of Novi Sad, Serbia*
[2] *Faculty of Science, Trg Dositeja Obradovića 3, 21000 Novi Sad, University of Novi Sad, Serbia*

Introduction

Bile acids are endogenous amphiphatic steroid molecules able to modulate transport of drugs and other xenobiotics across various membranes through both, paracellular transcellular routes (Djanic et al., 2016a; Golocorbin-Kon et al., 2009, Lalić-Popović et al., 2013, Mikov and Fawcett 2006, Stojancevic et al., 2013). The effect of bile acids on drug transporters is currently a challenging topic due to consequences on efficacy and safety profiles of drugs. Research on this topic has been mostly restricted to the impact of bile acids on the eukaryotic drug transporters what is confirmed by several studies (Yang et al., 2011). Up to date there has been no study on bile acids interactions with multidrug transporters in probiotic bacteria. Given that bile acids interact with intestinal microbiota as significant drug-metabolizing system that may be the cause of inter- and intra- individual differences in drug metabolism (Stojancevic et al., 2014) the purpose of this study was to use computational servers and softwares to assess the binding affinities of different bile acids towards bacterial multidrug transporters and to propose the mechanism of their influence on drug transport through bacterial membrane (Djanic et al., 2016b).

Materials and methods

As a representative of gut microflora, Lactobacillus acidophilus NCFM (LA) is chosen, as the most commonly commercially used probiotic bacterial strains and also abundant part of gut microflora. Docking study was carried out to estimate the binding affinities of three different bile acids: cholic acid (CA), 12-monoketocholic acid (MKC) and deoxycholic acid (DCA) to drug membrane transporters in LA. Docking step was performed using molecular docking program SwissDock web service which uses calculations performed in the CHARMM force field with EADock DSS. The lower estimated free energy of binding indicates the higher binding affinity. The list of multidrug transporters for LA was obtained from relational database – TransportDB, The amino acid sequence of all studied transport proteins were obtained from NCBI database in FASTA format. The 3D structures of proteins were predicted by I-TASSER server (Iterative Threading ASSEmbly Refinement). The obtained 3D structures are given a confidence score (c-score) which was in the range of [-5, 2] where a higher value indicates a model with a more reliable structure and vice-versa. Proteins with c-score below -1.5 were not taken into further consideration and were not analyzed in docking studies. 3D structures of CA and DCA in mol2 format were obtained from ZINC database. Structure of MKC was drawn in ACD/ ChemSketch, a freeware for chemical structure drawing and subsequent optimization from ACD Labs.

Results and discussion

According to data available at TransportDB database, the total number of multidrug transport proteins in LA was 30, 14 from ATP-binding cassette (ABC) superfamily and 16 from secondary transporters. The majority of predicted

* majadjanic@gmail.com

3D structures had a high confidence score reflecting the reliability of the obtained structures. Four proteins did not fulfill these criteria and were not further analyzed in docking studies. In other words, 26 transporters were included in docking studies.

The molecular docking results revealed that studied bile acids exhibit different binding affinities towards multidrug transporters in LA. The lowest binding energy for the majority of examined transporters was estimated for MKC. Namely, from all 26 examined transport proteins, 13 (50%) gave the lowest binding energy in combination with MKC. The most prominent effect of MKC was observed in the cases of LBA1821 from ATP family and LBA0753 from the family of secondary transporters.

Analyzing docking results, the second ranked bile acid was CA that gave the lowest docking result with 7 of 26 (26.9%) transporters. The most notable differences in docking score, regarding the CA was estimated for LBA0575, ABC transporter in LB. For only 6 transporters (23.0%), DCA was estimated to have the highest affinity compared to other examined bile acids. This is the most expressed in the case of LBA0552, secondary transporter.

The correlation between hydrogen bond interactions and estimated free binding energies for all studied transport proteins revealed weak inverse relationship between the variables. Based on the calculated Pearson's coefficient, it may be concluded that, in addition to the hydrogen-bond interactions, the hydrophobic interactions also contribute to the stability of analyzed complexes.

Conclusion

The molecular docking results presented in this study revealed that studied bile acids exhibit different binding affinities towards multidrug transporters in LA that may be the consequence of different chemical structures. The greatest effect of MKC for the majority of studied transport proteins suggests that keto group at the position 12 has a significant influence on the properties of MKC and consequently, on the interactions with membrane transporters. These findings might have a role in the prediction of bile acids and probiotics influence on drug pharmacokinetics. Computational techniques are expected to increase its contribution to structural pharmacology investigation of membrane transporters and their implication in drug metabolism. However, in order to confirm the predictive strength of these computationally obtained results, further in vitro and in vivo experimental validation is highly recommended.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant No. 41012.

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Macedonian pharmaceutical bulletin, **62** (suppl) 567 - 568 (2016)
ISSN 1409 - 8695
UDC: 615.213.03
Short communication

Therapeutic drug monitoring as a tool for good clinical outcomes

Suela Kellici[1]*, Anyla Bulo[2], Joana Mihani[3], Jera Kruja[4]

[1] *Department of Pharmaceutical Technology and Biopharmacy, Faculty of Pharmacy, University of Medicine, Dibra Street 376, 1005, Tirana, Albania*
[2] *Service of Clinical Biochemistry, Faculty of Medicine, University of Medicine, Dibra Street 376, 1005, Tirana, Albania*
[3] *Service of Neurology, Faculty of Medicine, University of Medicine, Dibra Street 376, 1005, Tirana, Albania*
[4] *Albanian Order of Pharmacists, Elbasani Street, 94, Tirana, Albania*

Introduction

The principles of therapeutic drug monitoring (TDM) were first discussed after the year1960 and since then, this concept has evolved and is implemented in different modalities in different countries (Hallworth, 1993). The objective of TDM is to evaluate the drug concentration in order to maximize the pharmacotherapeutic effects and minimize the toxic effects (Gross, 2001). Several countries developed a specific service dealing with the therapeutic monitoring of drugs. In other countries this service is provided by the pharmaceutic department, clinical pharmacy department, or the clinical biochemistry department (Murphy et al., 2007).

The Therapeutic Monitoring of drugs is considered as a helpful instrument in case of drugs with a narrow therapeutic index, drugs highly bound to the proteins, drugs with high potential of interactions ecc. There are many factors influencing the therapeutic drug monitoring such as diseases, non-compliance, habits, sampling time ecc. However it is possible to retroactively evaluate the appropriateness of the interventions undertaken through the results obtained from monitoring of the serum drug levels (Burton et al., 2006).

Carbamazepine is an antiepileptic drug presenting a narrow therapeutic range and a complicated pharmacokinetics, thus representing a candidate drug to undergo therapeutic monitoring (Levy et al., 2002; Neels et al., 2004).

The objective of this study was to evaluate the appropriateness of the interventions through the therapeutic carbamazepine monitoring in the neurology service in the University Hospital Mother Teresa in Tirana.

Materials and methods

The medical records regarding the monitoring of the serum levels for carbamazepine over 6 years (2008-2010 and 2013-2015) were evaluated. Data analyses were performed using Chi square test. Information was received from the Clinical Biochemical Laboratory and the Neurology Service in the University Hospital Mother Teresa in Tirana.

Results and discussion

We analyzed all medical records for patients who underwent the measurement of the serum level of carbamazepine for two separated periods: 2008-2010 and 2013-2015. All these requests for the measurement of carbamazepine serum level were performed by neurologists in order to identify whether the missing therapeutic effect or the presence of toxicity was dedicated to the abnormal serum level of the drug. The overall number of carbamazepine measurements in the Clinical Biochemical Laboratory was for the years 2008-2010 respectively 286, 249 and 227; and for the years 2013-2015 the measurements were 34, 49 and 51 respectively. All measurements were performed through immunoassay methods. Standard dose regimens for carbamazepine resulted in insufficient serum concentration in 37% of the cases for the measurements performed in the years 2008-2010 and in 23% of the cases for the measure-

* skellici@gmail.com

ments performed in the years 2013-2015. Standard dose regimens for carbamazepine resulted in overdose concentrations in 34% of the cases for the measurements performed in the years 2008-2010 and in 21% of the cases for the measurements performed in the years 2013-2015. Clinicians' response to the abnormal concentrations of carbamazepine were appropriate in 65% of the interventions in the first 3 years and this appropriateness raised to 98% in the second three years of evaluation ($p<0.01$).

A limitation of our study consists in the evaluation of restricted information. It would be helpful if we could analyze also the factors leading to the abnormal serum levels of carbamazepine, the concomitant therapies which patients were taking at the time of serum level measurement.

Our study demonstrated better therapeutic outcomes after evaluation of the first period of TDM application on carbamazepine levels. This finding is in accordance with similar studies where better results were obtained after critical evaluation of the experience gathered (Patsalos et al., 2008; Shakya et al., 2008).

The evaluation of the therapeutic interventions through the therapeutic monitoring of carbamazepine showed that the experience gained over the first three years' experience with this evaluation brought to the better understanding of the real need to perform the therapeutic monitoring of carbamazepine. This led to a significant cost containment in performing this evaluation. The cost of the therapeutic monitoring procedures has been a continuous concern of the TDM service worldwide. Several studies have shown the benefits both in terms of therapeutic benefit for the patient and of economic benefit due to the implementation of the therapeutic drug monitoring for different drugs. By the other hand there are other groups of drugs which do not need to undergo continuous therapeutic drug monitoring (Eadie, 1995).

The therapeutic drug monitoring is applied in Albania through the Clinical Biochemical Laboratory within the University Hospital, since there is not yet in place e specific service dealing with therapeutic monitoring of drugs. A future challenge for the therapeutic drug monitoring in Albania represents the inclusion of the clinical pharmacists in this process. It has been demonstrated through several studies that the pharmacists can play a critical role as part of the multidisciplinary team driving the TDM process as they have the skills and knowledge to resolve drug therapy problems (Ratanajamit et al., 2009; Steinman et al., 2011). The combination of different health professionals involved in the therapeutic drug monitoring process, together with the recent technology developments, facilitates new applications in this field (Eliason, 2013).

Conclusion

The service of therapeutic drug monitoring can provide important information about the appropriateness of the clinical prescriptions and can therefore influence the outcomes of the drug therapy. The continuous evaluation of the medical interventions through the therapeutic drug monitoring interventions can improve the drug therapy resulting in better outcomes for the patient and in cost containment. The involvement of the clinical pharmacist in the multidisciplinary team can improve the performance of the therapeutic drug monitoring service.

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Macedonian pharmaceutical bulletin, **62** (suppl) 569 - 570 (2016)
ISSN 1409 - 8695
UDC: 615.272.4:616.153.915(=18)
616.153.915-085.272.4(=18)
Short communication

Impact of SLCO1B1 521T>C and 388A>G polymorphisms on response to atorvastatin in the albanian population

Arlinda Daka[1,2], Aleksandar Dimovski[1], Aleksandra Kapedanovska[1], Marija Vavlukis[3], Aleksandar Eftimov[1], Nadica Matevska Geshkovska[1], Sashko Kedev[3], Dashnor Nebija[2], Pranvera Breznica Selmani[2], Blerina Koshi[2], Kristina Mladenovska[1]*

[1]*Faculty of Pharmacy, Center for Biomolecular Pharmaceutical Analyses, University "Ss Cyril and Methodius" in Skopje, Blv. "Mother Theresa" 47, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Medicine, University "Hasan Prishtina", Mother Theresa 10 000, Prishtina, Kosovo*
[3]*University Clinic of Cardiology, University "Ss Cyril and Methodius" in Skopje, Blv. "Mother Theresa"17, 1000 Skopje, Republic of Macedonia*

Introduction

Atorvastatin is a potent inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase and it is widely used in the treatment of hypercholesterolemia and hypertriglyceridemia (Fu et al., 2013). However, despite its clinical efficacy, great variability in clinical response exists. Much attention is paid to the genetic factors affecting inter-individual differences in clinical response and several studies in this area point to single nucleotide polymorphisms (SNPs) in the gene *SLCO1B1* encoding multiple organic anion-transporting polypeptide 1B1 (OATP1B1). As a membrane influx transporter, OATP1B1 regulates the cellular/hepatic uptake of a number of endogenous compounds and drugs, incl. atorvastatin (Giannakopoulou et al., 2014; Rodrigues et al., 2010; Chen et al., 2011). Hence, *SLCO1B1* SNPs are attractive targets for analyzing their influence on statin differential response (Giannakopoulou et al., 2014). SNPs 521T>C (*5, rs4149056) and 388A>G (*1b, rs2306283) that encode alanine substitution of valine at amino acid 174 (p.Val174Ala) and amino acid change at position 130 (p.Asn130Asp), respectively, are considered as the most prevalent *SLCO1B1* variants relevant for the variability of drug response. c.388A>G variant has been associated with increased OATP1B1 transport activity of several substrate drugs *in vitro* (He et al., 2011), unlike c.521T>C variant for which reduced transport activity *in vitro* and increased plasma concentrations of several substrate drugs in the human body were observed (Kalliokoski et al., 2010). The aim of this study was to investigate the influence of *SLCO1B1* c.521T>C and c.388A>G variants on the lipid lowering effect of atorvastatin in a sample of Albanian population.

Materials and methods

Subjects and study design

Briefly, 64 adults (53% males, av. age 58 yrs. and BMI 28) with primary hypercholesterolemia, all of Albanian origin participated in the study. All the participants used atorvastatin once daily in a dose range of 10-80 mg. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the ICH Note for Guidance on GCP (CPMP/ICH/135/95). The final clinical trial protocol as well as the informed consent and other information that required pre-approval were reviewed and approved by the Independent Ethics Committee according to the applicable regulations.

Methods

The genotyping of SLCO1B1 521T>C and 388A>G was performed at the UKIM-Center for biomolecular pharmaceutical analyses, using TaqMan allelic discrimination assay (TaqMan® Drug Metabolizing Assay; Applied Biosystems, Foster City, California, USA). Polymerase chain

* krml@ff.ukim.edu.mk

reaction was performed on the Real-Time PCR system Mx3005P (Stratagene, La Jolla, CA, USA) using TaqMan genotyping protocols (TaqMan®Drug Metabolizing assay; Applied Biosystems Foster City, Ca, USA) in total volume of 12.5 μL under the following conditions: one cycle of 2 min at 50 ºC, one cycle of 10 min at 95 ºC, and 50 cycles of 15 sec at 92 ºC and 1 min at 60 ºC. Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein A (ApoA) and apolipoprotein B (ApoB) were measured at beginning and after 3 months of treatment by standard accredited methods. All data were statistically analyzed using different statistical methods (independent t-test, Mann-Whitney and Kruskall-Wallis tests and Bonferroni correction, with 95%CI, IBM SPSS 19.0). Chi-square test was used to deduce deviations from Hardy-Weinberg equilibrium. The results were considered statistically significant at $p<0.05$.

Results and discussion

The frequencies of the SLCO1B1 521T>C and 388A>G variant alleles were found to be 11% and 41%, respectively. However, no carrier of c.521CC was identified. These frequencies were found to be similar to those observed in Macedonians (14.1% and 40%) (Daka et al., 2015) and Caucasians (15% and 37%) (Mwinyi et al., 2004). The observed frequency distributions did not show significant deviations from the Hardy-Weinberg equilibrium. Three-month treatment with atorvastatin significantly decreased the plasma levels of TC, LDL-C, TG and ApoB and increased the average values of HDL-C and ApoAI to the referent values. However, no statistically significant change in the levels of Lp(a) was observed and this parameter remained out of referent range (≥30 mg/dL; percent change -8.23±15.16%). In the carriers of c.521TC (n=14), higher decrease in the levels of TC, TG and Lp(a) (11%, 15% and 26%, accordingly) was observed in comparison with the c.521TT carriers (n=50), pointing to lower activity of OATP1B1 in the carriers of variant c.521-allel. This could be confirmed by the percent changes in the levels of HDL-C and ApoAI in different c.521 genotypes of this ethnic group. Namely, after 3-month treatment, the average percent change of HDL-C was lower in c.521TC carriers (7.46±9.66%) vs. higher average percent change of 15.96±93.68% in c.521TT carrier (53% lower increase). Similarly, the highest increase in the average percent change of ApoAI was observed in the carriers of c.521TT, 12.13±12.27% vs. 4.32±17.09% in the carriers of c.521TC (181% higher increase). No effect on the mean percentage changes of all these parameters after 3-month treatment with atorvastatin was observed among carriers of different SLCO1B1 c388A>G genotypes. These results are consistent with the findings from several studies conducted in the European (Giannakopoulou et al. 2014), Brazilian (Rodrigues et al., 2010) and Chinese population (Fu et al., 2013; Yang et al., 2010), where atorvastatin, simvastatin and pitavastatin as drugs were administered.

Conclusion

No significant association between different SLCO1B1 c.388A> G genotypes and atorvastatin response was observed in the studied Albanian population. Visible differences were observed between c.521TC and c.521TT carries, suggesting association between the c.521T>C SNP and atorvastatin response. Additional studies, with a large sample size, are needed to confirm statistical significance of this finding.

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Macedonian pharmaceutical bulletin, **62** (suppl) 571 - 572
(2016)
ISSN 1409 - 8695
UDC: 615.277
Short communication

New human Glutathione-S-transferase P1-1 inhibitors and their ligand binding site and GSH complex formation descriptions

Ismail Yalcin*, Tugba Ertan-Bolelli, Esin Akı-Yalcin

Pharmaceutical Chemistry Dept., Faculty of Pharmacy, Ankara University, Ankara, Turkey

Introduction

Glutathione-S-transferases (GSTs) are a family of dimeric multifunctional enzymes that play an important role in metabolism and detoxification of numerous xenobiotics, electrophilic chemicals (including drugs), environmental carcinogens, and products of oxidative stress in living organisms. GSTs are found in almost all organisms from mammals to plants and even in some prokaryotes. Human GSTs consist of three families: cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs. In mammalian cells cytosolic GSTs are further categorized into seven major classes according to their amino acid sequence: alpha, mu, pi, theta, zeta, omega, and sigma subfamilies, which have been identified in dimeric forms (Mathew et al., 2006).

For the GST-mediated detoxification reactions, a substrate/GSH conjugation (complex formation) is required. GSTs are a family of Phase II detoxification enzymes that catalyze the conjugation of the GSH to a wide variety of electrophilic compounds. GSTs catalyze the conjugation of the reduced form of glutathione (GSH) to electrophilic centers of endogenous and exogenous hydrophobic compounds. For GST-mediated catalytic reactions, the activation of the sulfur atom of the G site bound GSH to the thiolate anion, which is a strong nucleophile, is required in order to perform a GS-substrate conjugate by attacking to the electrophilic center of substrates bound to the H site. Furthermore, the GS-conjugated compounds may be actively extruded from the cell through specialized pumps; principally, the multidrug resistance proteins MRP-1 and MRP-2 (Wu and Dong, 2012).

Human glutathione-S-transferase pi1-1 (hGST P1-1) is a member of the pi class subfamily of cytosolic GST and composed of two homodimer GST P1 subunits. The structural analysis indicates that the hGST P1-1 is a soluble protein comprised of 209 amino acid residues. The crystal structures show that each GST subunit of the protein dimer contains an independent catalytic site composed of two components. The first is a binding site specific for GSH or a closely related homolog (the G site) formed from a conserved group of amino-acid residues in the amino-terminal domain of the polypeptide. The second component is a site that binds the hydrophobic substrate (the H site), which is structurally variable. Amino acid variations of the H-site, among the different GST classes, determine substrate specificity (Wu and Dong, 2012).

It is known that hGST P1-1 participates in a particular role in one of the mechanisms of the development of resistance in cancer cells towards the administration of anticancer agents in chemotherapy. Human GST P1-1 is overexpressed in many cancers and contributes to multidrug resistance by directly conjugating to chemotherapeutic agents including cisplatin, adriamycin, etoposide, thiotepa, and chlorambucil. It is suggested that this resistance is related to high expression of hGST P1-1 in cancers such as breast, lung, colon, pancreas and cervix, thereby contributing to resistance to chemotherapy. Studies have shown that hGST P1-1 levels correlate with resistance to standard chemotherapy and are elevated in biopsies of tumour tissues that have become resistant to therapy after administration of anticancer agents (Wu and Dong, 2012). Consequently, inhibitors of human GST P1-1 catalytic activity remain a potential therapeutic tool in cancer cell resistance to drugs. In order to overcome this resistance specific hGST P1-1 inhibitors are in demand.

Furthermore, a variety of sulfonamido-containing compounds are apparently accommodated by the H-site of GST and found to be the substrates for GSTs. Data given in the literature are clearly shown that GSTs can mediate the enzymatic cleavage of the sulfonamide bond displaying a sulfonamidase activity by catalyzing the GSH-mediated

* yalcin@ankara.edu.tr

hydrolysis of sulfonamide bonds. Groups capable of withdrawing sufficient electron density from the alpha carbon atom to the sulfonyl group are an absolute requirement in this enzymatic process. GST-mediated sulfonamide cleavage results in the formation of the GS-conjugate, the corresponding amine, and sulfur dioxide. The released GS-conjugate may provide a strong inhibitor against intracellular GSTs, thus facilitating cancer chemotherapy (Mathew et al., 2006).

The benzoxazoles have been the aim of many researches for many years because they constitute an important class of heterocyclic compounds exhibiting substantial chemotherapeutic activities.The newly synthesized 2-substituted-5-(4-nitrophenylsulfonamido)benzoxazole derivatives, which were screened for the in vitro inhibition of hGST P1-1, exhibited significant inhibitor activities. Among the tested compounds, 2-(4-chlorobenzyl)-5-(4-nitrophenyl-sulfonamido)benzoxazole displayed the most potent inhibitory activity for hGST P1-1 with an IC50 value of 10,2 μM, showing a similar potency with the used reference drug ethacrynic acid (Ertan-Bolelli et al., 2014).

The molecular docking studies performed by using CDocker method is revealed that the new synthesized 2-substituted-5-(4-nitrophenyl-sulfonamido)benzoxazoles are acting as catalytic inhibitors of hGST P1-1 by binding to the H-site and performing conjugates with GSH forming S-(4-nitrophenyl)-GS complex via nucleophilic aromatic substitution reaction (Ertan-Bolelli et al., 2014).

It is reported that the active site residues Tyr7 and Tyr108 play important roles for the activity of hGST P1-1. Different models have been proposed for the activation of GST, all highlighting the key role of active site residues Tyr7 and Tyr108 (Wu and Bong, 2012). In the mechanism for activation of GSH, Tyr7 acts as a general base, promotes proton abstraction from the GSH thiol, and creates a thiolate anion with high nucleophilic reactivity. Additionally, the hydroxyl group of Tyr108 appears to contribute to the catalytic mechanism in the conjugation reaction of GSH with the docked sulfonamide-substituted benzoxazoles (Ertan-Bolelli et al., 2014,).

Moreover, it was found that groups capable of withdrawing sufficient electron density from the alpha carbon atom to the sulfonyl group are an absolute requirement. The electrophilic substructure of the sulfonyl group is solely responsible for activation of the sulfonamide bond toward cleavage. On the other hand, the amine portion has little or no impact on the cleavability of sulfonamide substrates (Ertan-Bolelli et al., 2014).

In conclusion, the newly developed hGSTP1-1 inhibitory active 2-substituted-5-(4-nitrophenylsulfonamido)benzoxazoles are promising lead compound for further in vivo studies to develop a new chemotherapeutic agent for the treatment in MDR cancers. It is accepted that the hGST P1-1 contributes directly to drug resistance in some cell types via their catalytic activity. The development of chemotherapy resistant tumour cells is a significant problem encountered in cancer treatment. The finding of over expression of hGST P1-1 in many cancer tissues as well as in drug resistant cell line, suggests that elevated hGST P1-1 expression may be of direct relevance not only to acquired resistance, but also in natural resistance. hGST P1-1 has been shown to catalyse the conjugation of GSH with the alkylating agents chlorambucil and thiotepa, suggesting that overexpression of hGST P1-1 in cells exposed to these drugs would confer resistance. Elevated cellular levels of hGST P1-1 have been shown to accompany resistance to various common anticancer drugs, and the addition of the GST inhibitors will be restored sensitivity to alkylating agents in drug-resistant cells. Therefore inhibitors of hGST P1-1 catalytic activity remain as a potential therapeutic tool in MDR cancer chemotherapy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 573 - 574 (2016)
ISSN 1409 - 8695
UDC: 615.33.015
Short communication

Physicochemical properties of novel derivatives of norfloxacin: solubility and pKa

Pranvera Breznica-Selmani[1,2]*, Kristina Mladenovska[1,2,] Bozhana Mikhova[3], Arlinda Daka[4], Dashnor Nebija[4], Blerina Koshi[4], Z. Kavrakovski[1] and Emil Popovski[2,5]

[1]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, R. Macedonia*
[2]*Research Center for Environment and Materials, Macedonian Academy of Sciences and Arts, Krste Misirkov 2, 1000 Skopje, R. Macedonia*
[3]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev 9, 1113 Sofia, R. Bulgaria*
[4]*Faculty of Medicine, University of Prishtina, Mother Theresa 10 000, Prishtina, R. Kosovo*
[5]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Arhimedova 5, PO Box 162, 1000 Skopje, R. Macedonia*

Introduction

A major advance in the quinolones field came in 1980, when chemists at the Kyorin company reported the preparation of norfloxacin, in which the cyclic diamine piperazine found in pipemidic acid was combined with 6-flourine found in flumequine (Andriole, 2000). Later studies revealed that introduction of this atom serves both to increase quinolone activity against the enzyme target DNA gyrase and to facilitate penetration into the bacterial cell. Norfloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. The fluorine atom at the position 6 increases potency against Gram-negative organisms, while the piperazine moiety at the position 7 is responsible for anti-pseudomonal activity. As it is known, fluoroquinolones are fairly insoluble in water. Due to presence of basic amine and carboxylic acid they possess zwitter ionic character, which is reflected on their solubility, lipophilicity and acidity/basicity. At acidic pH, both amine and acidic group are protonated providing the molecule positive charge. At high pH values, amine group is in the free base form, while the carboxyl group exists as a carboxylate anion, providing the overall molecule negative charge. Therefore, the fluoroquinolones tend to be more soluble in water at acidic and/or basic pH (Andriole, 2000; Riley et al. 1989), which significantly affects their permeability. It has been well explained that solubility, dissolution and gastrointestinal permeability are fundamental parameters that control rate and extent of drug absorption and its bioavailability. In order to develop novel compounds based on quinolone core with favorable physico-chemical properties i.e. higher permeability, we have synthesized different derivatives of norfloxacin by incorporating (benzoylamino)methyl functions (BAM salts) on the free nitrogen of the 7-piperazinyl group. The aim of this study was to evaluate their solubility in water (logSw) and phosphate buffer solution (PBS) pH 7.4 (logS7.4) and pKa values as well.

Materials and methods

Norfloxacin was purchased from Fluka (Sigma-Aldrich ChemieGmbh Munich, Germany), while the compounds for preparation of phosphate buffer saline (PBS), NaH_2PO_4, NaOH and KH_2PO_4, were purchased from Merck KgaA (Germany).

Methods

The BAM salts and norfloxacin derivatives were synthesized by the methods already described (Breznica-Selmani et al., 2015). Five different derivatives of norfloxacin were synthesized: 1-ethyl-6-fluoro-7-{4[(benzoylamino)

* pranvera.breznica@uni-pr.edu

methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1a), 1-ethyl-6-fluoro-7-{4-[(4-methylbenzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1b), 1-ethyl-6-fluoro-7-{4-[(3-methylbenzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1c), 1-ethyl-6-fluoro-7-{4-[(2-chlorobenzoylamino)methyl] piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1d) and 1-ethyl-6-fluoro-7-{4-[(3-chlorobenzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1e). Solubility of the newly synthesized (benzoylamino)methyl derivatives of norfloxacin and norfloxacin as a reference was determined in water and in PBS (pH 7.4) using a static equilibrium method (Zhang and Wang, 2008). An excess of the compounds was exposed to the solvents, accordingly, the mixtures were allowed to be stirred in a thermostatic water bath at 37 ± 1 oC (Haake, SWB 20, Waltham, MA, USA) and the samples were assayed once every 4 h until the analyzing results were replicated three consecutive times. Afterwards, the supernatant solutions, obtained by ultracentrifugation at 12 000 rpm for 15 minutes, were filtered to ensure that they were free of particulate matter before sampling. The concentration of the synthesized compounds and norfloxacin was quantified by UV absorption at λmax 271-275 nm (Shimadzu UV – 1800, Japan). The whole procedure was light-protected. Sodium thiosulphate was added to the medium when PBS pH 7.4 was used to prevent oxidation. All the solubility experiments were repeated at least three times, and the mean values were considered as the measured results. The logScalc was calculated by online ChemAxon's software for fast and accurate predictions of basic physicochemical properties such as logP, logD, pKa and logS (www.chemicalize.org). ChemAxon'saqueous solubility predictor is based on the topology of the molecules. To determine the pKa's of all synthesized compounds and norfloxacin, a potentiometric titration was applied and for data analysis, second-derivative method was used. As it was reported by Qiang and Adams (2004), the second-derivative method, widely used to determine the pKa's of most drugs, is the most convenient for determination of pKa value among the three most applicable (Gran's plot, second-derivative and the least-square non-linear regression), because it is independent of titrate and titrant concentrations.

Results and discussion

The experimentally determined solubility data for the leading compound norfloxacin were in agreement with the pharmacopeia and literature data that point to very slight water solubility (0.1-1 mg/mL) and sharp increase in solubility at pH below 4 and above 10 due to the amphoteric nature (Dua et al., 2007). The water solubility of the synthesized derivatives, experimentally determined, was lower than the one of the leading compound norfloxacin, ranging from 0.005 (1b) to 0.030 mg/mL (1d). Considering the solubility in PBS (pH 7.4), similar values for norfloxacin derivatives were observed, ranging from 0.011 mg/mL (1e) to 0,036 mg/mL (1d), all being lower than the solubility of norfloxacin (0.447 mg/mL). However, it must be emphasized that the solubility of norfloxacin as hydrochloride salt was used as a reference. Lower solubility of these newly synthesized compounds can be explained by the lipophilic and inductive character of the (benzoylamino)methyl moiety that disfavors ionization. There was no agreement between the predicted and experimentally determined data for solubility both in water and PBS (pH 7.4) for all the compounds.

pKa values were readily determined by inspection of the second-derivative plot. The experimental values obtained for norfloxacin as well as calculated ones did not differ significantly from those reported in the literature (Qiang and Adams, 2004). For all the synthesized compounds, three pKa values were determined (around 4, 5 and 14), suggesting preservation of the zwitter ionic character of the synthesized compounds.

Conclusion

Five derivatives of norfloxacin were synthesized. All the synthesized compounds have lower water solubility and potentially higher lipophylicity than the leading compound norfloxacin. The experimental pKa values obtained for the leading compound as well as calculated ones did not differ significantly from those reported in the literature for norfloxacin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 575 - 576 (2016)
ISSN 1409 - 8695
UDC: 616.72
Short communication

Physicochemical properties of novel derivatives of norfloxacin: distribution coefficient

Pranvera Breznica-Selmani[1,2]*, Emil Popovski[2,5], Bozhana Mikhova[3], Arlinda Daka[4], Dashnor Nebija[4], Blerina Koshi[4], Maja Stevanoska[1] and Kristina Mladenovska[1,2]

[1]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, R. Macedonia*
[2]*Research Center for Environment and Materials, Macedonian Academy of Sciences and Arts, Krste Misirkov 2, 1000 Skopje, R. Macedonia*
[3]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev 9, 1113 Sofia, R. Bulgaria*
[4]*Faculty of Medicine, University "Hasan Prishtina", Mother Theresa 10 000, Prishtina, R. Kosovo*
[5]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Arhimedova 5, PO Box 162, 1000 Skopje, R. Macedonia*

Introduction

N-Mannich bases are of particular interest in drug chemistry because of their potential to be used as prodrugs of NH-acidic drugs containing amide, imide, ureide and amine moieties (Bala et al., 2014; Stella et al., 2007). With respect to the amines, N-Mannich bases are useful when an increase in their lipophilicity is required. Lipophilicity is one of the most important determinants of the pharmacokinetic characteristics of a drug since it is related to its absorption as well as distribution to the target site. Lipophilicity of the quinolones as an important factor in the quinolones intestinal absorption is considered responsible for the poor relationship between their in vivo and in vitro activity (Cabrera Perez et al., 2002). Therefore, in these compounds, formation of N-Mannich base could suppress the pKa, which means that an important proportion could remain unionized at the pH of intestine.

With an aim to increase lipophylicity of norfloxacin, we have synthesized five different derivatives of norfloxacin [1-ethyl-6-fluoro-7-{4[(benzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1a), 1-ethyl-6-fluoro-7-{4-[(4-methylbenzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1b), 1-ethyl-6-fluoro-7-{4-[(3-methylbenzoylamino)methyl] piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1c), 1-ethyl-6-fluoro-7-{4-[(2-chloro-benzoylamino)methyl] piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1d) and 1-ethyl-6-fluoro-7-{4-[(3-chlorobenzoylamino)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1e)], by incorporating (benzoylamino)methyl functions on the free nitrogen of the 7-piperazinyl group. In addition to the structural characterization of these novel compounds as potential N-Mannich base prodrugs of norfloxacin, distribution behavior of these compounds between n-octanol and buffer solution (pH 7.4) was mater of interest. In addition, logD7.4 values, experimentally obtained, were compared with the predicted ones by computational method developed by ChemAxon (http://www.chemicalize.org//).

Materials and methods

Norfloxacin and 1-octanol were purchased from Fluka (Sigma-Aldrich Chemie Gmbh Munich, Germany). The compounds for preparation of phosphate buffer saline (PBS), NaH2PO4, NaOH and KH2PO4, were purchased from Merck KgaA (Germany).

The experimental partition coefficient (D) between n-octanol and PBS (pH 7.4) was determined by shaking flask method described by Abuo-Rahma et al. (2009). Stock solutions of (benzoylamino)methyl derivatives of norfloxa-

* pranvera.breznica@uni-pr.edu

cin in PBS (pH 7.4) were prepared in concentration of app. 0.2 mg/mL for all tested compounds, respectively. Afterwards, equal volumes of each solution and n-octanol were mixed, vortexed for 3 min and agitated for 12 h in a shaking water bath at 25 ± 0.1 oC. The test tubes were protected from light by wrapping in aluminum foil. Before a partition coefficient was determined, the organic and aqueous phase was mutually saturated by shaking for 24 h at the temperature of the experiment. After equilibration, the octanol phase was removed with a Pasteur pipette and both phases were assayed spectrophotometrically at λmax 270-280 nm. The experimental partition coefficient D was determined from equation: D = Ci- Cw/Cw* Vw/ V0, where Ci represents the total concentration of solute in both phases after distribution, Cw the solute concentration in the aqueous phase after distribution; Vw represents the volume of the aqueous and Vo the volume of the organic phase. All partition coefficient determinations were made in triplicate. The logDcalc was calculated by online ChemAxon's software (www.chemicalize.org/), where both logP and logD predictions are based on a modified version of the method of Viswanadhan et al. (1989). The predicted partition coefficients are composed of the molecules' atomic increments and the applied modifications include the redefinition of selected atom types to accommodate electron delocalization and the addition of contributions of ionic forms. The logP value of zwitterions is calculated from the logD at the isoelectric point. Also, the effect of hydrogen bonds on logP is considered if the formation of a six membered ring between suitable donor and acceptor atoms can take place. As the logD values are pH-dependent, the logD calculation relies on the pKa prediction process.

Results and discussion

Generally, the experimentally obtained distribution coefficients of norfloxacin derivatives were higher than the one of the corresponding leading compound, with the logD7.4exp ranging from -0.91 to 0.58 (vs. logD7.4exp -0.89 for norfloxacin). To reveal a correlation between the experimental and calculated values, a linear regression was performed. Weak correlation R2=0.046 between the calculated and the experimental values for all synthesized compounds was observed. Similar data were reported from other researchers as well (Abuo-Rahma et al., 2009). One of the reasons for this weak correlation may be zwitterionic nature of the fluoroquinolones at physiological pH. It is clear that at pH corresponding to the isoelectric point, zwitterions and neutral molecules are present at their highest concentrations. Therefore, this ratio is remarkably important feature to describe the lipophilicity of fluoroquinolones (Bakhotmah et al., 2011). Our results revealed that the isoelectric points of the synthesized compounds are around pH 5-7. According to Kujawaski et al. (Kujawski et al., 2012), predicted logD is calculated only for neutral compounds. Programs for calculating logD are based on breaking molecules into fragments and summing these constant fragment values plus certain correction factors. Considering that, polyprotic equation becomes more complicated and potentially accumulates errors due to the logD and pKa predictions (Kujawski and Popielarska, 2012). This may be a reason for flimsy correlation between the predicted and experimental values of logD7.4 for the synthesized compounds in our study.

Conclusion

Novel (benzoylamino)methyl derivatives of fluoroquinolones were synthesized in one step and in high yields, starting from the leading compound norfloxacin. Addition of (benzoylamino)methyl functions in the piperazinyl ring of the leading compounds by substitution of hydrogen atom at position 4 resulted in formation of potentially useful antimicrobial compounds. The structures of the synthesized derivatives were confirmed by different spectroscopic techniques. The lipophilicity of the synthesized derivatives was higher than the ones of the leading compound, indicating possibility for improved bioavailability and penetration into bacterial cells.

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Macedonian pharmaceutical bulletin, **62** (suppl) 577 - 578 (2016)
ISSN 1409 - 8695
UDC: 615.277
Short communication

Methotrexate - an old drug with new pharmaceutical formulations and new indications

Svetlana Goločorbin-Kon[1], Nebojša Pavlović[1]*, Bojan Stanimirov[2], Saša Vukmirović[2], Boris Milijašević[2], Hani Al-Salami[3], Momir Mikov[2]

[1] *Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, Novi Sad, Serbia*
[2] *Department of Pharmacology, Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, Novi Sad, Serbia*
[3] *School of Pharmacy, Curtin University, Kent Street Bentley WA 6102, Perth, Australia*

Introduction

Methotrexate (MTX), also known as amethopterin is an antifolate and an antimetabolite drug, a chemical analogue of folic acid developed by Yellapragada Subbarao of Lederle (Rossi, 2013). MTX was first administered to children with acute lymphoblastic leukemia (ALL) in 1948. MTX became the first drug that induced remission. Remissions were short, but the principle was clear, antifolates could suppress proliferation of malignant cells, and could thereby re-establish normal bone-marrow function. MTX received FDA approval in 1953. Since that time it has been used worldwide for a variety of medical interventions.

Firstly, MTX has been used as a parenteral formulation for cancer treatment. The use of MTX in the treatment of psoriasis and rheumatoid arthritis dates from the 1960s. From the late 1970s to the early 1980s, many rheumatologists were reporting their experiences with MTX use in rheumatoid arthritis treatment (Ward, 1985). MTX can be given orally or by intravenous, intramuscular or subcutaneous injection (Jundt et al, 1993). Intramuscular, intravenous or subcutaneous administration is usually reserved for patients with poor oral bioavailability of the drug or poor adherence to oral therapy, or when the cost is an issue. Weekly dosing of MTX is recommended. More frequent administration than weekly increases the risk of toxicity. The entire dose can be administered at once or divided into three doses taken over a 24-hour period (i.e., every eight hours). MTX should never be given in daily doses. During the 1980's and 1990's many studies were done with MTX to determine if it could suppress the growth of a tubal or ectopic pregnancy. Researchers discovered that MTX could be given as a safe treatment for the ectopic pregnancy treatment cured without surgery. Further research found that MTX could also be used to induce a chemical abortion. A small percentage of the cases required surgical intervention. The benefit of a MTX-induced abortion is that if there is an unknown tubal pregnancy it will be treated. MTX has also been found efficacious in the treatment of other diseases, including psoriasis, asthma, systemic lupus erythematosus, Crohn's disease, myositis and vasculitis. Many physicians use MTX for its steroid-sparing properties in patients with asthma and others who may have side effects related to corticosteroid use. The key of the success of MTX in the treatment any of these diseases (with the exception of ALL) is the recognition that low-dose therapy achieves efficacy while minimizing side effects (Moss, 1995).

MTX is a toxic medication, but if it is dosed correctly and monitored appropriately, its toxic effects can be minimized. These effects are categorized as minor or major. Minor toxic effects such as stomatitis, malaise, nausea, vomiting, diarrhea, headaches and mild alopecia are not life threatening but occur in 20 to 30 percents of patients. Other effects in this category include fatigue, mood alteration, dizziness, fever, myalgia and polyarthralgia. Most minor toxic effects are associated with depletion of folate. Folate supplementation with 1 mg daily or 7 mg once weekly should be considered for all patients. Studies show that low-dose folate does not interfere with the efficacy of MTX (ACRM, 1996). Most rheumatologists advise patients to avoid taking the folate dose on the same day as the MTX dose. Often, minor toxic effects respond to a re-

* nebojsa.pavlovic@mf.uns.ac.rs

duction in the MTX dose or an adjustment in the dosing schedule. Major toxic effects of MTX, such as hepatic, renal, pulmonary and bone marrow disorders, occur less frequently than the minor effects but may be life threatening.

In order to improve therapeutic efficacy and the comfort of the application of MTX, new pharmaceutical formulation is ongoing. Controlled release of MTX has been achieved by several techniques, but mostly in in vitro and preclinical animal models. Formulations of MTX that have been recently developed in order to improve cancer treatment include injectable thermosensitive hydrogels containing MTX-loaded chitosan-based microspheres, folic acid-chitosan-MTX core-shell nanoparticles, thermosensitive systems prepared on biocompatible polymer Pluronic F-127 as a vehicle, MTX-loaded alpha-lactalbumin microparticles, MTX-monoclonal antibody conjugates, MTX intercalated in a nanoceramic vehicle magnesium aluminium layered double hydroxide, coated with poly(D,L-lactide-co-glycolide) (PLGA). Most of these pharmaceutical formulations have demonstrated superiority when compared to conventional formulations of MTX in terms of localized drug delivery, long-term sustained drug release, and good biocompatibility (Beidokhti et al., 2016). Novel topical formulations of MTX have been also evaluated for the potential use in the treatment of psoriasis. Some of these formulations with enhanced skin penetration of MTX include microemulsions, nanogels, niosomes, liposomal hydrogels, deformable liposomes and solid lipid nanoparticles (SLN) (Avasatthi et al., 2015). The latest topical formulations of MTX for the treatment of psoriasis prepared as nano-vesicles use mostly bioadhesive surfactant systems containing polysorbate 60 or 80 as surfactants. NLC-based smart gel of MTX composed of lipids, surfactant Tween 80 and co-surfactant PEG 400, was formulated for intra-articular administration that could give site-specific delivery of a drug to the rheumatic joints (Shinde et al., 2016). MTX was also formulated in transdermal patches with different ratios of ethyl-cellulose and hydroxypropylmethyl-cellulose, and permeation enhancers Tween-80, Span-80, dimethyl sulphoxide (DMSO), and isopropyl myristate. These transdermal patches were evaluated for in vitro release, ex vivo permeation and pharmacokinetics in vivo, and all formulations exerted improved bioavailability in comparison to MTX without enhancers (Rama et al., 2012).

In summary, MTX plays a significant role in the treatment of various diseases, but toxicity remains the main issue of the use of MTX. Therefore, novel pharmaceutical formulations, which have reduced toxicity, better pharmacokinetic properties and targeted delivery, have emerged. Promising results have been obtained, but mostly in in vitro and in vivo animal studies. Therefore, these new formulations and drug delivery systems of MTX need to be further evaluated, especially in clinical settings.

Acknowledgement:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 690876

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Macedonian pharmaceutical bulletin, **62** (suppl) 579 - 580 (2016)
ISSN 1409 - 8695
UDC: 576.311.33:616.379-008.64
Short communication

Targeting endoplasmic reticulum stress in diabetes

Bojan Stanimirov, Karmen Stankov, Nebojša Pavlović*, Maja Đanić, Svetlana Goločorbin-Kon, Momir Mikov

Faculty of Medicine, University of Novi Sad, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

Introduction

The endoplasmic reticulum (ER) represents a membranous labyrinth of branching interlinked tubules and flattened sacs extending from the perinuclear space throughout the cytoplasm, being responsible for an assortment of critical cellular housekeeping functions. The rough ER, assembled with ribosomes, plays a key role in protein synthesis, folding, posttranslational modification, and transport. The smooth ER has a central role in the biosynthesis of lipids and steroids, assembly of lipid bilayers, metabolism of carbohydrates, metabolism of drugs and xenobiotics and regulation of calcium intracellular homeostasis. Quality control mechanisms of the cell ensure that newly synthesized proteins are folded into their correct configuration according to their function and destination in the cell. Therefore, protein folding in particular represents an exquisitely orchestrated aspect of protein synthesis in the ER that involves pathways for folding, assembly, modification, quality control, and recycling. In addition to an oxidizing environment, protein folding requires the participation of chaperone proteins, glycosylating enzymes and adequately high calcium levels. Appropriate folding of the nascent polypeptide chain is achieved through the actions of a series of molecular chaperones and foldases, which keep the polypeptide in soluble form and facilitate folding into a thermodynamically favored structure. Since the ER provides high fidelity quality control in protein synthesis, maturation and transport, it is a highly dynamic organelle, whose complex function can be significantly influenced by various factors both inside the cell and in its microenvironment. Failures in control mechanisms lead to accumulation of unfolded, misfolded, insoluble or otherwise damaged proteins in the lumen of the ER resulting in a state known as ER stress (Stankov et al, 2013). Continued accumulation of incorrectly folded proteins can irreversibly and irreparably damage cellular functions leading to cell death. Therefore, several cellular sensors and pathways have evolved to respond to this threat and to reduce the risk. Prime among these is the unfolded protein response (UPR), a signaling pathway primarily aiming at protecting cellular integrity by restoring proper ER folding capacity and overall protein processing. However, terminally misfolded proteins that cannot be repaired may be removed from the cell by one of two separate processes. One process is ER-associated degradation (ERAD), which involves the retro-translocation of irreparably misfolded proteins from the ER back into the cytosol, where they are ubiquitinated and subsequently subjected to degradation via the proteasome. Furthermore, insoluble misfolded proteins may be assembled together with other cellular debris into aggresomes (juxtanuclear complexes that occur as a cell culture phenomenon, for sequestration of toxic, aggregated proteins) and then recycled via autophagy.

Material and methods

Detailed and comprehensive search of PubMed and Scopus databases was carried out for original and review articles using the key words: endoplasmic reticulum stress, unfolded protein response, diabetes mellitus, in order to determine patophysiological implications of ER stress in type 2 diabetes (T2D) and to identify potential ER stress-targeting antidiabetic agent.

Results and discussion

An imbalance in energy intake and expenditure leads to obesity, a major health threat that increases the risk of

* nebojsa.pavlovic@mf.uns.ac.rs

T2D, cardiovascular disease and cancer. Obese subjects show activation of UPR in metabolic tissues including adipose tissue, liver, and the pancreas. Obesity is also associated with both hepatic and peripheral insulin resistance, along with elevated levels of proinflammatory cytokines. Studies with genetically obese or diet-induced obese mice revealed elevated levels of ER stress-associated markers: RNA (PKR)-like ER kinase (PERK) and eukaryotic translation initiation factor-2 alpha phosphorylation, inositol-requiring enzyme-1 (IRE1)-mediated JNK activation, and higher amounts of ER chaperone protein, glucose-regulated protein 78 (GRP78) in the liver and adipose tissue. ER stress in obesity is thought to be induced by an augmented demand for protein synthesis under nutrient excess and by elevated levels of saturated free fatty acids (FFA). Excess in saturated FFA, especially palmitate has been shown to cause ER stress and to activate the UPR in pancreatic β-cells and hepatocytes by altering the integrity of ER membrane. It is well-recognized that hyperglycemia, high plasma levels of saturated FFAs and obesity in general are key risk factors for the development of T2D. The same conditions are identified as triggers of ER stress, particularly in organs such as the liver and pancreas. In addition, leptin resistance, a condition that has been documented in the majority of the obese population, has been shown to contribute to obesity-linked disorders via ER stress. Obesity induces T2D, a metabolic disorder characterized by a combination of insulin resistance, dysregulated hepatic glucose production, and inadequate insulin secretion by pancreatic β-cells. At the molecular level, it involves perturbations in insulin signaling, such as reduced insulin receptor function and reduced post–insulin receptor phosphorylation steps. ER stress parameters such as phosphorylation of PERK and IRE1, ER-associated protein kinases that determine cell fate during UPR, are increased in the liver and adipose tissues of T2D animals (Stankov, 2010). The three branches of the UPR: IRE1, PERK, and activating transcription factor 6 (ATF6), have been implicated in the cellular inflammatory processes. Increased activation of IRE1, X-box binding protein-1 (XBP1) and JNK results in decreased insulin receptor signaling and insulin resistance. Moreover, ER stress parameters including Grp78, XBP1s, phospho-eIF2α, and phospho-JNK, are increased in the liver and adipose tissues of obese insulin-resistant non-diabetic humans and these parameters are significantly reduced after weight loss. Interestingly, ursodeoxycholic acid (UDCA), a naturally occurring hydrophilic bile acid that has long been used to treat chronic cholestatic liver disease is emerged as the agent that mitigates ER stress at the cellular level (Stanimirov et al, 2015). The administration of UDCA resulted in normalization of hyperglycemia and restoration of hepatic and muscle insulin sensitivity in obese humans. Additionally, UDCA has been shown to act as leptin-sensitizing agent. Therefore, UDCA represents an ER stress-modifying agent with therapeutic potential in ER stress-induced complications of obesity. In accordance, UDCA has been shown to exert an anti-atherogenic activity in diabetic atherosclerosis by targeting ER stress. Additionally, by ameliorating ER stress-associated detrimental signaling, UDCA improves function of dysfunctional endothelial cells and prevents formation of atherosclerotic plaque. However, the detailed molecular mechanisms of UDCA-mediated alleviation of ER stress are still to be elucidated.

Conclusions

More detailed insight in ER stress mechanisms will provide opportunity to develop UPR-manipulating anti-diabetic therapeutic strategies. Therapeutic agents aimed at ameliorating ER stress by promoting proper protein processing and generally supporting proper ER maintenance may have useful effects in the therapy of obesity, T2D, and cardiovascular disease. Ursodeoxycholic acid administration showed some promising results so far; however, further studies are highly recommendable in order to decipher beneficial effects of its administration on ER stress signaling in diabetic patients.

Acknowledgment

Supported by the Ministry of education, science and technological development, the Republic of Serbia, Grant III41012.

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Macedonian pharmaceutical bulletin, **62** (suppl) 581 - 582 (2016)
ISSN 1409 - 8695
UDC: 616-056.7:575.224.23
Short communication

Detection of chromosomal abnormalities with multiplex ligation dependent probe amplification in patients with myelodisplastic syndromes

Tatjana Sotirova[1]*, Borce Georgievski[1], Oliver Karanfilski[1], Aleksandar Stojanovic[1], Sonja Genadieva-Stavric[1], Aleksandar Eftimov[2], Aleksandar J. Dimovski[2]

[1]*University Clinic of Hematology, "Ss Cyril and Methodius" University, Majka Tereza 17, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, "Ss Cyril and Methodius" University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia*

Introduction

Detection of chromosomal abnormalities in myelodysplastic syndromes (MDS) enables classification according to WHO and risk stratification according to IPSS and R-IPSS, providing physicians with data regarding personal therapy approach. Multiplex ligation-dependent probe amplification (MLPA) is a technique for targeted copy number variation (CNV) identification in many human genes simultaneously. MLPA is a multiplex polymerase chain reaction (PCR)-based technique that can quantify up to 50 different genomic targets simultaneously in a single experiment through amplification of specific hybridizing probes (Fabris et al., 2011). As the sequence recognized by an MLPA probe is only 50-70 nucleotides long, this assay is very useful for detection of deletions or amplifications of single exons (Schouten et al., 2002). The high specificity of MLPA is due to the ability of this method to distinguish sequences differing in length by only one nucleotide. Another advantage of this method is also the low amount of input DNA (minimum of 20-50 ng) required for a successful MLPA reaction (Schouten et al., 2002). Up to 40-50 small specific probes are directed at DNA regions of interest and to reference regions not associated with the disease, providing a resolution greater than FISH or BAC – based aCGH (Donahue et al., 2011). Each probe consists of two oligonucleotides (5´and 3´end-probes), that hybridize to adjacent sites of the target sequence. The short oligonucleotide contains a target-specific sequence and a universal PCR primer X. The long probe consists of a target-specific sequence, a universal PCR primer Y and a stuffer sequence of variable length in between (19-370 nucleotides) to generate the size differences necessary for electrophoretic resolution (Donahue et al., 2011; Sellener and Taylor, 2004). The MLPA reaction comprises five steps and requires a thermocycler and automated genetic analyzer for fragment analysis by capillary electrophoresis. A crucial step of the MLPA assay, especially when used in the diagnostic setting (i.e. gene deletion or when used in the diagnostic setting (i.e. gene deletion or and interpretation of results. The most widely used is the Coffalyser software, an Excel-based program able to perform data normalization steps and necessary corrections (Coffa et al., 2008; Jankowski et al. 2008). The results interpretation is based on the mathematical comparison between relative quantities of target DNA amplified from a tested (patient) sample vs. those of a normal (control) sample (Abdool et al., 2010). In general, signals are interpreted as aberrant at cut-off values below 0.7 (deletion) and above 1.3 (duplication/ amplification) (Eijk-Van Os et al., 2011). The utility of MLPA assay in the testing of acute leukemias and myelodysplastic syndromes has been analyzed, confirming excellent accuracy and specificity of MLPA as compared to FISH (Stippia et al., 2012).

Materials and methods

Our cohort consisted of 70 patients (pts) with 'de-novo' and therapy-related MDS (t- MDS), diagnosed at the University Clinic of Hematology, "Ss Cyril and Methodi-

* tatjanaso@hotmail.com

us" University, Skopje, Republic of Macedonia in the period of 52 months. Patients signed informed consent for entrance in the study. From each patient was collected 1 mL of material, obtained by bone marrow biopsy (BMB) and put in the test tube with an anticoagulant - K3EDTA. Deoxyribonucleic acid (DNA) was isolated with standard phenol chloroform extraction in the Faculty of Pharmacy, Department of biomolecular sciences, Skopje, Republic of Macedonia. The material from BMB was centrifuged at 3000 rpm, 4°C, 5 min. The watery upper layer was decanted, and the precipitated cells were washed with 0.9%NaCl, and later washed with lysed solution (1.55M NH4Cl, 0.1M NH4HCO3 and 1mM EDTA Ph 7.4, final concentration), centrifuged at 3000 rpm, 4°C, 5 min, in order to lyse the rest of red cells. The rest of the other cells were digested over night at 37°C with 0.1M NaCl, 0.05 M Tris, 1mM EDTA in final concentration. The next day was performed fluid-fluid extraction with saturated phenol (pH=8) and Chloroform : Isoamyl alcohol 24:1, after centrifugation at 3000 rpm, 4°C, 5 min. and removal of the upper watery layer, the precipitation of the DNA with cold 100% ethanol was performed and dissolved with TE puffer (Tris 10mM и EDTA 1 mM, final concentration). The isolated DNA was incubated overnight on 37°C for homogeneous dissolution in the puffer. The intactness of the isolated DNA was examined on gel electrophoresis. With NanoDrop 2000 spectrophotometer (Thermo Scientific) was determined the concentration of the isolated DNAs.

The SALSA MLPA MDS kits (P144 and P145, MRC Holland) were used to detect chromosomal abnormalities commonly associated with MDS. The probe mix contained probes for chromosomes 5, 7, 8, 11, 12 (ETV6), 17 (TP53), 20 and 21 (RUNX1). Analyses were performed with the application of P414-A1 MDS MLPA kits of MRC Holland, according to their protocol. The analysis of the results was performed by software - Coffalyser.

Results and discussion

Of 70 patients (pts), 33 women (47.1%) and 37 men (52.9%) participated in the study, aged 64.3 years (range 22-86 years). With primary MDS were 66 pts and with 4 pts with t- MDS. Chromosomal abnormalities were detected in 32.9% pts (5p+, 5q-, 7p-, 7q-, 8p+, 8q+, 11p-, 12p-, 17p-, 17q+, 19p+, 19p-, 20q- in different combinations), while 67.1% pts had normal findings. According to the WHO patients were classified as follows: RCMD – 34, RCED - 5, 5q syndrome - 1, RARS -1, RAEB 1- 6, RAEB -2 - 6, CMML-1 - 2, CMML-2 - 3, t-MDS - 4, MDS-u - 2, AML- 6 pts. According to IPSS, distribution was as follows: with low risk - 16 pts (22.9%), with intermediate 1 - 37 (52.9%), with intermediate 2 – 8 (11.3%), with high risk - 9 (12.9). According to R-IPSS, distribution was as follows: with very low risk - 5 (7.1%), with low risk 22 (31.4%), with intermediate risk - 24 (34.3%), with high risk - 10 (14.3%), with very high risk - 9 (12.9%) pts. Patients were treated based on IPSS and R-IPSS. Transformation in AML was registered in 24.3%, after 9.2 months. Overall survival (OS) was 30.2 months. In pts with chromosomal abnormalities OS was 25.7 months and in those without chromosomal abnormalities OS 34.2 months, but the difference was not statistically significant (p =0 .13101). 22 patients died.

Conclusion

MDS MLPA assay is sufficiently specific and reproducible to be used in routine diagnostic settings as the first-line genetic screening tool. Data concerning chromosomal abnormalities in MDS enable physicians to make treatment strategy at diagnosis. There are different treatment options for different risk groups including allogeneic transplantation for high and very high risk patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 583 - 584 (2016)
ISSN 1409 - 8695
UDC: 614.253.84:342.721
Short communication

Regulatory aspects of data protection and privacy requirements in interventional biomedical studies

Milica Zugic[1]*, Kristina Mladenovska[2]

[1]*Ewopharma AG, Representative Office, Anton Popov 1-2/3, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, Ss Cyril and Methodius University, Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

In order to obtain accurate, reliable and traceable results from the interventional biomedical studies, including clinical trials, and to come up with effective and safe medicinal products, and in the same time, to protect the confidentiality of personal health data, it is necessary to have a regulated access to the health information of the individuals. Data protection and privacy requirements are given in the Declaration of Helsinki (1964-2013) which states: "It is the duty of physicians who are involved in medical research to protect the life, health, dignity, integrity, right to self-determination, privacy, and confidentiality of personal information of research subjects." All precautions should be taken to protect the privacy of the subjects participating in biomedical research and to minimize the impact of study on their mental, physical and social integrity. Article 24 states: "Every precaution must be taken to protect the privacy of research subjects and the confidentiality of their personal information". One of the basic principles in the Guideline for Good Clinical Practice E6 (R1) (1996), section 2:11 states: "The confidentiality of records that could identify subjects should be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s)." To this end, each subject that has been enrolled in a clinical trial is assigned a unique identification number in order to protect his/her identity. This identification number is used instead of the name of the entity when the investigator submits reports of adverse events and/or other information related to the clinical trial. Personal data protection is a mandatory part of the Informed Consent, introduced as a compulsory element by the Nuremberg code. In the Informed Consent it is clearly stated that all medical records which contain personal patient data will be kept confidential within the limits of applicable laws and regulations and will not be available to the public. If the study results are published, the identity of the subjects will remain protected.

Data protection in EU

In the EU, personal data may be collected only under strictly controlled conditions and for legitimate purposes. Harmonized legislation is established among EU Member States regarding the protection of personal data. In addition, the European Commission keeps constant dialogue with non-EU countries to achieve high level of protection of personal data. Personal data protection in the EU is currently regulated by Directive 95/46/EC on the protection of personal data (1995) and Directive 2002/58/EC on the processing of personal data and the protection of privacy in the electronic communication (2002). Directive 95/46/EC provides the legal framework which strives to find the balance between the high level of protection of personal data and the free movement of personal data within the EU. The Directive sets strict limits on the collection and use of personal data and requires each Member State to appoint an independent national body responsible for the protection of personal data. Directive does not deal explicitly with biomedical research, but it is the basis for principles of protection of personal data in biomedical research. Directive 2002/58/EC provides an equivalent level of protection of fundamental rights and freedoms and especially of the right to privacy with regard to the processing of personal data in the electronic communications sector and the free movement of such data and the equipment for electronic communications and services in EU.

Rapid technological development and related regulatory challenges of privacy protection caused the European Commission to propose a new regulation on the pro-

* milicazugic@hotmail.com

tection of individuals with regard of the processing of personal data and their free movement - Data Protection Regulation ("DPR") (2015). After intense lobbying, the final text of DPR has been agreed on 17th of December 2015. It is expected to come into force in 2018. DPR will replace the current legislation and the various inconsistent national laws on data protection, with a transitional period of two years. Considering that the collection, processing and transfer of personal data, particularly the records of individual subjects/patients in accordance with applicable laws are critical to the success of biomedical research, the adoption of this regulation will affect sponsors, investigators and other participants in biomedical research/clinical trial. DPR mainly refers to: the unification/qualification of key-coded data of the subject/patient with which the subject/patient can be identified, clearer definition of the roles and responsibilities of the sponsor and the contract research organizations as responsible for the processing and control of data, clarification of the guidelines on existing restrictions on the transfer of data from the EU to parties outside the EU, the introduction of legal sanctions for violating laws on data protection, as well as administrative penalties.

Data protection in USA

In the USA, the medical information is regulated by the Health Insurance Portability and Accountability Act (HIPAA). HIPAA applies to health workers dealing with the processing of medical data and all other entities that come into contact with medical information. Standards for privacy of individually identified health information are given inthe HIPAA Privacy Rule and reffer to the collection, use and protection of health information or medical records. However, HIPPA-rules are related to defined entities only, they protect electronic data only and many of the entities are not or cannot be harmonized with the HIPPA safety requirements. Additionally, there are many guidelines that have been developed by government agencies and industry associations, which are not legally binding, used only for self-regulation and are considered "good practices".

Data protection in the Republic of Macedonia

In Macedonia, the Law on Personal Data Protection (2008) regulates the protection of personal data as fundamental rights and freedoms of individuals. The principles of this law are implemented in biomedical research. Article 2 defines special categories of personal data "data concerning health, including genetic data". Article 8 prohibits the processing of special categories of personal data, but an exception is made if "it is necessary for the purposes of medical diagnosis, treatment or management of a health institution and if it is carried by a person whose profession is to provide medical care under oath of secrecy of the information revealed to him in the course of his profession and if an appropriate safeguards are established in order to perform activities of public interest determined by law or by decision of the Directorate". In addition, according to the Article 31, the personal data shall be transferred in another state only if the second one provides the same level of protection. With this being stated it is safe to conclude that if biomedical research is performed in accordance with this Law, the code of ethics of health workers and an appropriate level of protection is provided and there is no barrier for scientific research.

Conclusion

It is clear that different countries have different approaches to personal data protection in clinical studies. In the USA there is no comprehensive national law regulating the collection and use of personal data. The novel DPR in EU is expected to be directly applicable to all Member States, to provide comprehensive and consistent level of protection, to be unique regimen for protection and in the same time simple but stronger executive framework in all Member States. When entering into force it is expected to affect national regulations, including the one in Macedonia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 585 - 586 (2016)
ISSN 1409 - 8695
UDC: 616-036.22:614.253.84]:342.721(497.7)
Short communication

Protecting personal data in (pharmaco)epidemiological research: international regulation and macedonian law

Milica Zugic[1]* and Kristina Mladenovska[2]

[1]*Ewopharma AG, Representative Office, Anton Popov str. 1-2/3, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

Introduction

The goal of the epidemiological research is to explain the natural causes of various diseases and conditions, their prevalence and the burden that they represent for society. Pharmacoepidemiology as a science evaluates the risks and benefits of drug use in large numbers of patients in everyday practice. Although (pharmaco)epidemiological studies are non-interventional there is still a risk of disclosure of sensitive medical information. The International Pharmacoepidemiological Society (ISPE) recognizes the need for balance between the protection of personal and medical data of patients and the necessity of relevant scientific and medical information to be used to solve medical and other problems related to public health. ISPE recommendations are based on existing laws on data protection and ethical guidelines, including inspection and approval by ethics committees. In accordance with these recommendations: (a) the studies should be designed in a way to protect the personal and medical information and confidentiality of medical records; (b) ISPE and other professional societies should continue to develop codes of conduct and "good practices" for epidemiological research and should encourage the researchers to follow them; (c) all pharmacoepidemiological studies that use personal data should be approved by ethics committees before commencing the study; (d) ethics committees may waive a requirement for written informed consent in cases where the direct harm to individuals is unlikely and individually identifiable data are not published; (e) the use of sophisticated information technology should be encouraged to ensure, where appropriate, that no identifying information remains on research datasets; (f) secondary research and statistical analysis of data already collected for other purposes should be approved, provided that the secondary research serves the public health and appropriate measures are implemented to protect personal data; (g) ISPE supports the notion that research using completely anonymize databases should not require IRB approval or specific patient authorization for each use of the data; (h) ISPE supports the development of guidelines and techniques for the anonymization of datasets; (i) the international transfer of data should be permitted under the condition that either a comparable legal level of data protection exists in the recipient country or special provisions are made by the person responsible for the data to ensure the same level of data protection exists in the country from which the data are transferred; and (j) unauthorized access to or distribution of personal and medical data should be subject to legal penalties sufficient to act as an obstacle (http://www.pharmacoepi.org/).

Data protection in EU

In January 2012, the European Commission presented the draft of a new General Data Protection Regulation (GDPR) to the European Parliament and the Council of the EU. The GDPR is planned to replace the Directive 95/46/EC (1995), which constitutes the present European legal framework for processing of personal data. The first Articles with specific relevance for scientific research are concerned with general principles (Article 5) and lawfulness (Article 6) of personal data processing. Article 5b lays down that personal data shall be collected for specified, explicit and legitimate purposes and may not be further processed in a way incompatible with those purposes. This corresponds to the same principle in the current Directive 95/46/EC. However, in the Directive 95/46/EC there is an exemption for research i.e. that further process-

* milicazugic@hotmail.com

ing of data for historical, statistical or scientific purposes is not to be considered as incompatible with the original purpose as long as Member States provide appropriate safeguards. This exemption was omitted in DPR, significantly reducing the scope for data sharing between research groups and severely restraining the use of retrospective study designs. If taken literally, the omission of the exemption threatens the (pharmaco)epidemiological research. In the final version of the DPR that has been agreed upon on 17th of December 2015 Article 5b has been extended: "further processing of personal data for archiving purposes in the public interest, or scientific and historical research purposes or statistical purposes shall, in accordance with Article 83(1), not be considered incompatible with the initial purpose". Introducing this exemption gives a legal basis for the (pharmaco)epidemiological research, especially the retrospective study designs.

Data protection in USA

Department of Health and Human Services (HHS) have realized that it is not always possible to get an authorization to use protected health information for research purposes, especially in research relating to public health and (pharmaco)epidemiological research.The HIPAA Privacy Rule (2004) acknowledges the possibility of selection bias if an authorization is sought. Due to these reasons, the Department of HHS decided that there are certain circumstances under which protected health information for research purposes can be used without an authorization, but certain criteria must be met and strict safeguards should be established. For the authorization waiver to be granted by the Ethics committee the following criteria should be met: (a) the research cannot be conducted without the waiver; (b) the research cannot be conducted without access to the protected health information; (c) use and disclosure of protected health information has minimal risk of disclosure of the privacy of the subject; (d) there is an appropriate plan to protect data from misuse and disclosure; (e) there is an appropriate plan for the destruction of all identifiers contained in the protected health information as soon as possible and in accordance with the research; (f) there is an adequate written assurance that the protected health information shall not be used or disclosed again to another individual or legal entity, unless required by law, in cases of authorized oversight of the research or for other research which has a license to use and disclose the protected health information.

Data protection in the Republic of Macedonia

In Macedonia, the Law on Protection of Personal Data ("Official Gazette of the Republic of Macedonia" No.7/05, 103/08) regulates the protection of personal data as fundamental rights and freedoms of individuals. Article 5 states: "secondary data processing for historic, scientific or statistical research shall be deemed in compliance with the primary purposes for collecting data, provided that appropriate safeguards are established which are in accordance with the law". This clearly shows that the use of personal medical data for purposes of (pharmaco)epidemiological research is justified and by this law allowed. Article 11 states: „Notwithstanding paragraph 1 of this Article, the controller has no obligation to inform the data subject about the processing of personal data for historical, scientific or statistical purposes, if it is impossible or requires a disproportionate effort or expense". This allows for the personal data to be used without Informed Consent if appropriate safeguard of the personal data is in place. The objectives of the (pharmaco)epidemiological research are by all means scientific and the methods of data processing are mostly statistical and the research itself serves the public health which deems (pharmaco)epidemiological research in compliance with this Law.

Conclusion

Non-interventional studies represent a significant portion of the combined (pharmaco)epidemiological literature and are of great public health significance. Restrictive interpretation of regulation could have devastating consequences for large (pharmaco)epidemiological studies where obtaining Informed Consent is not possible, or where non-participation in the study is a threat to impartiality and accuracy of the results.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 587 - 588 (2016)
ISSN 1409 - 8695
UDC: 616.33-02:615.276.036.8]:599.323.4
Short communication

Oxidative stress index in rat stomach as a measure of gastric tolerability of newly synthetized anti-inflammatory compounds

Jelena Savić[1]*, Marina Milenković[2], Jelena Kotur-Stevuljević[3], Zorica Vujić[1], Sote Vladimirov[1] and Jasmina Brborić[1]

[1]*Department of Pharmaceutical Chemistry, University of Belgrade-Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*
[2]*Department of Microbiology and Immunology, University of Belgrade-Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*
[3]*Department of Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, Vojvode Stepe 450, 11221, Belgrade, Serbia*

Introduction

Cyclooxygenase (COX) is an enzyme responsible for the initial step of arachidonic acid metabolism and prostaglandin production. There are two isoforms of COX: COX-1 which is considered to be physiological and constitutively expressed in many tissues and COX-2 which is considered to be inducible and pathological due to the fact that this isoform catalyzes production of prostaglandins which are mediators of pain and inflammation (Kiefer and Dannhardt, 2004; Goodsell, 2000). While the inhibition of COX-2 isoform is beneficial, the inhibition of COX-1 is considered to be associated with many side effects, but especially with gastric side effects. Conventional Nonsteroidal Anti-inflammatory Drugs (NSAIDs), like ibuprofen are non-selective and they inhibit both COX isoforms, thus producing gastric side effects which may vary from mild, like gastrointestinal irritation to very serious, like ulceration and bleeding (Radi and Khan, 2006). Ibuprofen is often an inspirational drug for developing novel compounds with anti-inflammatory activity, and often is used as a reference drug for comparison of anti-inflammatory activity.

We have previously reported that simple derivatives of β-aryl-β-hydroxyalkanoic acids exhibit good anti-inflammatory activity and good gastric tolerability (Savić et al., 2011). These compounds are interesting because their structure deviate from the usual structure of selective COX-2 inhibitor: their structure is simple, they contain carboxylic acid moiety and they lack sulphonamide or sulphone group. It is expected that these agents have comparable efficiency to ibuprofen, but a better safety profile.

Gastric tolerability can be assessed by method of Adami et al. (1963). Macroscopic observations can be supported by biochemical parameters which are oxidative stress markers: malondialdehyde (MDA) and glutathione (GSH).

It has been reported in literature that compounds showing less ulcerogenic activity also showed reduced MDA content, a byproduct of lipid peroxidation (Pohle et al., 2001). Therefore, MDA levels were determined in stomach tissues of animals which were treated with the highest dose of investigated compounds and obtained results were compared with result for ibuprofen. GSH is a tripeptide, a superoxide radical scavenger and it protects thiol protein groups required for maintaining the integrity of cell against oxidation. GSH is present in the stomach at high concentrations and plays an important role in maintaining the integrity of gastric mucosa (Hirota et al., 1989), so it is important to determine levels of this parameter in rat stomach. Quotient of GSH and MDA (in arbitrary units, AU) is referred as oxidative stress index (OSI) and can efficiently point out the compound exhibiting the best gastric tolerability (Petrovic et al., 2014). It is very convenient to introduce this index in order to precisely understand the results regarding compounds influence at oxidative stress mechanisms.

* jelena.savic@pharmacy.bg.ac.rs

Materials and methods

In order to assess the anti-inflammatory activity carrageenan-induced rat paw edema test was performed on adult male Wistar rats. After the animals were sacrificed, their stomach was removed, opened along the greater curvature and washed out with saline. Lesions were examined using illuminated magnifier (3X) in order to assess gastric damage according to a modified scoring system of Adami et al (1963). These stomachs were immediately frozen and stored at −80°C until determination of total protein content, MDA and GSH. To prepare the tissue homogenates, unfrozen stomach tissues were roughly chopped with liquid nitrogen using a pestle and mortar. The fragmented tissues (0.5 g each) were mixed with 4.5 ml of homogenization tris-buffer (10 mM, pH 7.4), homogenized on ice using an Ultra-Turrax homogenizer, filtered and centrifuged at 1000 g at 4 °C for 20 min. The supernatants were then used to determine the enzymatic activities (Mei et al., 2012). Both MDA and GSH are determined according to methods used by Uskoković-Marković et al. (2007).

Results and discussion

Seven acids [3-hydroxy-3-(4-nitrophenyl)-3-phenylpropanoic acid (1); 3-(4-(trifluoromethyl)phenyl)-3-hydroxy-3-phenylpropanoic acid (2); 3-(4-chlorophenyl)-3-hydroxy-3-phenylpropanoic acid (3); 3-hydroxy-3-(4-methylphenyl)-3-phenylpropanoic acid (4); 3-(3-(trifluoromethyl)phenyl)-3-hydroxy-3-phenyl-propanoic acid (5); 3-(3-chlorophenyl)-3-hydroxy-3-phenylpropanoic acid (6); 3-(3-methylphenyl)-3-hydroxy-3-phenylpropanoic acid (7)] were synthesized using already reported modification of Reformatsky reaction additionally optimized by increasing temperature of reaction to 65-69°C (Savić et al., 2011).

None of the synthesized substances produced any significant gastric lesions. The changes observed were in range of 0–1 according to the Adami's scoring scale. MDA values for all compounds except for compound 2 were lower than for ibuprofen. GSH level is increased in groups treated with ibuprofen, compounds 1, 2 and 6 compared to the control group. The level of GSH in groups treated with other compounds is similar to the control group. OSI was very high In gastric mucosa of animals received compounds 1 (186.45), 3 (132.6), 6 (137.4) and 7 (114.8) which means that these compounds produce good protection against oxidative stress in gastric tissue. In this respect compounds 2 (61.0) and 5 (48.0) produced more oxidative stress in gastric tissue than ibuprofen, while compound 4 (89.3) was on a pair with ibuprofen (79.9).

Conclusion

Although macroscopic examination of rat stomach showed that none of tested compounds produced any significant gastric lesions, biochemical parameters differentiated these compounds according to oxidative stress they produced. The obtained biochemical results indicated that ibuprofen produced significantly more oxidative stress in gastric tissue than four out of seven synthetized compounds. The results impose the conclusion that this four compounds exhibit better gastric tolerability than ibuprofen. Using OSI to describe results is much more concise way to perceive oxidative stress than using MDA and GSH values separately.

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Poster presentations

Macedonian pharmaceutical bulletin, **62** (suppl) 589 - 590 (2016)
ISSN 1409 - 8695
UDC: 615.277:616.24-006.6
616.24-006.6-085.277
Short communication

Antiproliferative effects of a betulin nanoformulation on a lung carcinoma cell line – A549

Ioana Zinuca Pavel, Iulia Pinzaru, Roxana Ghiulai, Stefana Avram, Marius Mioc, Codruta Soica, Dorina Coricovac*, Cristina Adriana Dehelean

"Victor Babes" University of Medicine and Pharmacy Timisoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., RO-300041, Timisoara, Romania

Introduction

Lung carcinoma is known as a malignancy with a high incidence and an increased mortality rate worldwide (Li et al., 2014). The discovery of a prophylactic and a curative treatment for this disease still represents a challenge for the researchers.

Another subject of scientific interest is the discovery of antitumoral agents of natural origin with potent anticancer activity and low/no toxic effects against normal cells.

Betulin is a natural compound, member of the pentacyclic triterpene family, obtained in high yields from the bark of the birch tree (Krol et al., 2015). Betulin is known for its multiple pharmacological effects, including: anti-inflammatory, hepatoprotective, anticancer and antiangiogenic effects (Dehelean et al., 2013; Krol et al., 2015). The use of betulin as an anticancer agent presents multiple advantages (potent anticancer effects and no toxicity in vitro against healthy cells and in vivo even at high doses), but one of its major disadvantages is its low water solubility, which limits its administration in vivo. A goal of the researchers was to find a proper formulation to increase betulin solubility in aqueous solutions what leads to increased bioavailability and higher antitumor activity.

The aim of our study was to obtain a silver nanoformulation of betulin and to verify its pharmacological effects in vitro on a human lung carcinoma cell line - A549.

Materials and methods

In this study we used A549 human lung carcinoma cells. The cell line was purchased from ECACC (European Collection of Cell Cultures) at passage no. 39. A549 cells were kept in liquid nitrogen and one week before the experiment started, we cultured in specific medium culture.

The cells were cultured in high glucose (4.5 g/l) Dulbecco's modified Eagle Medium (DMEM – Sigma Aldrich, Germany) supplemented with 15 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% foetal bovine serum (FBS). Cells were kept in a humidified atmosphere with 5% CO2 at 37 °C and were passaged every two days. Cells number was assessed by using Neubauer chamber in the presence of Trypan Blue.

For the cell viability assay, the cells(10000 cells/ well) were cultured in 96-well plates and left for 24h to adhere. The cells were stimulated for 24 h with different concentrations (10 and 50 uM) and were divided in the following groups: 1 - cells stimulated with DMSO (dimethylsulfoxide), 2 - cells stimulated with betulin dissolved in DMSO, 3 - cells stimulated with silver nanoformulation blank and 4 - cells stimulated with betulin silver nanoformulation. The cells viability was assessed by Alamar blue and MTT methods. It was also tested the effect of the new formulation against the migration capacity of the cells by scratch assay and pictures were taken at different time points: 0h, 3h, 8h and 24h.

Results and discussion

Stimulation of the A549 cells with different concentrations of betulin silver nanoformulation (10 and 50 uM) for 24h induced cytotoxicity of the cells in a dose-dependent manner. These results were compared with the results obtained after the stimulation with betulin dissolved in dimethylsulfoxide (DMSO), the solvent in which betulin has an increased solubility and the cytotoxic effect was higher

* dorinacoricovac@umft.ro
ioanaz.pavel@yahoo.com

in the case of the nanoformulation.

It was also tested the effect of these formulations against the capacity of migration of the A549 cells by the means of scratch assay and it was observed that at the smallest concentration used in the study, the nanoformulation was able to inhibit the migration of the cells.

Other studies developed on human lung carcinoma showed that betulin solved in DMSO had cytotoxic and anti-migratory effects (Rzeski et al., 2009). The anticancer mechanism of action of betulin is still unknown and several studies proposed as mechanism of action induction of apoptosis via the intrinsic mitochondrial pathway (Pyo et al., 2009). Another mechanism of action of betulin was the inhibition of tumor cells proliferation through activation of AMPK signaling (Li et al., 2014).

Conclusion

Our results showed that betulin formulated as a silver nanoformulation had a potent antiproliferative effect against human lung carcinoma cell line - A549 after 24h stimulation. Further studies will be realized in order to elucidate the anticancer mechanism of action of betulin and for the evaluation of the in vivo effects in animal models with human lung carcinoma.

This research was supported by the PN-II-RU-TE-2014-4-2842 grant, no. 180/2015.

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Macedonian pharmaceutical bulletin, **62** (suppl) 591 - 592 (2016)
ISSN 1409 - 8695
UDC: 616.11-002:616.61-008.64-78
Short communication

Treatment of uremic pericarditis treated with intermittent hemodialysis

Mirlind Behxheti[1, 2]*, Lutfi Zylbeari[1], Nasir Behxheti[1], Gazmend Zylbeari[1], Zamira Bexheti[1]

[1] *Faculty of medical sciences, University of Tetovo, M. Passa Derala bb, 1200 Tetovo, Republic of Macedonia*
[2] *Medical Faculty, University of Ss Cyril and Methodius, 50 Divizija 6, 1000 Skopje, Republic of Macedonia*
[3] *Special Hospital for Nephrology and Hemodialysis "Vita Medical Group"- Tetovo, Ilindenska bb, 1200 Tetovo, Republic of Macedonia*

Introduction

Pericarditis is inflammation of the pericardium, the serous membrane enclosing the heart and the roots of the great blood vessels. Richard Bright first described the association of pericarditis with renal failure in his landmark observation of 100 cases of patients with 'albuminous urine', which appeared in the Guys Hospital Report of 1836 (Bright, 1836). Autopsy studies demonstrated pericarditis or pericardial effusion in 37 of these patients. „Uremic pericarditis" is a term used to describe patients who develop clinical manifestations of pericarditis before renal replacement therapy or within 8 weeks of its initiation. „Dialysis pericarditis" is used for patients who develop clinical features of pericarditis after being stabilized on dialysis usually after 8 weeks of its initiation (Barach, 1922).Though several therapeutic modalities have been used to treat uremic and dialysis pericarditis and pericardial effusion, initial treatment of pericarditis is usually determined by the hemodynamic stability of the patient. In azotemic patients pericarditis is due to number of causes including infection, diseases like systemic lupus erythromatosis (SLE), or to uremic toxins itself. The incidence of acute pericardial disease punctuating chronic renal failure is decreasing because of effective dialysis and renal transplantation; approximately 20% of uremic patients requiring chronic dialysis develop pericarditis.It is surprising that even though pericardial involvement remains an important cause of morbidity and mortality in patientswith end stage renal disease (ESRD), the academic interest and the literature regarding the same has reduced over the past few years. During 1995 and 1998, less than 1% of the 650 authors discussing pericarditis were specifically related to uremic pericarditis uremia one of main complications in patients with terminal chronic kidney insufficiency is uremic pericarditis which usually is presented as acute uremic pericarditis but sometimes it can be chronic constrictive pericarditis with pericardial effusions.

Uremic pericarditis is caused from renal insufficiency either acute or chronic as result of inflammation of visceral and parietal layers by uremic toxin and disorders of urea and creatinine metabolism which are highly elevated in uremic patients. Uremic patients treated with hemodialysis can develop chonstrictive uremic pericarditis. Definitive cause of uremic pericarditis is still unknown. Many studies have documented that the onset of uremic pericarditis in uremic patients treated with HD is result of terminal renal disease and inadequate Hemodialysis(HD) sessions where enough amounts of fluid are not eliminated, volemic overload with secondary hyper-tension and overweight between HD intervals. Some authors suggest that uremic pericarditis can be result of retention of uremic toxins in organism.

Symptoms are pain pericarditis uremic presented with different intensity, which is alleviated when the patient is sitting or leaning forward. Clinical signs of pericarditis in patients with renal disease terminals (ESRD treat my chronic hemodialysis are similar to signs observed from other causes. Most patients complain of fever and pain pleural chest, the intensity of which is very different. the pain is localized in the region pericardial and may resemble the pain of angina pectoris. When developing effusionmassivepericardial may also appear dyspnea and heart failure.

* dr-luti@hotmail.com

Often pericarditis uremic accompanied with fever and high temperature. of particular importance is examination auscultation in the region of the heart after the notes sound of friction pericardial that are superficial. These is nonsystolic and diastolic and performances and typically three components of their appearance. When added effusion pericardial (the liquid) noise friction pericardial often disappear.Effusion pericarditis jugular veins cause strain (Kussmaul's sign-t) and can develop a paradoxical pulse (decrease of systolic pressure in inspiration). However, although there are documented facts that all patients with terminal renal disease treated me with recurrent pericarditis chronic hemodialysis may not manifest symptoms. In suspected cases of pericarditis, echocardiography should be done first so that Chiara's effusion pericardial highlights.

Aim of this paper is to verify and document the impact of terminal chronic kidney insufficiency and the impact from HD on the onset of pericarditis and its complications. Etiology of different types of pericarditis in patients with different diseases (cardiovascular, respiratory, infectious etc.) have been studied thoroughly except for manifestations and etiology of pericarditis in uremic patients treated with HD where not enough studies have been made therefore its etiology is still not definitely known. Therefore more and larger studies need to be made with more patients so the exact cause of this phenomenon with lethal consequences in uremic patients treated with HD can be found.

Materials and methods

In this clinical prospective observational study were analyzed 90 patients with terminal chronic kidney insufficiency treated with HD in Clinical Hospital of Tetovo on Department of Nephrology with mean age 62.40±5.20 identical for both genders. Initial evaluation includes a clinical history and physical examination, ECG, echocardiography(3D color Doppler) chest radiography, and lab studies.ECG can be diagnostic in acute pericarditis and typically shows ST elevation in all leads. The ratio of the amplitude of ST segment to the amplitude of the T wave in leads I, V4, V5, and V6 on electrocardiogram can be used to differentiate acute pericarditis (AP) from early repolarization (ER) and early repolarization of left ventricular hypertrophy (ERLVH), according to a recent study.

Statistical elaboration

The basic statistical methods used in this study were: arithmetical average value, standard deviation X±SD, Student "t" test, Mann Whitney U test, Wilcoxon test. The statistical significance of the differences between subjects of the experimented group and control group for the gained parameters was analyzed with "Anova Two Factor " with statistical value for *„p"* smallearthen 1% ($p<0.0001$).

Results and discussion

From 90 patients treated with HD, 30 patients (10 females, 20 males) showed symptoms of chest pain and tightening similar to pectoral angina (in small number of patients persistent temperature accompanied with fever was found). All patients who presented with symptoms of uremic pericarditis had fluid overload (between HD intervals) of 4000-6000ml.

Conclusion

In our work we verified that in 30% of examined patients predominate symptoms of uremic pericarditis, from those: in 25% pericarditis was caused as inadequate HD treatment, fluid overload between HD intervals, dietetic-hygienic disrespect of HD, termination of HD séance before required, disuse of antihypertensive and diuretic therapy (in 10% of patients with two weekly sessions of HD from 4 to 5 hours per séance), while in 5% of patients pericarditis was consequence of various infections. The hemodialysis patients also used the supp. Indomethacin, aspirin, corticotherapy and broad spectrum antibiotic.

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Macedonian pharmaceutical bulletin, **62** (suppl) 593 - 594 (2016)
ISSN 1409 - 8695
UDC: 616.12-008.331.1-085.225.2:616.61-008.64
Short communication

Treatment of arterial hypertension with ACE (Angiotensin-Converting-Enzyme) inhibitors for patients with chronic renal insufficiency

Dorontina Bexheti[1]*, Sadi Bexheti[1], Nexhbedin Beadini[1], Lutfi Zylbeari [1,2]

[1]*Faculty of Pharmacy, State University of Tetovo, R.M. Ul. D. Cara bb, 1200 Tetovo, Republic of Macedonia*
[2]*Special Hospital for Nephrology and Hemodialysis "Vita Medical Group"- Tetovo, Ilindenska bb, 1200 Tetovo, Republic of Macedonia*

Introduction

Cardiovascular diseases still count among the main causes of mortality in patients with chronic renal insufficiency (CRI). One of the risk indicators of cardiovascular factors considering this group of patients is Arterial hypertension (AH). Angiotensin Converting Enzyme inhibitors (ACE) are the most effective group of drugs used for the treatment of hypertension in patients with CRI (Brown et al., 1998) and cardiovascular diseases (CVD), based on the prevention of renal and CVD damages. ACE inhibitors are used primarily for the aforementioned diseases due to their effectiveness in slowing down the pace of progress and reducing the rate of morbidity and mortality in patients with these diseases. A large number of studies have been published where they show the positive effects of ACE inhibitors. These drugs remain further as the most preferred drugs in the treatment of patients with CRI and AH. AH and lipid abnormalities are among the most important causes that accelerate the progression of chronic renal disease (CRD) and the risk of CVD. The etiology of arterial hypertension is a multifactorial (near 20-25% of cases with AH the etiology is known, while other cases are due to many other disorders: hormonal, renal, cardiac, infectious, congenital diseases or inherited, different uropstruksionet, RVU (Reflux Vesico Uretral) etc.

AH remains one most important and common factor of diseases throughout the world. Between normotension and hypetension pressure not any precise definition persists, but based on the preferences and the World Health Organization all the values of systolic pressure higher than 140 mmHg and diastolic higher than 90 mmHg are treated as arterial hypertension. Recent years, a number of studies have verified and documented that between AH and lipid abnormalities and progress of CKD (Chronic Kidney Disease) there is a high positive correlation. There are facts documented that patients with CKD and other consequences besides AH, a large number of them suffer from a hipertregliceridemia and dyslipidemia, so it is very necessary examination, treatment, determination and correlation of lipids with AH, that in the initial stages of CRD with the sole purpose of preventing rapid pace of progress towards CRD uremia. Many contemporary studies testify a close connection between pressure and uremic dyslipidemia verifying that the AH with its oscillations significantly affect lipid disorder helping their stratification on the wall of blood vessels, thus increasing the risk of aterogenesis of coronary arteries, cerbrale with frequent manifestations of acute myocardial infarction.

Materials and methods

In the study 240 subjects were included (of which 140 male and 100 female respectively. The average age was 56.80 ± 12.50 years. The first group of 120 patients (50 female and 70 male) were treated over 12 months with the ACE inhibitor and the second group (50 female and 70 male) were treated with Angiotensin II AT1 receptor blockers and renin-angiotensin-aldosterone system. The patients were examined before and after the study. The examination was carried out for the analysis of proteinuria, serum urea, serum creatinine, uric acid electrolytes, profile of lipid and determination of the level of glomerular filtration

* dorentina.bexheti@cruk.cam.ac.uk

rate (GFR glomerular filtration Rate by Cockroft & Gault formula) before and after the therapy. The method used is standard deviation ± SD, two factor *ANOVA*.

Results and discussion

From the results obtained the blood pressure of the patients was lower in both treated groups, though statistically not significant. When comparing the groups treated with ACE inhibitors and the group treated with different group of medicines respectively there was statistically significant difference <0.005 for the concentrations of urea, creatinine, uric acid and with a stagnation of GFR in favor of the group that was treated with ACE inhibitors for over 12 months.

Conclusion

Many contemporary studies testify a close connection between pressure and uremic dyslipidemia verifying that the AH with its oscillations significantly affect lipid disorder helping their stratification on the wall of blood vessels, thus increasing the risk of aterogenesis of coronary arteries, cerebral with frequent manifestations of acute myocardial infarction, left ventricular hypertrophy, angina pectoris, heart failure congestive and cerebrovascular brain stroke (Antony et al., 1996). Common effects of AH and hyperlipidemia manifestly affect modification and reduce in renal functions causing nefroangiosklerosis with glomerulosclerosis. It is estimated that 10-13% of elderly patients in the US suffer from CKD and AH without taking to account the degree of CRD. AH during CRF is *volumic type* (with manifestations of cardiovascular complications with hypertensive cardiomyopathy etc. and despite that sometimes it may be as a result of other mechanisms like hipernatremia etc. Although more research needs to be carried out, our results suggest that ACE inhibitors, regardless of their antihypertensive effects, can reduce and slow down significantly the rate of chronic renal failure and cardiovascular diseases (Weiner et al., 2007). Inadequate treatment of HTA in patients with CRI symptoms will lead to risk of CVD and an increase in renal diseases.

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Macedonian pharmaceutical bulletin, **62** (suppl) 595 - 596 (2016)
ISSN 1409 - 8695
UDC: 616.72-002.77:616.153.915
Short communication

Treatment of apolipoproteinic profile in patient with rheumatoid arthritis

Nasir Behxheti[1,2]*, Lutfi Zylbeari[1,2], Mirlind Behxheti[1], Gazmend Zylbeari[2], Zamira Bexheti[1].

[1]*Medical Faculty, University of Tetovo, Ilindenska bb, 1200 Tetovo, Republic of Macedonia*
[2]*Clinical Hospital of Tetovo, 29 Noemvri bb, 1200 Tetovo, Republic of Macedonia*

Introduction

Disorders of lipoprotein metabolism in patients with rheumatoid arthritis (RA) even in early stages can be cause for the increased incidence of cardiovascular diseases, cerebrovascular events, perpheric artherial diseases and premature atherosclerosis (atherosclerosis praecox). In this study we wanted to display the apolipoproteinic abnormalities in patients with RA, their correlation and how RA causes changes of apolipoproteinic profile. RA is systemic disease of connective tissue (Boers et al., 2003; Dessein et al., 2006; Park et al., 1999; Gonzalez-Gay et al., 2005) with unknown ethiology, subacute or chronic with exacerbations and remissions, most commonly found in perypherial joints, symetrically, which with time can cause destruction, deformities and at the end ankylosis of joints. It is known fact that patients with RA are more prone to different inflamations which is confirmed from the fact that these patients have higher concentrations of C-Reactive Proteine, a biomarker who suggest the presence of inflammation who when is combined with apolipoproteinic abnormalities is count as additional factor on the onset of atherosclerotic changes of coronary and cerebrovascular arteries, with early onset of atherosclerosis in patients with RA.

Aim of study

Primary aim of this study is to show apolipoproteinic abnormalities and the concentrations of CPR in patients with RA, and their role on the progress of RA and their atherogenic effect on coronary, cerebrovacular and peripherial artheries.

Matherial and methods

In this cross-section study a total of 40 patients were included, from whom 24 were females with mean age of 48.0±18.6 and 16 males with mean age of 49.5±12.8, with verified diagnosis of RA according to criteria of American Association for Rheumatoid Arthritis of 1987. Control group was composed from 40 healthy individuals (voulentary blood donors) from whom 24 females and 16 males. In all patients (examined group and control group) beside routine analysis for lipid profile other analysis were made as well: total lipids (TL), total cholesterol (TCh), triglycerides (TG), HDL-ch,LDL-ch,ApolipoproteinA-1,Apo-B100, Apo-E, Lp(a)CRO and lipoprotein lipase (LPL) levels too.

Results and discussion

Our study confirmed that patients with RA are prone to have increased levels of: TL=8.20±2.70mmol/l;TCh=6.8±2.70mmol/l;TG=3.46±0.65 mmol/l; and LDL-ch=4.48±0.90 mmol/l whereas HDL levels were decreaed HDL-ch= 0.94 ± 0.60 mmol/l. From analysis of apolipoproteins we found low concentrations of Apo-A1=0.94±0.12 g/l and high concentrations of $ApoB_{100=}$ 2.98±1.85 g/l; Apo-E=6.50±1.70 g/l , Lp(a)=48.0±18.60, and CRP=18.0± 6.80 mg/l in contranst from the control group results: TL= 6.20±0.50 mmol/l; TCh =4,30±0.60 mmol/l; TG=1.24±0.70 mmol/l; HDL-ch= 1.28±0.40 and LDL-ch=2.90±0.90 mmol/l, and CRP= 4 mg/l. Lipoprotein lipase levels in patients with RA in our study showed low levels:LPL-12.80±4.20 u/L comparing with the control group LPL- 24.80±12.00 u/L. Decreased activity of LPL is believed to be result of increased concentrations of cyto-

* Nasir.behxheti@unite.edu.mk

cines: Interleukin 1, Interleukin 1β, Interleukin 1α, Interleukin 2,Interleukin 6, Interleukin 8, Interleukin 10, Interleukin 12 and Tumor Necrosis Factor. Disturbed apolipoproteinic profile, proinflamatory cytokines and increased levels of CRP are fact that patients with RA are prone to increased risk for atherosclerotic changes in blood vessels, with increased risk for cardiovascular diseases, cerebrovascular events, peripheric artherial disease and onset of early (premature) atheros-clerosis (atherosclerosis praecox).

Conclusion

It can be concluded that measuring lipidic and apolipoproteinic abnormalities in RA patients can help to take hygienic-dietetic measures as well as treatment methods which significantly would decrease prevalence of dyslipidemia and would also slow down the atherogenesis which would reduce in minimum the onset of cardiovascular, cerebrovascular, atherosclerotic macroarteriopathy and early atherosclerosis in patients with RA. Management of lipid abnormalities in patients with RA aims to normalize the atherogenic index (TCh/HDLch) with statines, fibrates, nyacine, cholestipol, cholestyramine or combined therapy, also prevention of inflamatory processes.

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Macedonian pharmaceutical bulletin, **62** (suppl) 597 - 598 (2016)
ISSN 1409 - 8695
UDC: 616.61-008.64-78-085.272
Short communication

Treatment with L-carnitine in uremic patients treated with chronic hemodialysis - reistant erythropoietin

Lutfi Zulbeari [1,2]*, Driton Selmani[1] Nexhibe Nuhii[1], Zamira Bexheti[1,2], Gazmend Zylbeari[1]

[1]*Special Hospital of Nephrology and Hemodialysis, Vîta Tetovo Medical Group, Ilindenska bb, 1200 Tetovo, Republic of Macedonia*
[2]*Faculty of Medical Sciences, State University of Tetovo, R.M. Ul. D. Cara bb, 1200 Tetovo, Republic of Macedonia*

Introduction

One of chronic disorders of terminal renal failure is also a metabolic disorder of L-Carnitines. L-carnitine in the body acts as an antioxidant fighting the harmful particles in the body known as free radicals, which damage the DNA and excite it. The lower concentration of L-carnitine(L-c) in patients treated with hemodialysis (HD) is a consequence of the loss of it through dialisant (Clutterbuck at al., 2002; Fornasini **et** al., 2007) digestion and reduce endogenous synthesis as a result of absence of the precursor (predecessor) amino acid Lisine. In the kidney, osmolytes including carnitine are crucial since hypertonicity is usual and the kidney must cope with fluctuations of diuresis (increased production of urine) and antidiruesis. Extracellular osmolarity of medullary cells may become more than four-fold that of isotonicity. In healthy individuals, carnitine is freely filtered and tubular resorption of free carnitine (FC) is almost complete. What is excreted in urine is carnitine ester, or acylcarnitine (AC). In healthy people, the renal clearance of AC is four to eight times that of FC.Impairment of excretion of AC occurs with deteriorating renal function leading to decreased carnitine clearance and resulting in elevated plasma levels of carnitine.Uremic patients have elevated levels of AC that occur as both elevated FC and total carnitine before (Arduini et al., 2006). Due to accumulation of metabolic intermediates, impaired carnitine biosynthesis, reduced protein intake, and increased removal of carnitine through hemodialysis (HD), patients who undergo routine HD usually present with plasma carnitine. (Cibulka et al., 2005) There are documented facts that the plasma levels of L-C at the end of each dialysis session slimmed down to about 70-80% on average. This reduction in plasma levels of L-carnitine affter HD is a consequence of epoetin-resistant anemia, intradyalitic hypotension,cardiomyopathy, fati-gue,muscle weakness, of various inflammation, reduced endogenous synthesis of cofactors of L-Carnitines and malnutrition. L-Carnitines affects remodeling of phospholipidic membrane of red blood cells, stimulates erythropoiesis in high concentrations>200 μmol/L, increases survival time of red blood cells, redu-ces oxidative stress through the hem oxygenasis-1, and It reduces inflammation, and reduces the value of C-reactive protein (PCR) and lipids. A large number of observers have documented studies of an inverse correlation between the dose of rHuEPO and carnitine level.

Randomized and controlled studies in a meta-analysis suggest that supplementation with L-Carnitines has shown positive effects in response to therapy with Erythropoietin in chronic uremic patients treated with HD. Uremic patients treated with HD due to low values of L-C often manifest anemia resistant to therapy with human Eritropoetin Recombinant (rHuEpo) with substitution with L-Carnitines significantly reduces the resis-tence to therapy with rHuEpo, with K/DOQI association recent years suggests that patients treated with chronic HD and resistant to therapy by the end of the session with HD must necessarily be treated with L-Carnitine (KDOQI Clinical Practice guidelines 2008-2009). Aim of this paper was to show the effects of L-Carnitine in uremic patients treated with HD.

* dr-luti@hotmail.com

Materials and methods

In total 100 patients treated with chronic HD were studied. Of the 100 patients treated with HD-46 (45%) were female- 54 (55%) were male, with an average identical age of 58.70±12.60, treated over 8 years in Tetovo Clinical Hospital and Clinic Skopje. Patients were divided into two groups: The first group comprised of 65 patients had a sensitive reaction to therapy with rHuEpo dose of 2000-4000 IU after HD subcutaneous and the second group consisted of 11 women and 12 men who after HD had the highest value carnitine clearence on even after increasing the dose showed a resistance to therapy after 6-8 weeks and we decided that this patients to be trea-ted with L-C ampoules of 1 g after each session of HD and 2x500 mg L-C in the form tablets days that have not HD. Within 8 months of this dose, we have seen a rapid increase in the level of carnitines, reaching levels two to three times higher than the concentration of physiological L-C and high impact to eritropoesis and stimulation of erythroblasts (11,12) as well as positive effects on therapy with rHuEpo which was manifested with adjustment of blood (Er, Hb, Htc) and improvement of symptoms of renal anemia . Our study confirms the elimination of L-C of 16.80 ± 28.40% over a period of 8-12 months of treatment with HD.

Results and discussion

Before starting the Epo therapy mean values of Hb in-female patients were 5.20±1.15 mmol / l, while in males were=5.60±1.10 mmol/l, the number of Er in female was:2.90± 0.86 x 10 12 / l, while in males were=3.20± 0.90 x 10 12/l. Values of HTC in female were=0:24 ± 0:04 while the men-12:28±12:06. After treatment with EPO therapy and amp. L-Carnitine a 1.0 g intravenously at the end of the session that the HD the desired target Hb was > 6.80 mmol / l and after 16 weeks the women target was Hb> 6:40 mmol/l, while the male target Hb>6.85 mmol/l, the Er number of female reached: 3.60±0.80x10 12 / l, while in the male were 3.85±0.80x1012 /l. The values of Htc in the female came up-0.32± 0.03 while the men-0.04± 0.35. The comparison of values of patients before treatment with L-carnitine and Epo therapy and after treatment showed a significant statistical difference of p <0.0001. Statistical processing: statistical methods that were used are: arithmetic mean value, standard deviation: X ± SD, Studentov,,t"test, Mann-Withney U test,Annova Two-Factor. The results obtained from the examination of Hb, Er, Htc are processed and presented in the form of charts, tables and diagrams.

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Conclusion

We can conclude that patients undernourished, anemic and resistent to therapy with rHuEpo and lower values of the concentrations of L-C after the session with HD, supple-mentation with carnitine (per os or ampular) can improve reanale anemia significantl resistant to Epo therapy. Our findings confirm that elimination of L-carnitines after application of L-C in HD decrease from 16.80 ±28.40% .But are necessary further studies with larger number of patients with longer duration so that can be documented and verified the effects of L-carnitine to uremic patients and resistant to Epo therapy.

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Macedonian pharmaceutical bulletin, **62** (suppl) 599 - 600 (2016)
ISSN 1409 - 8695
UDC: 615.217.22.074:543.544.5.068.7
Short communication

Prediction of blood–brain barrier permeation of α -adrenergic and imidazoline receptor ligands using different HPLC systems and quantitative structure-permeability relationship analysis

Jelica Vucicevic*, Marija Popovic, Katarina Nikolic, Slavica Filipic, Danica Agbaba

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Vojvode Stepe 450, 11000, Belgrade, Serbia

Introduction

In order to estimate central nervous delivery of novel compounds a lot of useful in vitro models have been developed including Parallel Artificial Membrane Permeability Assay (PAMPA) (Avdeef, 2005), Biopartitioning Micellar Chromatography (BMC) (Molero-Monfort et al., 2000), Immobilized Chromatographic Techniques (IAM), and cell based assays. BMC is a mode of micellar liquid chromatography that uses micellar mobile phases of Brij35 under adequate experimental conditions. It can be useful in mimicking the drug partitioning process into biological systems, because the characteristics of BMC system are similar to biological barriers and extracellular fluids (Molero-Monfort et al., 2000). On the other hand classical reversed phased HPLC system is recognized as the well-established system for estimation of compounds lipophilicity. The important role of lipophilicity and its use in prediction of blood-brain permeability of drugs have been demonstrated in several studies (Kaliszan, 2007). Retention data obtained by this method can be further used in correlation studies with some physicochemical and biological properties of tested compounds (Liu et al., 2011). The compounds examined in this study were imidazoline receptor ligands (IRs) (Ernsberger, 2000). IRs have been tested as novel centrally acting antihypertensives and drug candidates for treatment of various neurological disorders (Fenton et al., 2006; Finn et al., 2003). Since these ligands exhibit additional CNS effects, several structurally related CNS drugs were also included in this research. In this work, the retention data of 16 imidazoline/α-adrenergic receptor ligands and 15 CNS drugs, were examined using BMC and RP-HPLC systems and correlated with permeability coefficients (Pe) previously obtained in our laboratory using PAMPA (Vucicevic et al., 2015).

Materials and methods

Chromatographic conditions

Chromatographic analysis was carried out on an Agilent Technologies 1200 Series system (Santa Clara, CA, USA). BMC analyses were performed on a Zorbax Extend-C18 column (150 mm × 4.6 mm, particle size 5 μm), with a flow rate of 1 ml/min and a temperature of 36.5 °C. Micellar mobile phase was prepared by dissolving polyoxyethylene (23) lauryl ether Brij 35 (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) in buffered solution at pH 7.40 (which corresponds to blood pH of humans) to get a final surfactant concentration of 0.04 M. RP-HPLC measurements were performed using a XTerra® RP18 column (100 mm × 4.6 mm, particle size 3.5 μm). The flow rate was 0.8 ml/min, column temperature was set to 25 °C. Mobile phase was consisted of different ratios of methanol and sodium dihydrogen phosphate buffer (pH 7.40).

Computational method

The Quantitative Structure-Permeability Relationship (QSPR) models were developed using retention data from BMC system. PLS regression analysis was performed using the Soft Independent Modeling of Class Analogy SIMCA P+ 12.0 program (Umetrics AB, 2008), while STATISTICA 5.0 (StatSoft Inc., 1998) was used for the stepwise MLR and ANN analysis. The dominant molecules/cations species at pH = 7.4 of examined compounds have been ob-

* jelicav@pharmacy.bg.ac.rs

tained using the MarvinSketch 6.1.0 (ChemAxon, 2013). The geometry of all compounds have been minimized using B3LYP/6-31G(d,p) level of the Density Functional Theory (DFT) (Becke, 1993; Lee et al., 1988) included in ChemBio3D Ultra 13.0 program (CambridgeSoft Corporation, 2013). Constitutional, geometrical, physicochemical and electronical parameters of optimized molecular structures were calculated using following software: ChemBio3D Ultra 13.0, Dragon 6.0 (Talete srl, 2010) and ADMET Predictor 6.5. Additionally, HOMO and LUMO energies obtained using ChemBio3D Ultra 13.0 were used to calculate some quantum chemically based reactivity molecular parameters, such as chemical potential (l), electronegativity (v), hardness (g), global softness (S) and electrophilicity index (x) (Iczkowski and Margrave, 1961; Parr and Yang, 1989).

Results and discussion

Using ADMET predictor 6.5 (Simulation Plus, Inc., 2013) and ACD/i-Lab software (http://www.acdlabs.com/resources/ilab/) it was shown that those compounds are not substrates Pgp and their BBB-permeability could be accurately predicted by using non-cellular in vitro methods. Significant correlation was obtained between logarithm of BMC retention factor (logkBMC) and effective permeability (Pe) (R2=0.69), while for RP-HPLC system the correlation was lower (R2 logkwRP-HPLC / Pe = 0.49). Therefore it can be concluded that logkBMC parameter could be used as more reliable than logkwRP-HPLC to assess the BBB penetration of IRs/α-ARs ligands and CNS drugs in more time efficient manner. Further, retention factors (logkBMC) of α-adrenergic/imidazoline receptor ligands and CNS drugs were used as dependent (Y) variable in this QSPR studies, while their calculated molecular descriptors were selected as independent (X) variables. Partial Least Square (PLS) regression, stepwise Multiple Linear Regression (stepwise MLR), and Artificial Neural Networks (ANN) techniques were applied in order to create new mathematical models. Stepwise MLR analysis have been resulted in statisticaly better performance than PLS and ANN, and therefore MLR/QSPR-logkBMC model was selected as an optimal. The P_VSA_s_5, VE1_RG, P_VSA_p_2 and GATS1e were selected descriptors with the most significant influence on logkBMC and consequently on Pe, indicating on possible structural modifications which could enhance BBB penetration.

Conclusion

Formed models could be used as time and cost efficient screening tool for evaluation of blood-brain barrier permeation of novel α-adrenergic/imidazoline receptor ligands, as promising drug candidates for treatment of hypertension or neurological diseases.

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Continuing professional development

Macedonian pharmaceutical bulletin, **62** (suppl) 603 -604 (2016)
ISSN 1409 - 8695
UDC: 615.1
Short communication

Excellence in pharmacy practice – Quality indicators based on tradition, experience and innovations

Arijana Meštrović

Pharma Expert Competency Development Manager, Dunjevac 2, 10 000 Zagreb, Croatia

Introduction

Pharmaceutical care is the responsible pharmacists practice, which provide safe and best available therapy for the patient. It is the professional activity in which the pharmacist, using his knowledge and experience, revealing patients' needs, set priorities in the treatment process, and takes responsibility for a positive outcome of drug therapy (Hepler and Strand, 1989). That responsibility is shared with the doctor who determined the diagnosis and prescribed therapy, and with patients, encouraging them to the compliance, frequent check and counseling about responsible treatment. Today, many countries are trying to incorporate this new concept in its health care system, and although such attempts are of great interest to national and international pharmacy organizations, many challenges often appear in the implementation of this concept. Some of the difficulties may include: attitudes and opinions of other health professionals, lack of cooperation, and inadequate communication between them, an insufficient number of pharmacists, space or equipment for the provision of pharmaceutical care, including the structure and organization of health care (EDQM, 2012).

Pharmaceutical care derives from the principles and postulates of clinical pharmacy, which pharmacists recognize as the scientific basis for intervention in the treatment of patients (RPSGB, 2007). The concept of clinical pharmacy clarifies the role of the pharmacist in the process of providing health care. It involves different ways of cooperation of health professionals in which science and practice can be linked to patient care. But to make this impact had the biggest impact possible, it is necessary to develop clinical knowledge, but also communication skills, judgment and decision- making. Clinical practice should occupy an increasing role in the daily work of a pharmacist, instead of being just one of the possible options or specialty pharmacist. Clinical pharmacy practice means and includes the philosophy of pharmaceutical care that is focused on specialized knowledge and experience in the treatment. As a scientific discipline, clinical pharmacy includes the task of collecting and contributing to the creation of new knowledge that can improve the health and quality of life (McGivney et al., 2007).

Indicators of quality of pharmaceutical care are equally appropriate for in-patient and community settings, for hospital and community pharmacists, and other healthcare professionals, as applicable, in low-, middle-income and industrialized countries in Europe and other regions of the world. The indicators provide information about the range; quantity and quality of pharmaceutical care interventions/services delivered. The indicators also provide an opportunity to gather in-depth knowledge on pharmaceutical care practices regionally, nationally, and internationally that will permit the sharing and follow-up of experiences over time by professional disciplines and the health sector in general, regionally, nationally and internationally. These indicators are rather broad, and can be further developed and refined over time, but they are easily understood and will help pharmacists, other healthcare providers, and professional regulators to formalize and develop the pharmaceutical care philosophy and its working methods (EDQM, 2012).

Some of the terms, which are used in Indicators of quality, are:

1. Adverse drug reaction (ADR) means a response to a medicinal product which is noxious and unintended and which occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease or for the restoration, correction or modification of physiological function

* arijana.mestrovic@pharmaexpert.hr

2. Documentation – the detailed description of a patient-provider or provider-provider interaction. Documentation serves as a record for stating relevant participants, evidence, assumption, rationale, and analytical methods used in evaluating patient progress and quality of care or outcomes for individuals. Also functions as a means of communication among providers and analysis for billing purposes.
3. Customer loyalty - expresses an intended behavior related to the product or service. This includes the likelihood of future purchases or renewal of service contracts or, conversely, how likely it is that the customer will switch to another brand or service provider.
4. Follow up is maintenance of contact with or re-examination of a person (as a patient) at usually prescribed intervals following diagnosis or treatment
5. Medication review is an evaluation of patient‘s medicines with the aim of optimizing the outcome of medicine therapy by detecting, solving and preventing drug-related problems
6. Interprofessional collaboration – working together with one or more health care professionals who each make a unique contribution to obtain optimal patient medication outcomes, considering patient needs, expectations, and quality of life
7. Patient Medication Profile – is a comprehensive summary of all regular medicines taken by the patient.

Professional assessment of prescription is the assessment of whether the prescription includes an appropriate dosage form and appropriate route of administration; appropriateness according to patient's condition; dosage within therapeutic range; duration of treatment; appropriateness according to patient's parameters (age, weight, etc.) and previous medication; compatibility with other medication; consistency with formularies, clinical guidelines and protocols; possible side effects; risk of adverse drug reactions; potential for non-concordance, inappropriate use and misuse by patient; contraindications (RPSGB, 2007).

Pharmaceutical care is the responsible provision of drug therapy for the purpose of achieving definite outcomes that improve a patient's quality of life. These outcomes are:

- Cure of a disease
- Elimination or reduction of a patients' symptomatology
- Arresting or slowing of a disease process
- Preventing a disease or symptomatology

Pharmaceutical care involves the process that a pharmacist co-operates with the patient and healthcare professionals in designing, implementing, and monitoring a therapeutic plan that will produce specific therapeutic outcomes for the patient. This in turn involves three major functions:

- Identifying potential and actual drug-related problems
- Resolving actual drug-related problems and
- Preventing drug-related problems.

Pharmaceutical care is a necessary element of health care, and should be integrated with other elements (McGivney et al., 2007).

Pharmaceutical care is, however, provided for the direct benefit to the patient, and the pharmacist is responsible directly to the patient for the quality of that care. The fundamental relationship in pharmaceutical care is a mutually beneficial exchange in which the patient grants authority to the provider and the provider gives competence and commitment (accept responsibility) to the patient. These fundamental goals, processes, and relationships of pharmaceutical care exist regardless of practice setting and of professional background (Hepler and Strand, 1989).

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Macedonian pharmaceutical bulletin, **62** (suppl) 605 - 606 (2016)
ISSN 1409 - 8695
UDC: 615.1(497.7)
Short communication

Quality of community pharmacy service in Republic of Macedonia – professional supervision

Bistra Angelovska* and Jasminka Patceva

Pharmaceutical Chamber of Macedonia, 50 Divizija, 1000 Skopje, R. Macedonia

Legal framework

Pharmaceutical Chamber of Macedonia (Chamber) as a professional association of pharmacists with high education, beside public authorizations for issuing, continuing, renewing and revoking of working licenses and keeping registers, with the adoption of the Law on Health Care Protection in 2012 has obtained one more public authorization – professional supervision (LHP, 2012). According to this Law, supervision of the professional work of the healthcare institutions and other institutions that perform healthcare activities and of the healthcare workers and co-workers is being performed for the purpose of control of the professional work, the implementation of the professional guidelines, assessment of the professional work, as well as assessment of the conditions and the manner of provided healthcare (LHP, 2012). The final goal of the professional supervision is evaluation of the quality of the healthcare services provided by the healthcare workers, in this case pharmacists, and undertaking measures for their improvement. The quality of provided services by the pharmacists, as well as the conditions under they are provided have invaluable significance for the provision of pharmaceutical activities as a part of the whole health care protection, care for the patients, prevention of the diseases and obtainment health for the population. The Chamber has fully recognized the aims and the goals of the professional supervision, the need for determining the provision, quantity and sustainability of the quality of the healthcare services respectively, as well as determining the conditions for performing the supervision and their maintenance at the required level according to the current legal provisions.

Creating basis for implementation and developing acts and tools

The implementation of the professional supervision as a new public authorization in the Chamber started with forming a Commission for organizing the professional supervision with duties: to make proposed list of pharmacists – providers of the supervision, to develop criteria for performing professional supervision, to propose annual plan for conducting professional supervision, to keep evidence of performed supervisions and suggested and taken measures for improvement, to write annual reports, to give reports of performed supervisions to the Ministry of Health, to make orders for performing the supervision, to determine performers for supervision, all according to the Law, the Statute of the Chamber and the Code of Ethics (Rulebook, 2014).

During the 2013, the Commission for professional supervision created a Rulebook on the performing the professional supervision, which later was adopted by the Chamber's Assembly and published in the Official Gazette of R. Macedonia (Rulebook, 2014). In the Rulebook are determined the way of organizing the professional supervision and the procedures for performing according to the legal provisions. The conducted supervision can be regular supervision or professional supervision when necessary – extraordinary professional supervision (LHP, 2012). Regular supervision is conducted according to the annual plan, set by the Commission and adopted by the Executive Board of the Chamber (LHP, 2012). The extraordinary supervision is carried out in the case of a request for examination the operation of the health care facility and the work of the pharmacists during the provision of the pharmaceutical services (LHP, 2012). The request can be submitted by patient, member of the patient's family and governmental body (LHP, 2012). The professional supervision

* bistra_anglovska@yahoo.com

is conducted by the performers, who fulfill certain criteria such as (1) having specialization in the certain field of pharmacy or master degree, at least 5 years working experience in appropriate health care institution and valid license to work, or (2) having at least 10 years working experience in appropriate healthcare institution and valid license to work (Rulebook, 2014). During the professional supervision performers examine, evaluated and control the organization and the management of the work in the healthcare institution, the manner of work of the healthcare institution, professional qualifications of the pharmacists, the rights and the obligations of the pharmacists, professional work of the pharmacists, their continuing education, keeping official books and registers and provision of the legal requirements and professional guidelines (Rulebook, 2014). After completing the professional supervision, performers prepare minutes, which is submitted to the Commission. Commission further submits report to the Ministry of Health (LHP, 2012). If the performers determine irregularity in the work, they are obliged to give directions for eliminating the irregularity (Rulebook, 2014). They are also obliged to give instructions for improvement if needed. If pharmacists and the pharmacy don't carry out the instructions within the period laid down by the performers, then the Commission shall notify the competent authorities and bodies of the Chamber (Rulebook, 2014).

Furthermore, the Chamber announced call for selection of providers of professional supervision. After the performers' selections was completed, lists of providers of the professional supervisions for community pharmacy, hospital and clinical pharmacy, laboratories and other health care providers where pharmacists work were formed.

It was also decided professional supervision to start to be conducted in community pharmacies, and then to cover other healthcare institutions.

Because of fair, efficient and quality delivery of professional supervision the Commission has prepared List of indicators for the implementation of professional supervision over the work of community pharmacies and pharmacists who work in them. The List is a helpful tool to facilitate the conduction of the professional supervision without subjective influence on performers. The List is structured form of statements that determine certain obligation, criteria, condition or process needed to establish the quality of the provided healthcare services by the pharmacists as well as the conditions in which those services are provided.

Performing and initial results

Professional supervision began to be implemented in the second half of 2014. At the beginning was conducted on 8 pharmacists employed in 6 pharmacies on the territory of Skopje. The next year, were conducted 28 supervisions on pharmacists employed in 19 pharmacies in 9 communities in Skopje. In all performed supervisions it was found that the healthcare workers – pharmacists work professionally, respect legal requirements, educate continuously, respect patients' rights and confidentiality of the data, communicate with other healthcare workers, take care of the environment where they work, equipment and inventory. It was also determined that there were not irregularities in the overall work of the pharmacies as healthcare institutions. The condition settled in performed professional supervisions indicates several facts. Most of the pharmacies don't produce magistral preparations. They order them from other legal entities – galenic laboratories. Most of them have contract with Health Insurance Fund, and only for the medicines that are reimbursed they obligatory keep electronic record for the patients. Only 2 pharmacies have Standard Operating Procedures (SOPs) for the most of the processes performed in the pharmacy. In the supervised pharmacies, no one from the pharmacists has reported adverse reaction in the National center for reporting adverse reactions. No one at the pharmacies keeps evidence for shortages of the medicines. The performers of the supervision in individual cases have given instructions and guidelines, for example, haw to improve area for dissolving syrups, which data to be put in the electronic records, development and implementation of SOPs for individual processes in pharmacy, conflict management and else.

Professional supervision is being performed in 2016 according to the Plan for the current year.

Future developments and expectations

In the future, the Chamber regarding the public authorization – professional supervision aims: to increase the number of the performers to be able to carry out more supervisions simultaneously on the territory of Macedonia, to develop lists of indicators for other healthcare institutions where pharmacists work and to begin to conduct professional supervision

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Macedonian pharmaceutical bulletin, **62** (suppl) 607 - 608 (2016)
ISSN 1409 - 8695
UDC: 615.2:339.13.025.3
Short communication

Model framework for off label use of medicines

Blerina Koshi[1]*, Elizabeta Zisovska[2], Vasilka Nica[3], Maja Simonoska Crcarevska[4], Marija Glavas Dodov[4], Renata Slaveska-Raicki[4]

[1]*Department of Pharmacy, Faculty of Medicine, University of Prishtina "Hasan Prishtina", 10 000, Prishtina, Republic of Kosovo*
[2]*University Clinic for Gynecology and Obstetrics, Faculty of Medical Sciences, University "Goce Delcev", Krste Misirkov, bb, Stip, Macedonia*
[3]*Public Institution in the Health Sector, for the needs of PHI University Clinics, Institution and Urgent Centre, Majka Teresa 47, 1000 Skopje, Macedonia*
[4]*Faculty of Pharmacy, University Ss Cyril and Methodius, Majka Teresa 47, 1000 Skopje, Macedonia*

Background

The drug licensing regulatory system ensures that marketed drugs to be used, meet the high standards and requirements for quality, efficacy and safety. Unfortunately, in practice, prescribers are often obliged to deviate from granted medicine marketing authorisation, due to the lack of availability of appropriate medicines for patient's therapeutic needs and progress. This concept of medicines use not mentioned in the approved labelling (FDA Modernization Act) or outside of the terms of Summary of Product Characteristics regarding indication, age, dosage, pharmaceutical form and route of administration (British NHS Guideline) is defined as off-label use of licensed medicines.

On the global level, many supportive evidence and health care needs confirm that off-label medicines use occurs in every country and each level or specialty area of healthcare (Conroy, 2003). Moreover, it is an integral part of Good Medical Practice and may provide the best available option or even the standard of care in a particular health condition (Dresser and Frader, 2009). In general, this concept is legal and may be appropriate, but it can be associated with safety, clinical and ethical concerns, emphasizing the increased incidence of adverse events associated with off-label medicines uses in particularly vulnerable patient groups (Gazarian and Kelly, 2006).

A concerning issue is that the majority of all off-label uses have limited to no scientific support (Radley et al., 2006) and a considerable number of prescribers have no or limited knowledge about off-label medicine use or do not meet regulations regarding off-label use, if they exist. (Piñeiro Pérez et al., 2014).

Experience shows that to ensure the quality of off-label use of medicines, there should be a formal mechanism to assess the feasibility, monitoring the safety and efficiency of medication used based on this concept.Thus, in continuum, the off-label use of medicines has been an essential part of the ethical and legal considerations as well as, many regulatory initiatives.

The overall objective is to present a model regulatory framework setting out guidelines and recommendations for quality use of off-label medicines within the national profile of health care policy.

A literature search was undertaken to identify the issues and challenges related to off-label medicines use including clinical, safety and ethical concerns.

Recommendations for model framework

Principles of good practice for off-label use of medicines should include the following elements: identifying the medical needs; compilation of a consensus list of accepted, scientific based off-label uses; creating an official expert group for the evaluation and approval of specific off-label uses; and, providing a safe and effective supply. The main guiding principles and developed activities to support a responsible decision-making with regard to off-label medicines include: 1) the medical need- the best avail-

* sblerinakoshi@hotmail.com

able treatment in cases of specific characteristics when authorized medicines cannot meet the patients' need; 2) sufficient scientific basis and/or clinical practice experience to justify their action. Distinguish the routine off-label use, which is the use of these medicines based on "high quality" evidence and the use in specific exceptional circumstances; 3) information duty and a high degree of respect for patient rights, involving the patient/carer in decision-making process; 4) monitoring and reporting the outcomes, efficiency and adverse reactions; 5) considering self-monitoring of prescribing practices, liability and accountability. An additional special responsibility which among others falls on pharmacists should be to ensure that the prescriber is conscious for off-label prescribing and the reasons for that 6) production of compendia of certain medicines, enlisting those off-label uses judged to be legitimate.7) financial sustainability of an off-label use in medical practice. Before deciding to compound a patient-specific preparation, a step by step evaluation of alternatives should be made. These alternatives include a therapeutic alternative, dose rounding or manipulation of licensed dosage forms (splitting tablets, crushing tablets/opening capsules, dispersing their content in water or food, splitting suppositories, the use of a preparation designed for another route of administration).

Conclusion

Prescription, compounding, dispensing and administration of off label use of medicines should be regulated within the national profile of health care policy.

The regulation regarding the practice of off-label medicine use differs between countries. Some countries have this practice regulated by law, while in others it is covered by good practice regulations or general professional recommendations and ethical standards. Assuming that there is no any general rule to regulate the "accurate" off-label use of medicines it is of paramount importance for the countries to find a national solution to fulfil the ethical and legal demand, especially in the areas of pharmaceutical law and health insurance law. The common elements of these regulatory frameworks are the physicians' freedom to prescribe off-label medicines if the scientific evidence exists and the need to inform patients when making this decision. Making policy efforts, by adopting appropriate guidelines for off-label medicines use, based on scientific evidence, with specifications of healthcare professionals' responsibilities and a registry of off-label drug use in every day practice, would make possible a valuable approach towards ensuring a quality use of these medicines. Recommended solutions, as practiced in some countries, would support prescribes in more direct and active approach to handle the ethical and legal phenomenon associated with the off-label use of medicines

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Macedonian pharmaceutical bulletin, **62** (suppl) 609 - 610 (2016)
ISSN 1409 - 8695
UDC: 615.1(497.7)
614.27(497.7)
Short communication

Developing community pharmacy practice

Vesna Stavrova[1]*, Maja Simonoska Crcarevska[2], Marija Glavash Dodov[2] and Renata Slaveska-Raicki[2]

[1]*Pzu Apteka Sara farm Skopje, Teodosie Gologanov 58, Skopje, Macedonia*
[2]*Faculty of Pharmacy, University Ss Cyril and Methodius, Majka Teresa 47, Skopje, Macedonia*

Background

With the independence of the Republic of Macedonia (RM) in 1991, in parallel with the transition from planned to market economy, the Health System has also undergone through phases of transition from centrally planned towards market-guided. In 2005 with the amendments of the Law on Healthcare (LH), the Primary Healthcare Reform started to be intensively implemented, by primary health system transformation from Public to Private Sector. With this process which is almost entirely completed, all community pharmacies in RM have been privatized in the course of 2007, hence today, there are a total of 846 organizational units out of which 714 have concluded contract with the Health Insurance Fund of Macedonia (HIFM).

The Community Pharmacy is the basic unit in the pharmaceutical sector and it poses a primary level of pharmaceutical care. The contemporary community pharmacy practice in RM is clearly positioned in the Health Care System (LMMD, 2007) it acts in accordance with the principles of Good Pharmacy Practice (GPP), and it is organized in accordance with the requirements of the International Quality Management Standards ISO 9001:2000, as well as the Environment protection standards ISO 14001:2004. The activities for implementation of GPP in Community Pharmacy are also based on the established standards by the World Health Organization (WHO), in regards to the definition of health, illness, as well as the role of the pharmacist in the Healthcare system (WHO, 2006). In line with all these improvements in 01.07.2013 the system of e-prescription has been introduced.

Turnover of medicines in the Private Health Institution (PHI) community pharmacy

The pharmaceutical sector in RM has been regulated, in accordance with the Laws on: 1) medicines and medical devices; 2) Health care; 3) Health Insurance, further on, the Decree for Health Institutions Network, a series of rulebooks and other bylaws (LHP, 2004; LHI, 2010; LMMD, 2007). The work in the community pharmacies with the HIFM is regulated with a special Rulebook referring to the way of prescription and dispensing medicines and narcotic drugs (RB, 2010). In regards to the turnover of medicines, the medicines from "the list of medicines for primary healthcare" which are issued on prescription are provided through the PHI Community Pharmacies which have concluded contracts with the HIFM. The rulebook for determining the monthly funds that the PHI Community Pharmacies receive from the HIFM for the dispensed medicines on prescription from the abovementioned list funded by the HIFM, is in force. With the latest contracts and technical instructions of HIFM from 2014, electronic communication has been established between HIFM and the pharmacies by introducing a system of electronic way of invoicing of the performed healthcare services. The pharmacies which have concluded contracts with the HIFM, submit monthly invoices for the dispensed medicines on prescription which are funded by the HIFM in written and electronic form, together with the relevant supporting documentation. From the HIFM data on dispensed medicines on prescription, for the period 2013-2015 the trend of continuous growth in usage of medicines in the past years has been evident and this trend continues on in the first half of 2015 (HIFM, 2015a). Namely, most of the funds (27,5% from the total amount of HIFM) are foreseen for the ATC Group C – medicines for the cardiovascular system, while on the second place with about 15%, are the medicines for the R group i.e., for treatment of illnesses of the respiratory system. The medicines that affect the CNS (ATC Group N) are on the third place with a share of 14, 5%. The most dispensed medicine according to the number of prescriptions in the first half of 2015 is the medicine enalapril with a total of 971.540 dispensed prescriptions.

* apt.studentska@gmail.com

Medicines pricing policy

Another important aspect from the work of the Community Pharmacy is the medicines pricing policy. Namely, there is a national medicines pricing policy in RM for the medicines from the essential medicines list and the medicines from the list of medicines funded by HIFM (positive list). In addition, a rulebook has been adopted which determines the criteria and the procedure for determination of the reference prices of the medicines. In 2014, HIFM made the fifth in a row annual revision of all the reference prices from the positive list and determined new reference prices harmonized with the prices of medicines in the region and the new marketing approved process of the medicines from the National Agency of medicines and medical devices. The reference prices of medicines are guaranteed for all the insured persons.

As a result of the entire process, the latest data confirm that out of the total of 426 registered generic medicines from the Primary positive list, around 75% are without additional participation from the patients. The total number of the medicines without participation per nonproprietary name of medicines is still the highest until now and in 2014 it was 42% of the total number of dispensed medicines on prescription funded by the HIFM. Regarding the medicines per INN which are with participation, the largest part (82%) are with minimum participation or without participation. From the remaining medicines, 14% are with minimum participation and 4% are with maximum one. In the first half of 2015 the total increase of the number of dispensed prescriptions was around 10% compared to the same period of 2014 (HIFM, 2015b).

PHI community pharmacy inspection and most frequent irregularities detected in the work

The inspection of the PHI Community Pharmacy is performed on the bases of: 1) Law on Health Insurance, 2) bylaws, and 3) contracts concluded between HIFM and the PHI Pharmacies. The inspection is performed in the premises of the PHI Pharmacies and with a person authorized for the finances. Defined representative control sample is at least 15 randomly selected medicines. The licensed inspectors from the HIFM and the inspectors from the Pharmaceutical Sector have conducted a total number of 852 controls; 98% of the total number of pharmacies which have a contract with the HIFM have been controlled in 2014 (HIFM, 2015b).

Regarding the noncompliance in the work of a certain PHI Pharmacy – working unit according to the Legal regulations and the Contractual provisions, contractual penalties are foreseen from a measure reprimand to a measure one-sided termination of the contract. From the conducted inspections of the PHI Pharmacies most frequent determined irregularities in their work are the following: 1) the pharmacy does not have one or more medicines on prescription from the generic medicines for which it is obliged to have at all times; 2) irregularities for which a measure reprimand is issued (if these irregularities are not eliminated within 8 days the pharmacy is subject to contractual penalty); 3) problems with the documentation related to the pharmacy or the pharmaceutical staff; 4) irregularities in the way the medicines are dispensed; 5) the pharmacy does not have internet connection and 6) "other". Upon the concluded noncompliance in the work of the pharmacies determined through the inspections, HIFM undertakes adequate measures.

Pharmaceutical care in community pharmacy

In order for the pharmacist to successfully perform all the duties in the pharmaceutical practice, he/she must possess specific knowledge, competencies and skills and to continually upgrade them. The findings confirm that the pharmacist in a community pharmacy about 80% of his/her time devotes only to the process of medicines dispensing, while the remaining of the time is focused on giving information about the medicines. This data impose the need for significant changes in the profile of community pharmacist work. In regards to the pharmaceutical services, offered by the PHI Pharmacies which have significant importance and direct influence on the health state of the patients, as one of the strategic goal, is the continuous increase of the number of services and their modernization through monitoring of the impressions of the users related to the provided services. Namely, the community pharmacists actively participate in: promotion of the healthy lifestyle, providing advice to the patients and consumers and stimulate them to look after their health, they organize presentations, run health campaigns, etc. From the services offered by the PHI community pharmacies one can emphasize: measurement of bodyweight, blood pressure, taking pregnancy test, further on, running programs for quitting of smoking, for reducing of bodyweight etc.

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Macedonian pharmaceutical bulletin, **62** (suppl) 611 - 612 (2016)
ISSN 1409 - 8695
UDC: 615.15:37.035
Short communication

Lifelong learning - reality and perspective

Elizabeta Tomevska Ilievska[1]*, Jasmina Tonic Ribarska[2], Suzana Trajkovic Jolevska[2], Katerina Ancevska Netkovska[2], Goran Ajdinski[1]

[1]*Faculty of Philosophy, University "Ss Cyril and Methodius", Blvd. Goce Delcev 9A, 1000 Skopje, Macedonia*
[2] *Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia*

Introduction

Omnes, omnia, omnio.
(To teach everything, in-depth to everyone)
John Amos Komensky

Education is the basis for social transformation of human society. It represents a motivator, in its own right, towards democratization and the development of human society. Its effects on economic growth are indisputable, also on workforce mobility, as well as on the ability of integrating into international economic trends.

Current perspective shows that EU countries, and the whole world as well, are faced with challenges brought on by globalization, issues in demographic trends, and the enormous changes in labor market, based on technological development. The last challenge underlines the need for a society firmly grounded on learning (knowledge-based-society), where training and education must come first. "The purpose of lifelong learning is to maintain and preserve a positive and active approach towards learning at all ages, in the course of a lifetime" (MES, 2004).

Education and learning has enabled mankind an 'easier and more successful placement, not only concerning labor market, but also in knowledge in general, in a world of constant engagements' thereby, irrefutably imposing the necessity of an 'intervention' for improving the educational system in each country. Knowledge, skills, and attitudes, acquired and developed in surroundings varying from family to academia, are not permanent. A firm integration and learning in the life of an adult is a very important aspect of lifelong learning. From the viewpoint of lifelong learning (LLL), learning is defined as an 'endless continuum' alongside the building of a positive attitude towards learning in general.

From the standpoint of pharmacy, as a regulated profession, LLL is of great importance. LLL in pharmacy is self-directed and practitioner centered, and it emphasizes the importance of practice-based learning. Pharmacists, as health professionals, in order to perform their healthcare activities with patients, need a license to work, thus they are obliged to continuous education. The purpose is to ensure that pharmacists maintain and/or enhance their knowledge, skills, and competencies to practice throughout their careers in their specific area of practice.

The Republic of Macedonia, as one of the countries that signed the Declaration of Bologna, an in the same time, a candidate for EU membership, tends to follow every European trend, including education. As in other European countries 'the purpose of educational policy, of each one, is to provide the opportunity for a suitable educational level for everyone and for all' (MES, 2004) age groups, ensuring that they are in possession of knowledge, skills and attitudes, in accordance with the demands of society and the labor market, which is at the same time integrated into the concept of lifelong learning. The pharmacist of the 21st century should be in possession of a large amount of knowledge, but also personal skills, such as communication, critical thinking, problem-solving, the ability to work in teams and perceive lifelong learning.

In the time period between 2007 until the end of 2013, the leading EU program in the domain of education and training is the program called "Lifelong learning|, implemented by the European commission. On national level, the national agencies, formed in participant countries, have been granted the implementation and running of the program. Provided the main goals of strategic documents for education and training in R. M. have been analyzed, it is noted that their definitions are to a great extent, compatible with the goals of European policies, but also with the "Lifelong learning" program 2007 – 2013.

* beti@fzf.ukim.edu.mk

The Copenhagen protocol and the initiatives in the context of the Bologna protocol have been responsible for the beginning and significant progress in support of national reforms of lifelong learning, higher education modernization, and the development of communal European instruments, promoting quality, transparency and mobility (European Union, 2009).

The new strategic framework of European collaboration in education and training |Education and training 2020" has a particular focus on four strategic goals:

1. Making lifelong learning and mobility a reality;
2. Improvement of quality and efficiency of education and training;
3. Promoting equality, social cohesion and active citizenship;
4. Strengthening creativity and innovation, including entrepreneurship on all levels of education and training

Making lifelong learning and mobility a reality

Even though in the domain of lifelong learning, there can be new initiatives that will develop, reflecting future challenges, there is still a need of further improvement, particularly in the implementation of ubiquitous lifelong learning strategies (European Union, 2009). The need of "action" s stressed, through which the development of national frameworks for qualifications based on relevant educational gateways will ensure their development, and their connection to the European qualification framework, the creation of more flexible learning pathways, including better passages between different sectors of education and training, a more open approach to non-formal and informal learning and improved transparency and acknowledgement of educational gateways. Further efforts are at the same time the need of promoting learning among adults, the improvement of quality of guiding systems and learning, to make it generally more attractive, and including new forms of learning and implementation of new technologies of teaching and learning. As an indispensable element of lifelong learning, and also an important means raising employability and adaptability of people is the enlargement of mobility of all those learning, and the time periods spent in learning abroad, making them more of a rule than an exception. While at the same time, already established principles in the European protocol for quality and mobility should be implemented.

European programs for education and training have the tendency of promoting education that will strengthen the sustainability of economic growth. The ultimate gain is effectuating of the process of creating more and better positions and a better social cohesion, thus leading to a society based on knowledge.

At this very point we find the source of the claim that strengthening of educational capacities of the Republic of Macedonia, is largely conditioned by the use of European programs of education and training "Lifelong learning" and consequently the new program for education and training of youth and sports "Erasmus plus 2014 – 2020".

Useful links

Competences supporting lifelong learning and the "new skills for new jobs" initiative
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2010:135:0008:0011:EN:PDF
Towards more knowledge-based policy and practice in education and training
http://ec.europa.eu/education/lifelong-learning-policy/doc/policy/sec1098_en.pdf
Better integrating lifelong guidance into lifelong learning strategies
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2008:319:0004:0007:EN:PDF
European Qualifications Framework for lifelong learning
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2008:111:0001:0007:EN:PDF
Key competences for lifelong learning
http://europa.eu/eur-lex/lex/LexUriServ/LexUriServ.do?uri=OJ:L:2006:394:0010:0018:EN:PDF
European Qualifications Framework for lifelong learning - Implementing the Community Lisbon Programme
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2006:0479:FIN:EN:PDF
A new impetus for European cooperation in Vocational Education and Training to support the Europe 2020
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2010:0296:FIN:EN:PDF
Establishment of a European Quality Assurance Reference Framework for Vocational Education and Training
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2009:155:0001:0010:EN:PDF
Establishment of the European Credit system for Vocational Education and Training
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2008:0180:FIN:EN:PDF
Copenhagen Declaration
http://ec.europa.eu/education/lifelong-learning-policy/doc/policy/copenhagen_en.pdf
Action programme in the field of lifelong learning (2007-2013)
http://eur-lex.europa.eu/lex/LexUriServ/LexUriServ.do?uri=OJ:L:2006:327:0045:0068:EN:PDF

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Macedonian pharmaceutical bulletin, **62** (suppl) 613 - 614 (2016)
ISSN 1409 - 8695
UDC: 615.15:174
Short communication

Ethics, professionalism and autonomy of pharmacist – vision for the future

Arijana Meštrović

Pharma Expert Competency development manager, Dunjevac 2, 10 000 Zagreb, Croatia

Pharmacists in all practice settings (including community pharmacy, hospital pharmacy, academia, public health pharmacy, managed care pharmacy, clinical laboratory pharmacy, and industrial pharmacy) are confronted with ethical challenges, and those challenges are likely to increase in the future (IPF, 2013). There is not a consistent approach among countries in seeking compliance with pharmacist codes of ethics, and the level of pharmacists' professional autonomy, which varies greatly around the world, is influenced by many factors, including a country's history, social structures, social systems (e.g., economic, legal, political, and cultural systems), method of health care delivery and financing, and system of pharmacy education (FIP, 2002).

Issues of ethical behavior and autonomy are especially important to pharmacy practice because the profession is in transition, moving from largely a supply function to a patient-care function. This transition will be impeded if practitioners do not have sufficient autonomy to act in support of patients' best interests. If pharmacy practice were to limit itself strictly to a supply function, various forces - economic, technologic, social, and political - would likely coalesce over time to replace the pharmacist with other less expensive means of safely supplying medicines to patients. On the other hand, if pharmacists move toward assuming responsibility for helping patients and health professionals make the best use of medicines, they will be providing a higher value service than a mere supply function - a vital and complex service that is generally lacking in health care today. As pharmacist associations attempt to stimulate pharmacy's professional transition, they should help their members understand and address the ethical and moral dimensions of this transition. In countries in which the education of pharmacists has prepared them to enlarge their role in fostering responsible use of medicines, pharmacy practitioners have a moral obligation to put that education to its fullest use (FIP, 2002). In countries in which laws require pharmacists to own community pharmacies, the case for preserving those laws will be stronger if pharmacists are engaged in professional activities beyond the supply function and have demonstrated that they are a vital force in improving outcomes from the use of medicines.

The FIP identified the following four categories (with specific examples) of ethical issues experienced by pharmacists in all areas of practice (IPF, 2013):

1. Ethical challenges originating from individual and personal considerations.
   - Lack of a sense of professional responsibility.
   - Lack of competence.
   - Personal values in conflict with professional values, including conflicts that lead to refusal to provide service.
   - Stigma (e.g., denying service due to stigma [or inconvenience] towards illicit drug users or persons with disabilities).
   - Lack of awareness of principles of ethics in pharmacy.
   - Lack of care to apply ethical principles in practice.
   - Cultural, religious, or national interests in conflict with professional ethics.
   - Personal characteristics and traits (e.g., lack of moral courage).
2. Ethical challenges originating from economic considerations, either by limiting costs or by increasing revenues.
   - Managing resources – allocating limited resources.
   - Profitability and viability of business (greed vs. reasonable profit).
   - Advertising to promote inappropriate consumption.

* arijana.mestrovic@pharmaexpert.hr

- Insurance company policies that conflict with patients' best interests.
- Financial incentives offered by industry to sell certain products.
- Workload pressures.
- Products selected for sale in the pharmacy (e.g., tobacco, complementary medicines of unproven efficacy or quality, slimming products that don't work).
- Counterfeit drugs.
- Conflict of interest in continuing education presentations.
- Conflict of interest in publishing research findings.

3. Ethical challenges originating from human interactions (employer-employee or between colleagues).
   - Inter professional conflict.
   - Policy of the owner/employer.
   - Conflicts between the employer and the practitioner's commitments to engagement with professional organizations.
   - Reporting colleagues ("whistle-blowing").
   - Power imbalance and bullying/harassment (and subsequent job insecurity).
   - Lack of respect for colleagues.
   - Lack of good role modeling and initiative to teach younger practitioners.
   - Patient rights (e.g., privacy/confidentiality) (FIP, 2004).
4. Ethical challenges arising from the system or framework of practice.
   - Barriers imposed by institutional authorities.
   - Restrictions/challenges in adopting new technologies.
   - Lack of revision (updating) of codes of ethics.
   - Varying interpretations of codes of ethics.
   - Perceptions that codes of ethics are nonbinding.
   - Legislative or regulatory constraints.
   - Paradigm shifts; new scientific knowledge (e.g., pharmacogenomics).

Professional autonomy is the right and privilege granted by a governmental authority to a class of professionals, and to each licensed individual within that profession, to exercise independent, expert judgment within a legally defined scope of practice, to provide services in the best interests of the client.

Professional autonomy helps pharmacists fulfill their societal mission. That mission, as expressed in FIP's Centennial Declaration, is to help patients make responsible use of medicines.

Throughout its history, the profession of pharmacy has served humanity well around the globe. Although pharmacy has great potential for extending its record of service, it faces many obstacles in attempting to do so, not the least of which are challenges related to professional ethics and autonomy. Pharmacy cannot achieve its full potential, and patients will not benefit from that potential, unless pharmacists are committed to the highest standards of professional conduct and have sufficient autonomy to serve patients' best interests (The Tokyo Declaration, 1997).

In every country, the appropriate association of pharmacists should produce a Code of Ethics for pharmacists setting out their professional obligations and take steps to ensure that pharmacists comply with the provisions of that Code. The obligations of pharmacists set out in these codes should include to act with fairness and equity in the allocation of any health resources made available to them, to ensure that their priorities are the safety, well being and best interests of those to whom they provide professional services and that they act at all times with integrity in their dealings with them, to collaborate with other health professionals to ensure that the best possible quality of healthcare is provided both to individuals and the community at large (IPF, 2013).

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Macedonian pharmaceutical bulletin, **62** (suppl) 615 - 616 (2016)
ISSN 1409 - 8695
UDC: 615:174
Short communication

Ethical dimensions of pharmacy

Kiril Temkov

Faculty of Philosophy, Ss. Cyril & Methodius University, Goce Delcev 9A, 1000 Skopje, R. Macedonia

In the recent world all human activities are subjected to the ethical evaluation. The values of Good and Evil consist part of thinking as well as the behaviour of the people, affirming and seeking the goodness, criticizing and escaping from bad purposes and actions. So is in all kinds of living, in the professions (which are almost 20.000) and in the sciences (more then 400), as everyday and special appearances of human acts. Professional and scientific ethics are very important moral issues and visions of the modern civilisation.

Pharmacy is one of the most important and the most interest contemporary activity and science. For this, its philosophy and ethics are instructive for understanding the world and the human orientations (on pharmacy and its ethics I wrote in 2008-th in text "Bioethics, the Good Pharmaceutical Practice, and Protection of Moral Principles", in my book *Professional Ethics*, 2011, pp. 68-83). Basic for the pharmacy is that it (although one of the oldest occupation and one of the very first science) is not one-dimensional, but one of the most complex new science with inter-disciplinary, even more correct with poly-disciplinary approach. It is part of medicine, as the most important and the most developed recent science and practice for saving and improving the human health, but part of biology too, as well as of the chemistry, of the economy and the others different kinds of human thought and interests. The pharmacy is also defining by its relation to the bioethics, as modern and in the same time the most pompous orientation in the people's spiritual and social life, with whom moral aims and purposes are treating as model for almighty and particular human practice in every region and act. So pharmacy present oneself as exceptional instructive type of activity to-day which can to show us on problems and necessery values in many districts of human services which reflect to the human condition. With that ethics of pharmacy is one of the most eminent and the most influential for the new (bio)ethical sights and axiological principles.

For understanding the ethics of pharmacy is very considerable its complexity of activities-from definition the most difficult tasks before mind and the scientific inquirers, from numberless and capable laboratory examinations and improvings of medicines worldwide, from highqualified (offen industrial) performance of medicines, from its rigorous (the most frequently international) control for employment approval, from very advance organization of trade as well as the daily distribution for medical practice and for individual necessary. Every of this steps of the large pharmaceutics activity are not only structure part of the medical practice as well as of the medical ethics also, they have special meaning and particular professional ethical norms, which compose general ethics of health. Pharmaceutics stores are indispensable portion for connection and realizing of medical treatment with personal aims of patients, but also centers for high higienical purposes, distributors of healthy food, vitamins, and minerals, necessary services for small healthy aids etc.

Every pharmaceutics levels have its very severe ethical sense and values. Biotechnology and practical pharmacy are expanded worldwide. But forfrom the pharmacy are not important only its simple and pointed norms. It helps to rethink the problems of human existence but also the ethics which concern us with the universal visions of human unity with nature. In this case, in modern pharmacy we sense the tracks of classical alchemy, which is not lost for the humankind. As was said in Renaissance, we stand on the shoulders of countless people who worked of this fields– and this can not be forget and wipe out. To the ethics of pharmacy that give new and very interesting dimensions.

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* etika1958@gmail.com

Macedonian pharmaceutical bulletin, **62** (suppl) 617 - 618 (2016)
ISSN 1409 - 8695
UDC: 615.15:37.035
Short communication

Continuing professional development - challenge for professional organization

Svetlana Stojkov[1]*, Dragana Rajković[1], Bistra Angelovska [2], Zahida Binakaj [3]

[1] Pharmaceutical Chamber of Serbia, Mutapova 25, Belgrade, Serbia
[2] Pharmaceutical Chamber of Macedonia, 50 Divizija 34, 1000 Skopje, Macedonia
[3]Pharmaceutical Chamber of Federation of Bosnia and Herzegovina, Tuzlanska bb, 71000 Sarajevo, Bosnia and Herzegovina

Introduction

Professions, as one of key sectors of social systems, bear a leading role in the existing social work organization. Free professions take up a special place and significance, all the way from Roman artes liberales to our times.

Pharmaceutical profession, as one of the oldest, led by ethical principles, is regulated by postulates accepted by the profession members, and in modern times established through legislations. Typical determinants of the regulated professions, which also refer to pharmacists, as chamber members, are as follows: following ethical principles, specific skills and knowledge, professional development, autonomy at work, continuing improvement, competencies development, professional associations, licensing. Updated legislations in Europe recognize chambers as most widespread professional organizations of pharmacists as healthcare professionals. Specificities of the field, as well as responsibilities in providing healthcare, ask of the pharmacists to possess optimum level of knowledge, skills, attitude, and competency. The need for continuing professional development is recognized by professional authority, employers, practices, as well as professional associations, which, through their activities and projects, represent support to pharmacists' professional development. Tools that allow development and competencies assessment of the pharmacists, are accepted by international associations (WHO, FIP) and are applied in the South-East Europe (Stojkov et al., 2014).

By analyzing legislations of the countries in the region – Serbia, Federation of Bosnia and Herzegovina and Macedonia, a mutual platform can be noticed, which emphasizes pharmacists' professional identity, profession protection and care for patients' interests. It was confirmed through the Memorandum on cooperation, signed in Sarajevo in 2015.

Goals of the paper are to perceive difficulties and possible solutions when renewing the license for the first time and to collect data on pharmacists' continuing education and professional development, their attitudes and preferences of the fields and types of education, form of organization, limiting and facilitating factors.

Materials and methods

Data that were used are the ones on the number of pharmacists who need to renew their license with Pharmaceutical Chamber of Serbia (FKS) in accordance with already existing legislations, and the number of pharmacist who do not fulfill set conditions. The causes and possibility of overcoming this problem were also discussed.

A research was conducted on pharmacists' continuing education and professional development, attitudes and preferences of the fields and types of education, form of organization, limiting and facilitating factors, structured in electronic questionnaire, put up on the FKS website during 2015. Electronic questionnaire was voluntarily filled in by 490 pharmacists.

The results were presented in percentages, depending on the total amount of participants, discussed and compared to relevant literature data on similar conducted research.

* snezanacupara@yahoo.com

Results and discussion

During 2015, FKS has initiated preparations for pharmacists' license renewal. According to the available data, from the total of 5500 Chamber members, 2500 pharmacists needed to renew their license, after the expiration of the first seven-year period. The key condition for work permit -license renewal is the number of points gathered during licensed period, acquired through participation in accredited education. Preliminary analysis, which included data on pharmacists' education, pointed out that a significant number does not fulfill the required conditions (over 70%). In order to prevent the difficulties in the unobstructed professional improvement and work permit renewal, the Chamber has launched a legislation modification.

Pharmacists who participated in the poll, primarily employed in community pharmacies (93.9%), recognised professional competencies as the most needed professional improvement (55.7%), whereas expressed needs for personal competencies and organization, as well as management competencies are significantly lower (26.4%; 17.4%). Pharmacists' familiarity with competencies and competencies framework, is the result of a project, which lasted for several years, on assessment and competencies development of pharmacists in Serbia (Stojkov et al., 2014), as well as the National document (www.farmkom.rs/index.php?) enacted by the FKS Assembly. The Chamber, as a professional organization, provides continuing education of pharmacists, and their areas of interest, so the poll included a question on the fields that are of interest to pharmacists, and the preference was given to safety and efficiency of nutrients in polytherapy (28.7%), as well as special populations (children and elderly) and medicine safety (27.2%). The participants in the poll have shown high preference towards the following forms of learning: showcase and workshops; and when it comes to study models, the internet and interactive workshops were the first choice. Day of the week that suits most participants was Saturday (49.5%), and the limiting factors were expenses and time (34.9%; 51%). The research conducted in Australia (Mariot et al., 2007), stated availability and relevance of the content as the limiting factors, next to time. Also, more flexible education programmes of education were suggested, and they are becoming more significant in Serbia as well – digital formats available on the internet are dominant as first choice of learning (48.6%). Access to the internet and foreign language knowledge were not recognized as relevant limiting factors among the participating pharmacists: 48.6% and 32.6% of participants respectfully marked these factors as least relevant in the sense of limitation. From the answers to the question: "How many continuing educations did you participate in last year", it can be determined that pharmacists took an active part in their professional improvement: over 40% took part in 2-5, and 45.3% in more than 5 continuing educations. Only 5.1% stated that they did not participate.

Conclusion

The road that leads professions of the modern times towards modern formatting is drafted through several stages of professionalism: modification of legislation and protection of professions by means of law, improvement in practice through professional development, establishing special trust relationship between free professions representatives and beneficiaries, providing professional associations with wider authorization, and finally cooperation on a regional level. The chambers, as most widespread pharmaceutical organizations should provide support to continuing professional development, protection of profession's interests, as well as be important factors in improvement of pharmaceutical healthcare.

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Macedonian pharmaceutical bulletin, **62** (suppl) 619 - 620 (2016)
ISSN 1409 - 8695
UDC: 615.12:614.35
Short communication

Implementation of standards for good compounding practices in hospital pharmacy

Vasilka Nicha[1*], Maja Simonoska Crcareska[2], Marija Glavas Dodov[2], Renata Slaveska Raichki[2]

[1]*Public Institution in the Health Sector, for the needs of PHI University Clinics, Institution and Urgent Centre, Majka Teresa 47, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, University Ss Cyril and Methodius, Majka Teresa 47, 1000 Skopje, Republic of Macedonia*

Background

Compounding in a hospital pharmacy (HP) setting is a comprehensive essential function of the pharmacy practice and, also important core element of the provision of healthcare tailored to the needs of an individual patient. In general, for the purpose of compounding, many unit operations and decision making processes are required that, on the one hand, more fully define this activity in several ways; one of the simpler definitions is as follows:"The act of preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner–patient–pharmacist–compounder relationship in the course of professional practice (GSP, 2016). As acknowledged, compounded medicines, medicinal products and devices in HP are not approved by competent regulatory body thus, the regulatory approval process for medicines do not verify their quality, safety or effectiveness. All these underlining reasons can serve as further evidence of the paramount importance for establishing contemporary compounding practice in HPto comprise not only the art, current scientific principles, good pharmacy practice, facility, equipment, personnel and etc., but also provisions for pharmacists to comply with policy, regulations and guidelines in this filed. Additionally, it is required to put the focus on issues and challenges related to the implementation of the official standards for Good Compounding Practice (GCP), if they exist. In continuum, with the development of the sciences related to the dosage form formulation and advanced therapies, the position and responsibilities of hospital pharmacists (HPs) requires enhancing their knowledge and skills in the field of patient centered pharmaceutical care.

Recommendations for implementation of standards

It is known that compounding of medicines in HP is followed by clinical and technical risks. Therefore, for the purpose of risk reduction, guideline or algorithm adopted on national level to facilitate decision making process within this function is strongly recommended. For example, in many cases the HPs should have to verify whether an equivalent medicine is available on the national market or not, and consequently, to consider certain alternative therapies including risk evaluation concept. In line with this, the HPt, for instance, can suggest usage of a less potent steroid molecule or dosage form instead of dilution of certain formulation or in other case, to advice different route of drug administration for patient (GMC, 2013). When all solutions are exhausted, HPs have to compound a dosage form compliant with the best health care needs of patient. Additionally, the basic principles of good practices in compounding demand HPs to comply with national regulations and with all relevant international standards were developed by the World Health Organization (Good Manufacturing Practices-GMP Guide), EudraLex and the Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S-Good Preparation Practices-GPP Guide) including requirements in relevant Pharmacopeias. To start with, an appropriate quali-

* vasilkanica@yahoo.com

ty assurance system has to be established with the aim to reduce the risks linked with compounding medicinal products- whether it is sterile or non-sterile. Therefore, a risk assessment for compounded medical products should always be carried out in order to define their quality assurance level. According to the references presented in the Resolution CM/Res (2011)1 on quality and safety assurance requirements for medicinal products compounded in pharmacies for the special needs of patients (Council of Europe, 2011), there is a need of establishing a "risk evaluation system" for the assessment of "high- risk" and "low-risk" compounded medicinal products. In this context, it is recommended that the GMP guideline has to be used as a reference for an appropriate quality assurance for "high risk preparations", while the PIC/S GPP Guide can be used for so called, "low-risk preparations". However, the application of other guidelines with an equivalent quality level is possible, depending on the national legislation or guidance. According to GMP and GPP guides, there are integral elements needed for reducing risk linked with compounding, including: standard operation procedures affirmed by quality assurance unit, calibrated equipment, quality control of starting materials, evaluation of starting materials and compounding process by means of health hazards, documented independent check of pharmaceutical calculations, trained personnel involved etc. To underline as an example, the organization of services for total parenteral nutrition in many cases is a real challenge for HPs due to the unavailability of commercially prepared products. As it is known, this service of HP may be followed by risk in calculation errors, stability and incompatibility connected with product compounding. However, since the aforementioned GCP guidelines are more general they cannot cover in detail all aspects thus, some countries developed additional national guidelines on compounding adopted by certain professional bodies (PB, 2015).These guidelines include both, steps in compounding process and competences required. Another important compounding aspects related to algorithm for decision making process when there is a need to choose suitable dosage form for the patient need. For example, firstly, it is looking for standardized, validated dosage form; secondly, if it is not available, then compounding according to scientific principles and practice is recommended and thirdly, whenever suitable and possible, to consider compounding of the simplest pharmaceutical formula (Glass and Haywood, 2006). The next step is the process of dosage form evaluation which includes several GCP related aspects that must be considered such as: ingredients quality, palatability, irritability, toxicity and stability; possibility of extensive API metabolism by first pass, therapeutic index data and bioavailability; packaging, storage, and labeling, assessment for possible interactions development between API-excipients-containers, quality control, stability, expiration, and beyond-use dating stability testing date etc. Additional important steps tailored to compounding process in HP environment include: evaluation of toxicity and safety of compounded dosage form and monitoring of the clinical effect ad and adverse drug reactions etc. When medicines with marketing authorization have to be modified to facilitate administration there is a benefit for compounding pharmacists to acquire information from the manufacturer about: physical and chemical data about API, excipients, critical functional characteristics of dosage form, possibility for manipulation in order to change the dosage form, possibility to obtain API from manufacture to compound quality dosage form, relevant information about testing and analyzing API on request by pharmacist (EMA/CHMP/PEG/194810/2005).

Conclusion

HPs should maintain at least four sets of records for compounding. An existing development requires reference library and other sources of information relevant to compounding to be included in the guidelines for GCP.

As a provider of pharmaceutical care, the HPs have to be well educated and trained in the field of art and science of pharmacy compounding for creating personalized medicines solutions. In addition to the policy and good practices, further guidelines supporting the creation of safe and effective medicines have to be put in place.

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Macedonian pharmaceutical bulletin, **62** (suppl) 621 - 622 (2016)
ISSN 1409 - 8695
UDC: 616-002.2-085(497.7)
Short communication

Role of community pharmacists in chronic disease management in the Republic of Macedonia

Donka Pankov*, Maja Simonoska Crcarevska, Kristina Mladenovska, Renata Slaveska Raicki, Marija Glavas Dodov

Institute of Pharmaceutical Technology and Biopharmacy, Faculty of Pharmacy, University "Ss Cyril and Methodius", Mother Theresa 47, 1000 Skopje, Republic of Macedonia

Introduction

Chronic diseases are increasing in global prevalence and seriously threaten developing countries. They become dominant health burden, with 70-80% of health-care costs spent on their management. Chronic diseases are interrelated, have common risk factors and are largely preventable. However, in their prevention, only 3% of health expenses are presently invested (Commission of the European Communities, 2007; The Economist Intelligence Unit, 2012). Management of chronic diseases has been also pointed as a serious concern, with WHO's statement that approximately 50% of chronic patients do not adhere to therapy and reports on the economic magnitude of this problem (WHO, 2003), 194,500 deaths and estimated costs of €125bn annually in the EU due to miss-dose and non-adherence to prescribed medication (PGEU, 2008). Therefore, a successful and sustainable chronic disease management program requires a collaborative approach at the system level, both professional-directed and patient-oriented (NEHI, 2009).

The current primary care models implicate that the strong physician-patient-pharmacist relationships are not promoted; training for chronic disease management is rarely sufficient and there is insufficient continuity and coordination among health-care providers. Among the healthcare professionals, community pharmacists are the most accessible and are in an ideal position to assist the management of patients with chronic diseases. However, despite the recent developments in pharmacy practice there still appears to be an overall unawareness of their potential within this area of health-care system, with "the potential for pharmacy being untapped" (Petrushevska-Tozi and Mladenovska, 2012; WHO, 2011). Therefore, WHO has been called pharmacies upon to engage further in health promotion, chronic disease prevention and rational use of medicines, specifically by supporting people with chronic conditions, improving their quality of life and self-management, supporting better knowledge about pharmacotherapy, etc (WHO, 2011). However, in order to take up the challenge, it would be necessary to examine what have already been done in chronic disease management by community pharmacists in the Republic of Macedonia, from both patients and health-care providers perspectives, which was the aim of this study.

Materials and methods

For the patients and health-care providers, two separate cross-sectional descriptive questionnaires were designed, where combined pre-coded open ended and multiple choice closed questions were used with type of response format A-agree, B-partly agree, C- disagree, covering two essential components: need of providing patient education by a pharmacist to prevent chronic diseases and disability by promoting healthy life-style and need of expanding pharmacy services and interventions in chronic disease management. The questionnaires were pre-tested by involving the target groups in the design (10 community pharmacists (PHs) from different pharmacies, 10 general practitioners (GPs), 10 specialists in internal medicine (SPs) and 10 patients (PTs), to evaluate the apparent (logical) validity of the questionnaires (instructions, coverage of the issue of research, format and questions sequences, legibility and readability, too much/little offered answers, measurability of the questions, time for completing, etc.). The average age (±SD) of the health-care professionals

* dpankov0@gmail.com

was 47.3 ±10.5 yrs, of which 76.7% were females. Related to the profile of patient group, the average age (±SD) was 67±9 yrs, with most of them females (80%) and 60% having university degree diploma.

The responses were collected face-to-face in the GPs' and SPs'offices and community pharmacies, from January 3rd to February 28th, 2016, with the surveyors being pharmacists. Only 10% of the respondents stated that some of the questions were unclear and these items will be further reconsidered when generating direct anonymous final questionnaire.

Obtained data were tabulated using Microsoft Excel® (Microsoft Corp. Redmond, WA, USA), computed and consequently evaluated using statistical software STATGRAPHICS Centurion XVI evaluation (Stat Point technologies Inc., USA).

Results and discussion

Unlike GPs, who think that patients are either well- or partly educated about the chronic diseases, their prevention and management (30% and 70%, respectively), 20% and 40% of the PTs, 20% and 70% of the SPs, and 50% and 50% of the PHs believe that patients are not properly or partly educated, respectively, pointing to the necessity of pharmacists to use their competences and skills in educating patients about prevention and management of chronic disease. This necessity was approved by 60% of the PHs and SPs, while 40% of them it partly approved. Interestingly, 30% of GPs were not agreed with this statement, unlike 100% of PTs, who expect best pharmacy practice and care in this area. In this respect, 100% of PHs and 90% of PTs are agreed that engagement of pharmacists in chronic disease management would improve patients' use of medicines and adherence and their understanding about the chronic diseases they have. However, only 20% of the GPs and 40% of the SPs think that CPs can improve patients' use of medicines, pointing that the pharmacists cannot improve patients' understanding about their chronic disease.

Within the chronic diseases management, engagement of pharmacists in education of chronic patients for using medical devices for drug administration or therapeutic monitoring was also evaluated. The most of responders were agreed that this activity contributes to efficacious diseases management by a patient itself and improve outcome of the treatment. Only 20% of the PHs and 30% of the GPs were not agreed with one or both of these statements, unlike PTs, who were all agreed. Considering engagement of the community pharmacists in disease/therapeutic monitoring, 70% of PHs, 60% of GPs, 50% of SPs and 100% of PTs acknowledge the need of these additional services. Interestingly, 20% of PHs are not interested and 10% of GPs and SPs think the same.

Interest and willingness of pharmacists to be engaged in education, care and control of patients with chronic diseases were also a matter of interest. For 90% of PHs, 60% of GPs and 100% of SPs, pharmacy structure was identified as a key factor/barrier. All health care responders were consent that it is necessary to determine the resources and content for education and training of pharmacists in managing chronic diseases and to implement and develop mutual cooperation between pharmacists and physicians for a better control of chronic diseases.

Conclusion

From the processed answers one can conclude that the role of community pharmacists in chronic disease management is indisputable. Further thorough research is needed to identify the key areas for intervention for medication adherence to be improved, pharmacy services to be introduced or their scope expanded, interprofessional collaboration to be strengthened, and education and training of pharmacists in managing chronic diseases to be implemented. In addition, a strategy for all stakeholders should be developed, in order awareness and understanding of the role of pharmacists in providing pharmaceutical care and additional services in patients with chronic diseases to be increased.

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Macedonian pharmaceutical bulletin, **62** (suppl) 623 - 624 (2016)
ISSN 1409 - 8695
UDC: 615.15:614.256(497.7)
Short communication

The role of the community pharmacist in self-medication with over-the-counter drugs: R. Macedonia survey

Marija Glavas Dodov[1], Ana Poceva Panovska[2], Maja Simonoska Crcarevska[1], Andrea Puzderliski[3], Vladimir Indov[3], Aneta Dimitrovska[2]

[1]Institute of Pharmaceutical Technology, Faculty of Pharmacy, University of "Ss. Cyril & Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia
[2]Institute of Applied Chemistry and Pharmaceutical Analyses, Faculty of Pharmacy, University of "Ss. Cyril & Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia
[3]Alkaloid AD Skopje, PC Pharmaceuticals, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia

Introduction

Responsible self-medication with over-the counter (OTC) drugs assumes that individuals treat minor ailments and conditions with medicines approved by national regulatory agencies and are available without prescription, safe and effective when used as directed (Bakic-Miric, 2009; Cavaco and Pereira, 2012). Literature data indicate that self medication is prevalent form of medical care in the world, with estimation that 80% of all medical symptoms are self-recognized and self-treated without professional care where more than 92% of all consumers used at least one OTC drug in the past year, and 55% of which have used more than one (Wertheimer and Serradell, 2008).

Community pharmacists working on the frontline of health-care system are primary and easiest accessible source of professional information for OTC drugs as most commonly they are supplied in community pharmacies, although in some countries there are OTC drugs that can be sold elsewhere. Community pharmacists - OTC consumers interface is of substantial importance for ensuring proper and safe OTC drug use as community pharmacists have more experience with self-care products than any other health care professional (Gazibara et al., 2013).

The aim of the present study was to investigate community pharmacists' attitudes, perceptions, practice and perceived barriers/obstacles in ensuring the proper pharmaceutical care with OTC medicines in the community pharmacies in Republic of Macedonia (RM).

Materials and methods

Educational course entitled "The role of pharmacist in self-care and self medication" was conducted by pharmaceutical company Alkaloid AD, Skopje in a two months period, February to March, 2014. Participant of this course were mainly community pharmacists and minority of pharmacy technicians, from different regions in RM. Survey was completed by 465 participants of the course.

For the purpose of this survey, a structured anonymous questionary was used. The questionnaire included 26 items related to the community pharmacists` attitudes and perceptions in everyday practice with OTC drugs as well as obstacles in ensuring the proper pharmaceutical care.

The anonymous questionnaire covers data related to: *i)* socio-demographic factors (city, age, gender, work experience in the pharmacy), location and workforce in the community pharmacy and average number of patients and prescriptions per week; *ii)* attitudes (job satisfaction, importance of OTC counseling in everyday practice, willingness to provide OTC recommendations) and *iii)* probable barriers (lack of time, lack of trainings, continuing education courses, etc.)

Obtained data were tabulated using Microsoft Excel® (Microsoft Corp. Redmond, WA, USA) and were computed and consequently evaluated using statistical software STATGRAPHICS Centurion XVI evaluation (StatPoint technologies Inc., USA).

* magl@ff.ukim.edu.mk

Results and discussion

A vast majority of the surveyed participants were graduated pharmacists (89.2%) while pharmacy technicians with 5.4% were significant minority. The rest 5.4% did not provide information about educational attainment. All regions in RM were represented, with prevalence of the capital, Skopje, with 28.9% followed by Pelagonija Region (Bitola, Prilep, Resen) with 16.1%, South-Eastern Region (Gevgelija, Bodanci, Dojran, Radovis, Strumica, Valdandovo) with 12.3%, South-Western Region (Debar, Kicevo, Ohrid, Struga, Vevcani) with 10.6%, Polog Region (Gostivar, Tetovo) with 9.7%, Eastern Region (Stip, Kocani, Berovo, Delcevo, M.Kamenica, Vinica) with 9%, Vardar Region (Veles, Kavadarci, Sv. Nikole, Negotino) with 7.3% and North-Eastern Region (Kratovo, Kumanovo) with 5.9%. Female gender was represented with 90.5%. The dominant cohort (52.3%) was in age group of 23 – 35 years. The average community pharmacy experience was 11.88±9.28 years. Nearly 42% were community pharmacy owners. Most of the surveyed pharmacists (53.12%) reported that community pharmacy they work in, has more than 2000 prescriptions and 29.03% reported 1000-2000 prescriptions dispensed per month. Overall job satisfaction was rated on a scale of 1 to 5 where 1 refers to highest and 5 to lowest satisfaction. The determined average satisfaction was 2.24±1.2 with mode of 1 in 30.32% and only 4.1% were not satisfied by their job as community pharmacists. The highest percentage of respondents (28.8%) reported counseling patients on OTC medications 16-20% of their working time, which equates to the approximately 75 to 90 minutes during an eight hour shift. Slightly over 50% of the respondents reported that they were educated for OTC drugs during their undergraduated studies, however still high percentage - 46.67% were not. Approximately one third of the surveyed community pharmacists always advice patients for OTC drugs use while the majority (60.43%) frequently does that.

Solely, continuing education courses were source of OTC drugs knowledge for 29.89% of surveyed participants, working experience (practice) for 26.67%, undergraduated studies for 5.81% and pharmaceutical companies for 4.73%. Combined, continuing education courses/practice was reported by 17.85% and undergraduated studies/practice by 7.96% of the respondents as OTC drug knowledge source. More than a half (57.2%) of the surveyed pharmacist agreed that OTC drug counseling is important component of community pharmacy practice, while 42.6% strongly agreed with this statement. Nearly equal percent responded that they have enough time for OTC drugs counseling (41.94%) and opposite (not enough time - 42.79%). A majority (80%) agreed that patients accept their advices in respect to OTC drugs selection. Almost equally either agree or strongly agree (app. 50% each) that they are willing to provide OTC recommendations when given the opportunity. Confidence in own competentiality (knowledge) related to adequate OTC drugs selection was expressed by 65.6% of the respondents. Participants were asked to rank their knowledge about certain OTC drugs classes, where 1 referred to highest and 7 to lowest rank. The highest scores were determined for cough/cold OTC drugs 1.89±1.31 and pain/analgesics OTC drugs 1.96±1.34, while the lowest scores were related to eye/ear and first aid OTC drugs, 5.16±2 and 4.82±2.24 accordingly. The results were comparable with the ranking related to the patients' demands for classes of OTC drugs counseling (rank of 1 indicated most frequently and 7 - least frequently). Advices for cough/cold OTC drugs were requested in average of 1.71±1.12 and pain/analgesics OTC drugs, in average of 1.95±1.35 with a mode of 1, 52.9% and 39.35% respectively. Patients` demands for first aid and eye/ear OTC drugs counseling were lowest with average of 5.45±1.96 and 5.35±1.8 consequently.

Respondents agreed (52.7%) and strongly agreed (44.52%) that patient counseling positively influence community pharmacy practice. Most frequently (63.87%) questions and advices for OTC drugs are initiated by the community pharmacists, while 27.3% are instigated by the patients.

Majority of the surveyed participants, 61.94%, were interested for input into OTC drug counseling while 33.98% articulated high interest.

Participants were asked to rank six quoted barriers/obstacles that might influence proper pharmaceutical care with OTC medicines in the community pharmacies. Rank of 1 referred to the most significant and 6 to least significant barrier/obstacle. Lack of time and personal scarcity with average of 1.55±1.22 and 2.78±1.57 respectively, were identified as the most significant barriers/obstacles.

Conclusion

Outcomes from the conducted survey provided an insight to the main attitudes, willingness, barriers/obstacles and educational requirements of pharmacists in Republic of Macedonia related to self-medication with OTC drugs. The results obtained are valuable for the pharmaceutical community and can be used in order to improve and advance community pharmacy practice by further promoting the pharmacist's role in self-care.

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Macedonian pharmaceutical bulletin, **62** (suppl) 625 - 626 (2016)
ISSN 1409 - 8695
UDC: 661.12:[005.32:331.101.32
Short communication

Relationship between management style and pharmacist job satisfaction in marketing strategy departments (MSDs) in headquarters of ten pharmaceutical companies in Bangladesh: a cross-sectional study

Dilshad Noor Lira*, Abu Shara Shamsur Rouf

Department of Pharmaceutical Technology, University of Dhaka, Dhaka-1000, Bangladesh

Introduction

Providing optimum public health care are the main goals of a health system that requires integration of human resources at all levels. Therefore, any low esteem or dissatisfaction conceived by any of the key components of the service chain may relatively threaten the comprehensive health care. Pharmacists as the key players in this multidisciplinary sector are no exception to this rule. In Bangladesh a study was conducted to identify the factors affecting employee job satisfaction of pharmaceutical sector (Parvin and Kabir, 2011). Given the noticeable lack of studies involving management styles with the job satisfaction of pharmacists this study attempts to address the gap in the literature. In 2007 Jovic-Vranes A. et al. found that job satisfaction was associated with good interpersonal relationships, working environments and a feeling of being able to provide good quality care. Other studies opine that there is a strong association between low levels of job satisfaction and organizational factors (Piko et al., 2006). A study by Krogstad U. et al. 2006, reflected that support and feedback from one's immediate supervisor was the main explanatory variable for job satisfaction among nurses. Buciuniene et al. 2003, concluded that low salaries result in low job satisfaction among Lithuanian primary health care doctors. Rad, Yarmohammadian, 2006 and De Stefano et al. 2005, exerted that the greater the chances for development within the organization, the greater the likelihood of a higher level of job satisfaction. The results of Bodur, 2001, study showed low levels of job satisfaction mainly due to working conditions and salary. A significant relationship between job satisfaction and management style was exhibited in a master's' thesis by Nakata, 1992.

The research questions of the present study are:

1. What are the management styles (MS) of first-line managers as perceived by staff pharmacists (SP) in the marketing strategy departments in selected pharmaceutical companies?
2. What are the management styles (MS) of first-line managers desired by staff pharmacists (SP) in the marketing strategy departments in selected pharmaceutical companies?
3. Is there a relationship between the perceived management style (MS) of first-line managers and the job satisfaction (JS) of staff pharmacists (SP)?

The purpose of this study is to determine the SP's perception on MS and its relationship to their JS working in MSDs in reputed pharmaceutical companies of Bangladesh. This study also investigates the MS that SPs desire in their organizations. The broader purpose of the study is to provide information that might assist pharmaceutical companies in providing the best working environment for pharmacists' job satisfaction, efficiency and retention.

Materials and methods

The conceptual framework for this cross-sectional study design is based on Likert management theory which describes a continuum of four types of management styles and the adaptations of Maslow's hierarchy of needs. Per-

* dlira.1125@gmail.com

ceived and desired management styles (independent variables) were measured by the Profile of Organizational Characteristics, Form SLM-1978 by Rensis Likert Associates, Inc. with response choices from l to 4. Each question requires two responses, one that describes the management style on the participant's working unit at the present time and one that describes how the subject would like the management style on the working unit to operate. Job satisfaction (dependent variable) was measured by a job satisfaction tool based on a tool developed by Munson and Heda (1974) which is based on the conceptualization of Maslow's hierarchy of needs. The subscales measure: authoritative satisfaction, interpersonal satisfaction (belonging), involvement needs (ego needs), training/other facilities satisfaction, workload satisfaction and satisfaction regarding payment/promotion. Additionally, demographic data, such as educational level, age range were collected. So, there were three types of interview questionnaires used for this study: Questionnaire A- written in English, consisting of 19 questions regarding job satisfaction among the pharmacists, Questionnaire B- written in English, consisting of 10 questions, each of them having 2 parts, one was for the perceived management style and the other for desired management style, Questionnaire C-7 items elicited socio-demographic data including the respondents' age, gender, level of education. A sample of 384 individuals was randomly selected from 10 pre-selected pharmaceutical companies which belong to top 20 companies of the country. Data was gathered through face-to face interviews with individual respondents between July and October, 2015 and it was analyzed with the program SPSS v22.0.

Results and discussion

Among the respondents 63.6% were male and 36.4% female. The data indicated that the SP currently perceive an overall benevolent-authoritative style of management on their units represented by an overall mean score of 4.66 which reflects less fear and punishment than in the exploitative-authoritative style but does not use staff involvement. The SP desired an MS approaching the participative group style with an overall mean score of 7.2 which expresses the necessity of a style that uses ideas solicited from the staff and asks for the staff's opinions on matters that affect their work.

The JS subscale with the highest mean score was interpersonal satisfaction (4.27) which is associated with the concept of belonging and warmth in relationships at their workplace. The JS subscale score having the lowest mean was payment (3.42, where 3=Disagree slightly) which conveys that SP were in general less satisfied with the income and opportunities and scope for promotion in their jobs.

Null hypothesis H0: There is no relationship between perceived management style and job satisfaction. Alternate hypothesis Ha: There is significant relationship between perceived management style and job satisfaction. The Pearson Product-Moment Correlation score for these two variables was r=0.436 (p=0.00000000000876) which convincingly rejects the null hypothesis and is further intensified by Spearman rank correlation and Kendall rank correlation coefficient $\rho = 0.317$ ($P = 0.0000000156$) and $\tau = 0.239$ ($P = 0.0000000159$) respectively.

Conclusion

The strong correlation between management style and job satisfaction emphasizes the need to intervene with an appropriate organization-specific MS (i.e. participative group style) to promote JS among pharmacists.

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Student session

Macedonian pharmaceutical bulletin, **62** (suppl) 629 - 630 (2016)
ISSN 1409 - 8695
UDC: 616.53-002-085.89:638.135
Short communication

Porous microparticulated system for topical delivery of natural bioactive compounds

Lea Taneska*, Elena Risteska*, Blagorodna Koprivica, Marija Glavas Dodov, Maja Simonoska Crcarevska

Institute of Pharmaceutical Technology and Center of pharmaceutical nanotechnology, Faculty of Pharmacy, University of "Ss. Cyril & Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Acne vulgaris is a common chronic skin disease affecting ~85% of adolescents and young adults, although all age groups could be affected. Active pharmaceutical ingredients (API) and commercial dosage forms that are commonly used for acne treatment are characterized by lack of efficacy and number of side and adverse effects mainly related to the inability for targeted and controlled drug delivery. Currently, there is extensive ongoing research for finding alternative approaches for acne treatment. Topical application of propolis (natural bioactive compound) has shown notable efficacy against microorganisms etiologically connected with acne. However, often propolis extracts due to distinctive color, strong and aromatic odor and stickiness fail to ensure patient compliance. One approach that might be used for improvement of acne treatment efficacy and patient compliance is to incorporate API into drug delivery carriers (micro/nanoparticulated) thus enabling controlled drug release at the site of action. Having in mind previous, our group is working on development of porous microparticulated systems (microsponges) for topical delivery of propolis with certain physico-chemical and biopharmaceutical properties favoring prolonged release, enhanced efficacy and patient compliance in acne treatment. In the previous work of Dodov et al. (2016) microsponges loaded with 20% propolis ethanolic propylene glycolic extract were formulated. The current work is addressed to formulation and characterization of microsponges prepared with 25% propolis ethanolic extract using two different hydrophilic surfactants.

Materials and methods

Materials

Ethylcellulose (EC) and Chitosan (CTS) (high viscous) were purchased from Sigma- Aldrich, USA. Tween 80 (T80), Tween 20 (T20), Span 80 (S80), Aceton (ACT), and Dichlormethane (DM) were obtained from Merck, Germany. Propolis ethanolic extract (PE) (25%) was supplied from Apimel, Macedonia. All other chemicals and reagents were of analytical grade and used without any modifications.

Preparation of microsponges loaded with propolis extract

The PE loaded microsponges (MPL) were prepared using previously optimized double emulsion solvent diffusion technique (Crcarevska et al., 2015; Dodov et al., 2016). This method involves preparation of a primary W/O emulsion, and a subsequent second emulsification resulting in a W/O/W multiple emulsion. The primary W/O system was prepared by continuous dropwise addition of internal water phase (CTS (0.8% (m/v) in 3% acetic acid (v/v)), water (W1) and ACT) to oil phase (EC, S80, DHM and PE) during a period of 4 min under continuous rotor–stator homogenization (Ultra-Turrax1 T-25, Dispersing element S25N-10G, IKA, Germany, 24000 rpm, ambient conditions). After the addition of internal water phase was completed, mixing was continued for additional 1 min. Multiple W/O/W emulsion was prepared by dropwise addition of primary W/O emulsion to outer water phase (water (W2) and T80 or T20 as surfactant), for time period of 10 minutes at the same conditions as for W/O primary emulsion.

The removal of ACT, DHM, ethanol and the solidifica-

* lea.taneska@gmail.com, risteska_eka@live.com

tion of MPLs was finalized under continuous mixing (24000 rpm, 40 °C, 30 min). The resulting MPL dispersion was cooled down to room temperature under magnetic stirring (200 rpm, 20 min, Variomag, Multipoint HP 15, Germany).

Composition of the prepared formulations was as follows: CTS (0.8%):W1:ACT (ml) = 1:0.67:5, S80/EC=0.33, T20/W2 or T80/W2 = 0.015. S80/DHM:PE/EC was 0.0039:0.5 (MPL1), 0.004:1 (MPL2), 0.0043:2 (MPL3) and 0.005:4 (MPL4). A total of 8 formulations were prepared. 4 formulations were prepared with T20 (MPL-T20) and 4 with T80 (MPL-T80) as hydrophilic surfactant.

Nonencapsulated PE was separated and prepared MPLs were freeze dried (Freeze-dryer, Labconco, USA, 0,05 mBar, - 49 °C, 24h) in presence of cryoprotecting agent (20% trehalose/g EC)

MPLs characterization

Characterisation of particle size and particle size distribution of MPLs was done by laser diffractometry (Crcarevska et al., 2015). Particle size was expressed in micrometers (μm) by volume diameter Dv50±SD, while particle size distribution was expressed in term of SPAN factor±SD.

The non-encapsulated fraction of PE was separated from the MPLs, and the propolis content (PC) was determined according to procedure described by Dodov et al. (2016). PC was expressed as dry PE (g)/EC (g)±SD.

MPL`s *in vitro* release studies were performed by non-membranic dissolution model (Dodov et al., 2016). In brief, accurately weighted amounts of freeze-dried MPLs with known and equal content of PE (0.002 g dry PE/ ml) were placed in closed glass tubes on thermostat water-bath horizontal shaker (4h, 32 °C, 40 rpm, (Unitronic OR, Selecta, Barcelona, Spain)). In appropriate time intervals the samples were centrifuged (4000 rpm, 5 min) (Tehtnika Centric 322B, Slovenia) and 8 ml of the total volume of supernatants were collected. This volume was replaced with 8 ml of distilled water (32 °C) and dissolution studies continued.

Drug release data were fitted into zero order, Higuchi, Korsmeyer-Peppas and Peppas and Sahlin model (DD-Solver 1.0 - Microsoft Excel menu-driven add-in program).

Results and discussion

Dv50 for formulations prepared with T20 as hydrophilic surfactant was 26.67±2.15 μm (MPL1-T20), 34.79±6.46 μm (MPL2-T20), 89.72±5.64 μm (MPL3-T20) and 157.05±31.86 μm (MPL4-T20), while for those prepared with T80, Dv50 was 31.56±1.19 μm (MPL1-T80), 75.56±1.02 μm (MPL2-T80), 96.15±4.01 μm (MPL3-T80) and 475.47±17.24 μm (MPL4-T80). Particle size for freeze dried MPLs was 9.14±0.27 μm (MPL1-T20), 20.41±0.87 μm (MPL2-T20), 36.25±1.89 μm (MPL3-T20), 117.68±4.19 μm (MPL4-T20), 25.26±0.0.66 μm (MPL1-T80), 22.87±1.35 μm (MPL2-T80), 33.50±3.32 μm (MPL3-T80) and 117.09±11.51 μm (MPL4-T80). Obtained results pointed that particle size increased with increment of PE/EC. When particle size of freeze dried MPLs was compared significant difference was determined in MPL1-T20/MPL1-T80 (one way *ANOVA,* Fischer LSD, $p<0.05$).

PC expressed as dry PE (g)/EC (g) was 0.37±0.07 in MPL1-T20, 0.74±0.02 in MPL2-T20, 1.47±0.04 in MPL3-T20 and 2.93±0.1 at MPL4-T20, while in MPLs prepared with T80 as hydrophilic surfactant it was 0.33±0.05 in MPL1-T80, 0.73±0.12 in MPL2-T80, 1.41±0.22 in MPL3-T80 and 3.35±0.23 in MPL4-T80. It can be concluded that increment of PE/EC resulted with increased PC most likely due to the higher amount of PE available for encapsulation into MPLs. Significant difference was observed among all samples except MPL1-T20/MPL1-T80, MPL2-T20/MPL2-T80 and MPL3-T20/MPL3-T80 (one way *ANOVA*, Fisher's LSD, $p<0.05$).

Dissolution studies pointed that release of PE from MPLs was continued for 3-4 hours, indicating prolonged release compared to PE alone (immediately dissolved). Dissolution profile comparison using pairwise procedure - f2 similarity factor, resulted with f2 values >50 in all tested formulations except MPL1-T20/MPL1-T80. It can be anticipated that lack of similarity is most likely related to the difference in their particle size.

Drug release data best fitted to model of Peppas and Sahlin, a model accounted for the coupled effects of Fickian diffusion and case II transport. However, a negative K1 value indicated insignificant effect of Fickian diffusion on drug release.

Conclusion

MPLs loaded with 25% PE were prepared by double emulsion solvent diffusion technique. Influence of the amount of used propolis (PE/EC= 0.5, 1, 2 and 4) and type of hydrophilic surfactants (T20 and T80) on MPLs properties was evaluated. By subsequent incorporation of prepared MPLs into gel system additional prolongation of PE release could be achieved, thus resulting with a topical dosage form with appropriate controlled released properties in favor of efficient acne treatment.

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Macedonian pharmaceutical bulletin, **62** (suppl) 631 - 632 (2016)
ISSN 1409 - 8695
UDC: 582.684.1-119.2: 543.544.5.068.7
Short communication

HPLC determination of hypericin content in Hyperici oleum

Veronika Stoilkovska[1]*, Jelena Acevska[2], Gjose Stefkov[1]

[1]*Institute of Pharmacognosy, Faculty of Pharmacy, University ,,Ss. Cyril and Methodius'', Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University ,,Ss. Cyril and Methodius'', Majka Tereza 47, 1000 Skopje, R. Macedonia*

Introduction

St. John's Wort Oil (Hyperici Oleum) is a traditional herbal preparation which is prepared by fatty oil maceration of the flowering aerial parts of Hypericum perforatum Linn. (fam.Hypericaceae) in sunlight for several weeks (EMEA/HMPC/101303/2008). It is used mainly externally for the treatment of wounds, especially burn wounds, bruises and swellings. Internally, it is used to treat dyspepsia (Kovar and Maisenbacher, 1992).

In the Ph. Eur. official are St John's Wort (Hyperici herba) and St. John's Wort dry extract, quantified (Hyperici herba extractum siccum), but not St. John's Wort Oil (Hyperici oleum) (Anyżewska et al., 2010). St. John's Wort Oil (Hyperici Oleum) is official according to the German Commission E and Swiss Pharmacopoeia. These pharmacopoeias suggest spectrophotometric determination where hypericin content is expressed as sum of all derivatives of hypericin like hypericin, pseudohypericin, protohypericin and isohypericin (Anyżewska et al., 2010, EMEA/HMPC/101303/2008).

Hypericin which is naphtodiantrone, has specific protonation and deprotonation behavior. The protonation behavior of carbonyl groups is assigned to the pKa= -6, deprotonation of OH groups in position 3 and 4 to the pKa= 2 and second deprotonation assigned to the pKa= 9, which generates diphenolate ion by ionization of one of the OH groups in position 1, 6, 8 or 13. This indicates that hypericin is present as an anion under most circumstances due to its rather low pKa value of 2 (Wirz, 2000). Specific conditioning of Solid Phase Extraction (SPE) cartridges is crucial since it positively charges the stationary surface with which bonding of hypericin is enabled and separation from the vegetable oil is possible, which is one of the main challenges.

The aim of this study is to develop HPLC method for quantitative determination of hypericin in St John's Wort oil using solid phase extraction as sample preparation method and compare it to the official spectrophotometric pharmacopoeial method.

Materials and methods

Plant material: Aerial flowering parts of wild H. perforatum populations were collected from locality Stracin, R. Macedonia.

Preparation of Hyperici Oleum: Herbal drug (crushed, fresh flowers and dry upper aerial part) and vegetable oil (sunflower oil) ratio was 1:4. The transparent glass jars containing the herbal drug and vegetable oil were left at sunny place for 6 weeks. Afterwards the herbal drug was pressed out and oil extracts were collected in dark, cylindrical flasks (EMEA/HMPC/101303/2008).

Primary reference standard hypericin was purchased from HWi Analytik GmbH, Rülzheim, Germany with 89.73% purity. Methanolic solution of the standard (0.006 mg/mL) was prepared (Hm). Solution of hypericin standard in sunflower oil (0.006 mg/mL) was prepared (Ho). For SPE, n-hexane dilution of Ho was done (H1=0.006 mg/mL and H2=0.003 mg/mL).

2 mL of each, commercial St. John's Wort oil (B1) or St. John's Wort oil (crushed fresh flowers in sunflower oil) (B2) were diluted to 10 mL with n-hexane. For the spiked sample (B3), 1 ml Ho and 1 ml B1 were diluted to 5 mL with n-hexane.

Sample preparation was done using solid-phase aminopropylsilica extraction cartridges (Sep-Pak®, 6cc, 500 mg sorbent), which were conditioned sequentially with 10 mL each of 0.01 N NaOH, methanol, acetone and n-hexane (Kovar and Maisenbacher, 1992). Samples were loaded on SPE-NH2 cartridges using vacuum manifold and

* veronika.stoilkovska@gmail.com

then the cartridge bed was rinsed with 12 mL of n-hexane. Elution was done with 4 mL 5 % (m/V) oxalic acid in acetone:methanol (1:1 V/V) (Eoa) or with mobile phase (Emp). For HPLC analysis the following eluates were obtained: EmpH1, EmpH2, EmpB1, EoaB1, EmpB2, EoaB2, EmpB3 and EoaB3.

The chromatographic analyses were carried out using Agilent 1200 HPLC equipped with DAD G1315D, quaternary pump G1311A, column thermostat G1316A and thermostatted autosampler G1329A. Separation was achieved using Agilent ZORBAX SB-C18 column (4.6 x 250 mm, 5 µm). The isocratic mobile phase at a flow 1 mL/min consisted of 78 mL ethyl acetate, 82 mL sodium dihydrogen phosphate solution adjusted to pH 2 with phosphoric acid and 360 mL methanol. Injection volume was 20 µL and the wavelength of detection was 590 nm. The separation was performed at 40˚C (Monograph of St. John's Wort dry extract; Ph. Eur.8).

For spectrophotometric measurements a Perkin- Elmer UV/ VIS spectrophotometer Lambda 16 was used.

Results and discussion

With SPE procedure, the vegetable oil was successfully separated and the phytocomponents were clearly eluated into the eluates, with characteristic red colour.

With HPLC analysis of EmpH1, EmpH2, EmpB3 and EoaB3 eluates a peak of hypericin was identified, with Rt and UV/ VIS spectrum corresponding to the results obtained by HPLC analysis of the methanolic solution of the hypericin standard (Hm). Relative peak intensity of the target component- hypericin was higher in Emp eluates compared to Eoa eluates. By analysis of EmpH1, EmpH2, EmpB3 and EoaB3 satisfactory selectivity of the SPE procedure and HPLC method for hypericin were proven.

HPLC analysis of EmpB1, EoaB1, EmpB2, EoaB2 did not reveal any peak corresponding to hypericin, suggesting that target component may not be present in form of hypericin in St. John's Wort Oil.

Possible reason for missing peak from hypericin in the HPLC analyses might be due to photolysis when exposed to sunlight several weeks during preparation of Hyperici oleum. This thesis is also suggested from Maisenbacher et al. (1992) who had examined a solution of hypericin in acetone after irradiation with a daylight lamp for 40 h and by UV/ VIS, IR and 1H NMR spectroscopy have given assumption of the basic structure of lipophilic hypericin- derivatives generated by photolysis of hypericin. It was suggested that St. John's Wort Oil red colour comes from the lipophilic breakdown products of hypericin (Kovar and Maisenbacher, 1992).

On the other hand, with spectrophotometry method proposed in Ph. Helv. 11.0 the obtained results complied with the pharmacopoeial criteria and indicated presence of hypericin in the samples H2, B1 and B2.

Presence of unidentified compounds detected in the HPLC chromatogram of St. John's Wort Oil indicated that further research should be done in order to explain the possible chemical modification of hypericin like derivatives.

Conclusion

Within this research, SPE procedure was successfully applied for separating hypericin and other phytocomponents from vegetable oil solution. Hypericin was not detected in St. John's Wort Oil by the proposed HPLC method, although the content of hypericin derivatives, determined by spectrophotometric method, was in compliance with the Ph. Helv 11.0. Analysis of the obtained results suggests possibility that the target component hypericin may undergo chemical modification as unidentified peaks were detected in the chromatogram of St. John's Wort Oil. Further analysis of different preparation methods for St. John's Wort Oil and their affection to hypericin content, stability testing of hypericin in vegetable oil, as well as quantitative and qualitative determination of the possible degradation products of hypericin are required

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Macedonian pharmaceutical bulletin, **62** (suppl) 633 - 634 (2016)
ISSN 1409 - 8695
UDC: 543.544.5.068.7
Short communication

Optimization of HPLC method for determination of related substances in metamizole sodium using core-shell columns

Belma Asanova*, Filip Cvetanovski, Gabriela Petrovska, Marija Zafirova, Katerina Brezovska

Institute of Applied chemistry and Pharmaceutical analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

A new core-shell silica particles were developed as a material for chromatographic stationary phases that can separate small and large molecules and complex samples, providing fast and high efficiency separations at pressures compatible with conventional HPLC equipment (Guiochon and Gritti, 2011). Core shell particles (also called fused-core™ particles, shell particles or controlled-surface-porosity particles) consist of a non porous silica core and a narrow shell of porous silica. Compared with conventional columns with fully porous particles, core shell particles provide shorter diffusion paths of the analytes into the stationary phase which significantly influence the separation parameters (Berger, 2011; Destefano et al., 2008; Wu et al., 2013). Core-shell columns have been mostly used for reversed-phase HPLC, as well as in hydrophilic interaction liquid chromatography (HILIC), chiral separation, two dimensional liquid chromatography and capillary electrochromatography separation (Kirkland et al., 2013; Ruta et al., 2012). Transfer of the HPLC methods from columns packed with fully porous silica particles to core-shell columns provide significant time and cost savings, while maintaining column performance and minimizing operating difficulties (Hayes et al., 2014).

The aim of our work was to transfer the method for determination of related substances of metamizole sodium using the method described in the Ph.Eur. monograph (01/2008:1346) from conventional to core-shell column and to optimize the method for determination of related substances of metamizole in tablets.

Materials and methods

Chemicals and standards

Metamizole sodium and metamizole impurity A, certified reference standards, were purchased from EDQM (Strasbourg, France). Sodium dihydrogen phosphate, (Ph. Eur., grade for analysis) and triethylamine were purchased from Merck, Germany. Methanol (HPLC grade) was purchased from Carlo Erba Reagents, France. Water R, was obtained with a TKA-LAB Reinstwasser system (Niederelbert, Germany).

Chromatographic Conditions

HPLC separation was performed on Agilent 1200 LC Systems, using ChemStation software, Version B.04.03 for data acquisition and instrument control. The column used for the separation was Kinetex XB-C18, 50 mm x 2.1 mm, 5 µm (Phenomenex, Inc). Mixture of 28 volumes of methanol R and 72 volumes of a buffer solution containing 1000 volumes of a 6.0 g/L solution of sodium dyhidrogen phosphate R and 1 volume of triethylamine R, pH 7.0, was used as a mobile phase. The column temperature was 25°C. Flow rate was 0.2 mL/min. Injection volume was 0.5 µL. UV detection was performed at 254 nm.

Preparation of solutions

Reference solution e, containing metamizole sodium (0.3 mg/mL) and metamizole impurity A (0.4 mg/mL) prepared as described in Ph.Eur. monograph (01/2008:1346) was used as system suitability solution.

Test solution was prepared by dissolving a quantity of powdered tablets containing 500 mg metamizole and dilu-

* belma.asanova@hotmail.com

tion to 100, 0 ml of methanol.

Reference solutions used for calculations were prepared according to the method for related substances described in Ph.Eur. monograph 01/2008:1346.

Validation of the method

Validation of the method was performed according to International Conference on Harmonization (ICH) Guideline on Validation of Analytical Procedures: Text and Methodology (ICH, 2005).

Results and discussion

The method for simultaneous determination of metamizole sodium and its two impurities (A and C) described in Ph.Eur. monograph (01/2008:1346) was transferred from conventional fully porous column (base deactivated octadecylsilyl silica gel for chromatography R, 0.25 m x 4.6 mm; 5 µm) to column based on core-shell technology (XB-C18, 50 mm x 2.1 mm, 5 µm) and optimized for determination of related substances of metamizole in tablets.

Satisfactory system suitability requirements were obtained in total analysis time of 9 minutes (4.5 times of the retention time of metamizole, Rt = 2.15) and the value for resolution (Rs) between metamizole and impurity A (Rs = 3.11) fulfills the requirement of the Ph.Eur monograph (Rs ≥ 2.5). The purity ratio was within the calculated threshold limit (1.000) for both impurity A (0.128) and impurity C (0.0197), confirming the purity of the peaks and specificity of the method. The obtained values for number of theoretical plates (N=10187), retention factor (k'=1.88), symmetry factor (As=1.28) and relative standard deviation (RSD(Rt)=0.16%; RSD(peak area)=0.53%; n=6) for the peak of metamizole impurity A, indicate on a satisfactory column efficiency and adequate performance of the chromatographic system.

The results obtained from the regression analysis of the peak area (y) versus concentration (x), confirmed the linearity of the method (y = 6,476x + 2,417; R2 = 0.9999) in concentration range from 5 µg /mL to 50 µg /mL of metamisole impurity A (5, 10, 20, 25, 30, and 50 µg/mL). The values for limit of detection (DL=0.33 µg/mL) and limit of quantification (QL= 0.99 µg/mL), determined from the residual standard deviation of the regression line and the slope are below the disregard limit (1.25 µg/mL) given in the Ph.Eur. monograph, indicating on satisfactory sensitivity of the method.

The repeatability of the method was confirmed from the values for the relative standard deviation (RSD), obtained from determination of the test solution (RSD = 2.26 %, n=6). Recovery values obtained from the determination of metamizole impurity A (81.3 % - 90.4 %) in the test solution (spiked with metamizole impurity A, at three different concentrations) confirmed that the method was accurate in the range of 80-120% of the working concentration.

The results from the determination of the robustness of the method indicated that small but deliberate changes in the analytical conditions did not affect the system suitability parameters and that the method can be applied in routine work.

Conclusion

The results have shown that the optimized method using core-shell column is suitable for determination of related substances of metamizole in tablets containing metamizole sodium, providing fast and highly efficient separation on conventional HPLC equipment, available in almost any laboratory.

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Macedonian pharmaceutical bulletin, **62** (suppl) 635 - 636 (2016)
ISSN 1409 - 8695
UDC: 615.12(497.7)
Short communication

Survey of community pharmacy practice in Republic of Macedonia

Angela Arsovska[1]*, Maja Simonoska Crcarevska[1], Marija Glavas Dodov[1], Tatjana Sterjeva[2], Renata Slaveska Raicki[1]

[1]*Institute of Pharmaceutical Technology, Faculty of Pharmacy, University of "Ss. Cyril & Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia*
[2]*Euro-farm, Ognjan Prica 5, 1000 Skopje, R. Macedonia*

Introduction

The pharmacy profession is facing continuous evolvement toward patient (PT) oriented health care services in order to ensure the safe, effective and rational use of medicines. The areas of pharmacist practice are versatile - from activities concerned with PT care and public health to drug design and development, supply and dispensing (Traulsen and Almarsdottir, 2005). Currently 2413 pharmacists are licensed at the pharmaceutical chamber of R. Macedonia, where 1107 are employed in CPs across the country (data obtained from Pharmaceutical chamber of Macedonia on 22.02.2016). Having in mind that a major area of licensed pharmacist (LP) settlements in R. Macedonia are CPs (~46% of all LPs), we conducted a survey (20.10.2014-20.01.2015) in order to establish a baseline in respect of CP activity, staffing, infrastructure etc. We hope that the results of the conducted survey will help for further development of CP sector.

Materials and methods

Focus-methodology group approach was applied in the process of design and structuring of the questionnaire. It was conducted between 1-10 October, 2014 on 10 LP employed at CPs across the country in order to explore the level of understanding, acceptability (questions wording, complexity and ambiguity of proposed answers) as well as the time required to complete the questionnaire. Afterwards the final questionnaire with 29 items was structured.

The anonymous questionnaire covers data related to socio-demographic factors (city, age, gender and length of time registered as a LP), average number of PTs and prescriptions (Px) in the CP per week, location and workforce in the CP, services offered, continuing education and experiences and opinions related to e- Px.

The questionnaire was distributed to 150 CPs across the country and 124 were returned. Only one questionnaire was in a group with more than 20% missing values and therefore 123 questionnaires were analyzed.

Obtained data were tabulated using Microsoft Excel® (Microsoft Corp. Redmond, WA, USA) and were computed and consequently evaluated using statistical software STATGRAPHICS Centurion XVI evaluation (StatPoint technologies Inc., USA)

Results and discussion

According to the data found on the web site of Macedonian Agency for Drugs and Medicinal Products (http://malmed.gov.mk) there are 1019 registered pharmacies in R. Macedonia of which 846 (83%) are CPs (web site accessed at 24.02.2016). The analyzed sample of 123 CPs covers 14.54% of all CPs in R. Macedonia pointing to satisfactory margin over target of necessary 10% to have reliable results.

Related to the profile of LP employed in CPs the average age was 36.24 ± 9.89 years of which 96.7% were females. The average length of time registered as a LP was 10 ± 8.9 years. Only 2.4% have degree of postgraduate health-care specialist, and 1.6% were enrolled in such study at the time of survey.

Most of the surveyed CPs (48.4%) were visited by up to 1000 PTs per week followed by those with 1000-2499 PTs (41.8%). The average number of dispensed Px items per week 500-999 (47.5%) preceded the ones with lower than 500 (40.8%). Majority of the respondents (45.4%) stated that the number of dispensed Px per week issued by general practitioners was 500-900 while the specialists in 55.4% cases issued in average 100-499. Most of the CPs

* angela.arso@gmail.com

(60%) were visited by medium number of PTs from all age groups (56.9%). The average working time per day (Mon. to Sat.) was 13.22±1.35 hours, where 30% have opening hours from 8:00-21:00 h, 15.83% from 8:00-22:00 h and 10% from 7:30-21:00 h. Sunday was working day in 35.83% of surveyed CPs with 7.15±2.03 working hours on average with only 3.33% working 12 or more hours. Only 8.1% of the respondents worked within small chain (up to 3 CP), where majority (69.7%) worked in large chain and the rest of 22.1% worked in single CPs. CP location in 46.3% of the cases was in central city region and in 21.1% near the general practitioner and/or hospital (15.4%).

The findings related to workforce indicated that average number of total staff per CP was 4±1.52 persons. On average, there were 2.34 ± 1.04 LPs reported as working in each CP with the most common response (mode) being 2 (39.2%) while the maximum reported number of LPs was 5. In 21.7% of cases only one LP was employed. Isolated consultation/counseling area for PTs was not available in 86.3% of the CPs. Only 7.4% CPs dispensed in-house compounded medications.

Related to medication (MED) therapy management, 61.7% of the LPs would like to have information about previous use of the certain MED, 37.4% if the PT knows how to use the MED, 34.6% were there any allergies or intolerances noted, 33.6% about other used MEDs and only 4.7% were asking the PT if he/she was familiar with possible side/adverse effects of the drug dispensed. Preferences about the drug manufacturer were asked in 19.6%. LPs in 93.5% of the cases audited possible physicians' errors, 71.5% check out drug interactions, 74% allergy to the prescribed drug or its excipients, 51.2% other allergies present and something else in 14.6%. MED therapy errors were solved and evidenced in 64.4%, while in 32.2% were solved but not evidenced. Only 9.4% of the respondents reported adverse drug reactions.

More than 90% of the respondents advised PTs for eye drops and inhaler use, ~82% for pregnancy testing, ~40% for insulin pen and ~28% for other matters. Drug use instructions were written on MED package by ~97% of the LPs.

Respondents' opinion about the availability of information needed for efficient MED therapy management was as follows:

- all of them would like to know if there is history of drug related allergies and ~80% about other allergies present, where more than 56% appreciated to obtain the information both from the PT and medical dossier,
- diagnosis was stated to be valuable by ~87% while medical history (past 6 months) was valued by 58.5%. Medical dossier as source was selected by more than 50%,
- adverse drug reactions was found to be valuable for 97.3%, and both medical dossier and PT as source by 39.1%,
- discharge summary and results from screening and laboratory tests were valued by ~41%, 29% and 52% respectively and medical dossier as source by ~64%, 60% and 71% of the respondents accordingly.

PT adherence to chronic therapy was followed via direct PT questioning by 70% of the respondents.

Blood glucose could be monitored in 18.7%, while blood pressure in 35.8% of surveyed CPs. Health promotions (eg. colon cancer, nutrition dietetic regimens) and pregnancy advices were practiced in 18.7% and 36.6% of the cases respectively.

Nearly 84% responded that PTs were seeking advices for smoking cessation and nutrition, ~65% for vaccines, and ~55% were asked regarding alcohol drinking as well as physical activity. More than 95% of the respondents stated that PTs sought an advice concerning drugs currently used, side/adverse drug reaction, change of therapy/treatment, health related issues in general and herbal drugs. Urgent contraception, dietetic regimen in time of ongoing therapy, MED storage conditions and need of physician visit were issues of PTs` interest in more than 80% of the cases. Nearly 60% of the respondents stated that they were asked about therapy monitoring and ~66% about new approved drugs.

Only 26.2% responded that unwanted medicines were returned to CP for disposal.

Survey for PTs` experience from CP work and activities in the past year was conducted in 16.1%, but however 51.3% stated that they were planning to conduct such a survey in the future. PTs ` compliant procedure was established in 26.5% of the surveyed CPs.

Continuing education preferences were expressed by 75% of the respondents, but however when it comes to the type and topic, response rate from the surveyed LPs was relatively low, 52.8% and 37.4% respectively. From those responded 84.6% preferred conventional lectures, seminars, symposia, congresses, and only 10.8% e-education. Topics related to new approved drugs and CP practice & communication were preferred by 21.7% and 19.6% from the respondents, accordingly.

The vast majority of the respondents were satisfied by introduction and use of e- Px.

Only 25% of the surveyed LPs provided their opinion in relation to CP work improvement where 29% of the respondents suggested less administrative tasks, e- Px only and PTs` part in the e- Px to be automatically filled by data already present in the system and 22.6% suggested to make clear distinction between duties of LPs and pharmaceutical technicians working in CPs, as well as different person responsible for Px and OTC drugs.

Conclusion

This study enabled baseline establishment for the current situation in CP practice in R. Macedonia. The results could be used for further development and improvement of CPs sector in our country.

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Macedonian pharmaceutical bulletin, **62** (suppl) 637 - 638 (2016)
ISSN 1409 - 8695
UDC: 615.33.015.8
Short communication

Antimicrobial resistance to antibacterial agents in common respiratory tract pathogens in pediatric population

Stefan Matik*, Ana Vavlukis*, Aleksandra Kapedanovska Nestorovska, Zorica Naumovska, Aleksandra Grozdanova, Zoran Sterjev

Institute of pharmaceutical chemistry, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, Skopje, Republic of Macedonia

Introduction

Background and Aims: Antibiotics have always been considered one of the wonder discoveries of the 20th century. This is true, but the real wonder is the rise of antimicrobial resistance in hospitals, communities, and the environment concomitant with their use. The extraordinary genetic capacities of microbes have benefited from man's overuse of antibiotics to exploit every source of resistance genes and every means of horizontal gene transmission to develop multiple mechanisms of resistance for each and every antibiotic introduced into practice clinically or otherwise (Davies and Davies, 2010). Resistance mechanisms have been identified and described for all known antimicrobials. Acquired bacterial antibiotic resistance can result from the mutation of normal cellular genes, the acquisition of foreign resistance genes, or a combination. The most common resistance mechanisms employed by bacteria include enzymatic degradation or alteration of the antimicrobial, mutation in the antimicrobial target site, decreased cell wall permeability to the antimicrobial, and active efflux of the antimicrobial (Harbottle et al., 2006). With respect to the pediatric population, respiratory tract infections (RTIs) are a common reason for health care provider visits and the primary reason for antimicrobials prescribing in this population. The overuse of antibiotics in children is becoming a major public health problem. Although most of the common childhood infections, such as the upper respiratory tract infections, are caused by viruses, large volumes of antibiotics are prescribed for these infections in children in the primary care setting. It is estimated that 90% of upper respiratory tract infections are self-limiting viral illnesses and even bacterial infections like acute otitis media often run a self-limiting course. Clinical trials have shown that antibiotic use to treat common upper respiratory tract infection like sore throat, nasopharyngitis and otitis media has no or minimal benefit on the clinical outcome (Kutty, 2011). Given the proven relationship between the resistance rate of a specific pathogen and the rate of antibiotic usage (community setting and nosocomial), the aim of this study was to evaluate the resistance rate to specific antibiotics in common respiratory tract pathogens in pediatric population in R. Macedonia.

Materials and methods

Retrospective analysis of medical data from 7079 patients was done in the period July 2013 – January 2014. The analyzed isolates were taken from: nose (2141), throat, epipharynx, pharynx, deep swab from pharynx, tonsils (2752); drainage secretions (2186). The testing was performed by the Cabinet for microbiological analysis, Institute for respiratory diseases in children - „Kozle" (Skopje). Data from sensitivity tests (antibiograms) were analyzed for 6 RTIs pathogens - *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*. The isolates were obtained using standard microbiological procedures and screened using disc-diffusion test. Sensitivity was tested to penicillins (Amoxicillin, Oxacillin), cephalosporins (Cefadroxil, Cefuroxime, Cefixime, Ceftriaxone) and macrolides (Azithromycin, Clarithromycin, Erythromycin). The antibiotics were selected based on the institute's prescription practices for RTIs. The pathogen sensitivity to a specific antibiotic in the antibiogram reports was numerically labeled - 3 (sensitive), 2 (moder-

* stefanm_bt@hotmail.com

ately sensitive), 0 (resistant). Data from oxacillin test (3/0) were analyzed for *Streptococcus pneumoniae*. A basic statistical analysis was performed to calculate the resistance rate (percentage value) of every individual pathogen to every specific antibiotic. The resistance rate (lack of susceptibility) was calculated by dividing the number of isolates with an identified resistant pathogen (numerically labeled as 0 in the antibiogram reports) and the total number of isolates of that given pathogen.

Results and discussion

Moraxella catarrhalis demonstrated 9.5% resistance rate to Cefixime, and an average resistance to macrolides - 10-12%. *Streptococcus pneumoniae* had a sizeable (30.9%) resistance rate to Cefixime, 38-43% to macrolides, and a dramatically high (70.1%) resistance rate on the Oxacillin test. The oxacillin test for *S.pneumoniae* is considered a test for penicillin susceptibility in the first place, but unsusceptible isolates are also considered unsusceptible to extended-spectrum cephalosporins (e.g. Ceftriaxone or Cefotaxime). *Haemophilus influenzae* demonstrated significant resistance to Cefixime (10.6%). There were no cases of resistance to Amoxicillin. *Staphylococcus aureus* showed a substantial (14-23%) resistance rate to the macrolides. *Streptococcus pyogenes* showed no resistance to any of the tested antibacterial agents. *Escherichia coli* showed a very high resistance rate to the class of chepalosporins: Cefadroxil - 77.8%, Cefuroxime - 61.5%, Cefixime - 71.3%, and Ceftriaxone - 67.3%. The study revealed a 28-35% resistance rate of *Klebsiella pneumoniae* to the group of cephalosporins. *K. pneumoniae* is considered intrinsically resistant to amoxicillin.

If on the other hand, we take into account the data for antimicrobials consumption in Europe for the aforementioned period - a causative relationship between the antimicrobials use and the rate of antimicrobial resistance could clearly be established. For example, in 2011, Greece was on the first place with antimicrobials consumption in Europe (35 DDD/1000/day), and at the same time the data for antimicrobials resistance rates in Greece demonstrated very high values for various isolates. The same could be concluded for Cyprus and other countries that are positioned on the top of the list for antimicrobials consumption. Contrary to this, Netherlands, Estonia, Latvia and the Scandinavian countries, which are positioned on the bottom of the same list, are also the countries in Europe with the least percentage of antimicrobial resistance (EARS-Net Annual Report, 2013).

The situation in Macedonia was much like that in Greece and Cyprus. The consumption of antimicrobials for the period 2008-2012 was approximately 32 DDD/1000/day. For that period, the most commonly utilized antibiotics were the beta-lactams. During this time, the consumption of antimicrobials dropped compared to the previous period for all groups of antibiotics, except for the penicillins, where an increase was noticed. The most commonly prescribed anti-infective agent for systemic use was Amoxicillin + clavulanic acid. Amoxicillin followed on the second place, whereas Cefuroxime and Cefixime placed fourth and fifth. Ciprofloxacin was positioned on the third place (Annual report 2012, Health Insurance Fund of Macedonia). Furthermore, also to be considered is the fact that in the time of conduction of this research, Cefixime was the only oral third-generation cephalosporin registered in Macedonia, and even more, available as a pediatric oral suspension. To this, could partially be attributed the observed great percentage of resistance to Cefixime.

If we take into consideration the data obtained in this research, we can clearly postulate that the high consumption of antimicrobials in Macedonia could be in a great manner responsible for the substantial percentage of antimicrobial resistance, by constantly increasing the ever-growing positive pressure on microorganisms to continuously acquire new means of resistance.

Conclusion

The results from the susceptibility tests on one side, and the total antibiotics use (32 DDD/1000/day) in the Macedonian population on the other, proved the previously stated proportional relationship between the population antibiotics consumption level and pathogen resistance rate.

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Macedonian pharmaceutical bulletin, **62** (suppl) 639 - 640 (2016)
ISSN 1409 - 8695
UDC: 615.214.015
Short communication

Risperidone loaded nanostructured lipid carriers: formulation optimisation and characterisation

Nikola Lazarevski*, Hristina Litovin, Maja Simonoska Crcarevska, Marija Glavas Dodov

Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Risperidone (RPD) is an antipsychoticdrug commonly used for the treatment of schizophrenia. The treatment for this disease is generally life-long, so the oral route is the most convenient and non-invasive way of drug administration. However, RPD is a poorly water-soluble BCS class II drug that undergoes extensive first-pass hepatic metabolism and has poor oral bioavailability. Moreover, it binds about 90% to plasma proteins, which renders its delivery across the blood-brain barrier (Kumar et al., 2009).

Literature data suggests that nanoparticles could avoid the first-pass effect and provide prolonged release, thus enabling significant reduction of the RPD dose and minimising its undesired side-effects (Patel et al., 2011). Lipid nanoparticles are a promising strategy for drug delivery to the brain due to their rapid uptake, bio-acceptability, biodegradability and lower toxicity compared to polymeric nanoparticles. Nanostructured lipid carriers (NLCs) are an improved generation of lipid carriers that are preferred over conventional lipid formulations and solid lipid nanoparticles due to their enhanced properties for drug loading, modulation of the drug release profile and improved physical stability (Alam et al., 2015). Additionally, the small size, unique drug incorporation abilities, steric propertiesand possibilities for surface modification of NLCs favour brain targeting and delivery.

The presented study focuses on the formulation optimisation of surface stabilised RPD loaded NLCs and their characterisation in terms of particle size, particle size distribution and drug encapsulation efficiency.

Materials and methods

Materials

Cutina® GMS V PH (GMS) was kindly donated by Cognis, Germany. Miglyol 812 and Phospholipon 90G (Ph 90G) were purchased from Sasol, Germany and Lipoid, Germany, respectively. Sodium glycocholate (SGH) and Tween 80 were obtained from NutriScience Innovations, LLC, USA and Merck, Germany, respectively. Poloxamer 407, Poloxamer 188 and PEG 6000 were a generous gift from BASF, Germany. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO, USA). Risperidone (RPD) was purchased from Teva-Tech, Israel. All the other chemicals and reagents were of the highest purity grade commercially available and used as received.

Preparation of NLCs

Different formulations of blank NLCs were prepared using melt emulsification and rotor-stator homogenisation technique. Optimised formulation (sample 1) (central composite design: blocked cube star) composed of 2.5% GMS, 2.5% Miglyol812, 0.75% Ph 90G and 0.2% SGH was selected by minimising both responses, mean particle diameter (D_{50}) and particle size distribution expressed in terms of SPAN factor. Briefly, the lipid phase was melted by heating to ~80 °C and dispersed in an aqueous phase containing SGH, preheated at the same temperature, under high shear homogenisation (7 min, 24.000 rpm) using Ultra-Turrax® T25 (Ika-Werke, Germany). Subsequently, the dispersion was allowed to recrystallize at 5 °C for 1 h.

In order to turn the hydrophobic NLC surface into a more hydrophilic one, sample 1 was further modified by incorporating different concentrations (4.0, 4.5, 5.0 and

* nkllazarevski@yahoo.com

5.5% w/w) of Tween 80 (samples 1A1-4), Poloxamer 407 (sample 1B1-4), Poloxamer 188 (sample 1C1-4) or PEG 6000 (sample 1D1-4) in the aqueous phase, prior to the high-shear homogenisation. Sample 1A4, prepared with 5.5% Tween 80 was selected for preparation of RPD loaded NLCs, based on the results obtained from *in vitro* protein adsorption studies. Drug loaded NLCs were prepared using the same procedure as described above, by adding different concentrations of RPD (drug/lipid mass ratio 1, 10 and 20%; samples R_1, R_{10} and R_{20}, respectively) to the melted lipid phase prior to the high-shear homogenisation.

Characterisation of NLCs

The objective of the protein adsorption studies was to determine the amount of adsorbed BSA on the surface of NLCs prepared with different types and concentrations of stabilisers. Briefly, 100 µl aliquots of the prepared sampleswere incubated with 2mg of BSA in 2 ml of phosphate-buffered saline (pH=7.4) for 1 h on shaking water bath (37 ˚C, 75 rpm). After adsorption, the unbound protein was separated by centrifugal ultrafiltration through three washing cycles using Vivaspin 20, 1000 KDa (15 min, 4000 rpm; Hettich, Rotofix 32, Germany). Afterwards, the volume of samples was condensed to the initial volume of NLC dispersions. The adsorbed amount of BSA/mg lipid was calculated indirectly by determining the total amount of protein remaining in the filtrate by UV spectroscopy (595 nm; Bio-Rad's Model 3550-UV Microplate Reader) after reaction with Bio-Rad protein assay (Bio-Rad, USA), based on the method of Bradford (microtiter plate protocol) and subsequent subtraction from the initial BSA amount. The particle size (D_{50}) and particle size distribution (SPAN) of the prepared NLCs were determined by laser diffractometry (Mastersizer 2000, Hydro 2000S, Malvern Instruments Ltd., UK).

In order to determine the drug content (mg RPD/g lipid) and encapsulation efficiency (EE%) of RPD in the NLCs, the particles in the suspension were separated by centrifugal ultrafiltration through three washing cycles using Vivaspin 20, 1000 KDa (15 min, 4000 rpm; Hettich, Rotofix 32, Germany) and brake-opened using methanol. The solution was then passed through a 0.45 µm filter and analysed by reverse-phased HPLC method (Agilent HPLC system, 1200 Series; column: Waters Spherisorb ODS-2; 150 mm x 4.6 mm, 5 µm). The used mobile phase was consisted of methanol, acetate buffer (0.05M, pH=4.6) and triethanolamine (60:40:0.02, *v/v/v*) with flow rate of 1 ml/min at 25.0±0.5 ˚C. The injection volume was 50 µl and the detection was carried out at 280 nm. The EE% was calculated from the total amount of drug in the nanoparticle suspension and the quantity of RPD added initially during the preparation.

Results and discussion

In the development of site-specific drug delivery systems it is a prerequisite to identify the factors determining the carriers' *in vivo* behaviour and disposition. For effective brain targeting, NLCs should have a hydrophilic surface, reduced protein adsorption pattern and prolonged blood circulatory time in order to permit efficient and satisfied interactions and penetration in the brain endothelial cells.

Considerable differences in BSA adsorption onto NLCs surface were detected for different stabilizers in a concentration-dependent manner and in all cases the total protein adsorption was lower compared to unmodified NLCs (sample 1). NLCs prepared with Tween 80 (samples 1A1-4) resulted with lowest amount of adsorbed BSA (surfactant concentrations: 4.0, 4.5, 5.0 and 5.5%*;* unbound BSA: 46.5, 65.4, 68.2 and 94.9%, respectively). The obtained results are in accordance with the literature data pointing that the presence of Tween 80 could minimise the interaction of the nanoparticles with serum albumin and prolong their half-life in the blood circulation (Göppert and Rainer, 2005), potentially giving NLCs best stealth characteristics compared to other formulations. Therefore, sample 1A4, prepared with 5.5% of Tween 80 was selected for preparation of RPD loaded NLCs.

The obtained unmodified NLCs (sample 1) had D_{50} of 107 nm and SPAN factor of 0.804 with unimodal narrow size distribution. There were no significant differences in D_{50} between unmodified and surface-modified particles. The D_{50} and SPAN of RPD loaded NLCs were 116 nm and 0.832 (sample R_1), 127 nm and 0.959 (sample R_{10}) and 123 nm and 1.012 (sample R_{20}), respectively.

The determined drug content in the prepared formulations was 8.8, 99.9 and 227.5 mg RPD/g lipid and the EE was 87.2, 89.9 and 91% for R_1, R_{10} and R_{20}, respectively. It is obvious that higher drug concentration used for preparing the samples resulted with increased drug content, thus pointing that the drug/lipid ratio was a critical parameter for efficient RPD loading into the NLCs.

Conclusion

In an attempt to realise the 'magic bullet' concept postulated by Ehrlich, the presented study indicates that Tween 80 stabilised NLCs could be suitable risperidone carriers with prolonged blood circulation time and site-specific brain delivery.

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Macedonian pharmaceutical bulletin, **62** (suppl) 641 - 642 (2016)
ISSN 1409 - 8695
UDC: 615.21:[615.322:547.565
Short communication

Formulation and characterization of rosmarinic extract loaded PEGylated liposomes for brain delivery

Ljubica Cambuleva*, Dushko Shalabalija*, Ivana Cvetkovikj, Maja Simonoska Crcarevska, Marija Glavas Dodov

Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia
Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Human brain is one of the most intricate organs because of its hundreds of billions of neurons continuously transmitting electrochemical signals to each other. With the aging of the world population, brain-based disorders, especially neurodegenerative diseases like Alzheimer's and Parkinson's are becoming one of the major health problems. However, brain is the most difficult organ for drug delivery and poses unique challenges. Specific anatomic and physiological features of the cerebrovasculature and cerebral tissue fluids result in barriers, *i.e*. blood–brain barrier (BBB), which limits the penetration of many molecules/substances including drugs to the central nervous system. Molecular characteristics, which are known to reduce the ability for partition/penetrance into the lipids of the cell membranes (through the BBB) are, a significant polarity, high potential for hydrogen bond formation, molecular weight of more than 500 kDa, as well as molecular structures which contain rotatable bonds and those that are highly branched (Haque et al., 2012; Mutlu et al., 2011).

Accordingly, the development of brain drug-delivery systems is imperative. Among different particulate carrier systems, liposomes have gained much attention because of their biocompatibility and structural similarity to that of cells. Liposomes can fuse with cells, facilitating the transport of drugs across biomembranes. Also, liposomes possess excellent criteria as drug carriers, because they are closed vesicles composed of an internal aqueous compartment and an external lipophilic bilayer of phospholipids molecules. External surface of liposomes can be modified by polyethylene glycol (PEG) to produce stealth properties. Stealth liposomes are characterized by long circulation time *in vivo* and could be easily coupled to different targeting moieties in order to improve the site-specific drug delivery.

Having in mind the limitations of currently available drugs for treatment of neurodegenerative diseases, development of phytopharmaceutical based liposomes could be considered as an attractive alternative approach for efficacious treatment of brain disorders. Natural polyphenols are well known organic compounds with antioxidative, free radical scavenging and anti-inflammatory characteristics. In this connection, polyphenols are thought to be potential candidates in prevention and treatment of diseases caused by oxidative damage, such as neurodegenerative disorders (Mignet et al., 2013). However, they exhibit low bioavailability and poor solubility, which limits their use.

Therefore, the aim of this study was design and formulation of PEGylated liposomes containing rosmarinic extract in order to enhance the bioavailability and efficacy of polyphenols in treatment of neurodegenerative disorders.

Materials and methods

Materials

Soybean lecithin (SL) was purchased from Vitalia, Macedonia. LIPOID PE 18:0/18:0-PEG 2000 (PEG) was kindly donated from Lipoid, Germany. Cholesterol (CH) was obtained from Sigma Aldrich (St. Louis, USA). Rosmarinic extract (RA-E) was a generous gift from the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia. All the other chemicals and reagents were of the highest purity grade commercially available and used as received.

* lj.cambuleva@gmail.com; d_salabalija@hotmail.com

Preparation of liposomes

During the preliminary studies, different formulations of liposomes were prepared in order to obtain vesicles with mean size of ~200 nm suitable for brain delivery (Geldenhuys et al., 2015). Optimized process parameters' were further used for formulation modification. Samples were prepared by the modified lipid film hydration technique. Briefly, required amounts of SL and CH (mass ratio=5:1, 10:1 and 20:1; samples 1, 2 and 3) were dissolved in chloroform/methanol mixture 4:1 (v/v). Afterwards, the organic solvents were removed by evaporation under vacuum using a rotavapor (25 °C, 50 rpm; Buchi 215, Switzerland). Thus obtained dried lipid film was hydrated with aqueous phase, phosphate buffer pH 7.4, under three hydration steps: ultrasonication (50/60 Hz), vortexing and gentle shaking. Every step lasted 5 min and four cycles of hydration were performed. Obtained liposomes were submitted to high shear homogenization (24 000 rpm, 5 min; Ultra-Turrax T25, Ika-Werke, Germany) and were allowed to stand for 24 h at 4 °C. For preparation of RA-E loaded liposomes (200 mg initially added) two different approaches were performed, *i*). RA-E was dissolved in organic solvents mixture required for lipid phase formation (samples 1a, 2a and 3a) and *ii).* RA-HE was dissolved in an aqueous phase (sample 1b, 2b and 3b).

From results obtained from particle size analysis and drug encapsulation efficiency (EE%), sample 2a was selected for preparation of PEGylated liposomes (sample P2a), where PEG was incorporated into the lipid phase (SL:CH:PEG mass ratio=10:1:0.5).

Liposome characterization

Prepared liposomes were characterized in terms of mean particle size (D_{50}) and particle size distribution (SPAN factor) by laser diffractometry (Mastersizer 2000, Hydro 2000S, Malvern Instr. Ltd., UK). EE% was calculated on a basis of non-encapsulated portion of RA-E by HPLC. Analyses were performed on Merck Hitachi HPLC system (Darmstadt, Germany), equipped with Ellite LaChrom L-2200 autosampler, L-2130 pump and L-2450 diode array detector. The column used was Zorbax Eclipse XDB RP C-18 column (150 mm × 4.6 mm, 5 μm, Agilent, Germany). The used mobile phase was: A - 1% formic acid (pH 3.0) and B - acetonitrile (90:10 v/v). Chromatographic conditions were set according to the method described by Cvetkovikj et al. (2013) with small modifications. Quantification of rosmarinic acid was performed using UV/VIS DAD at 330 nm.

Results and discussion

Obtained blank liposomes had D_{50} of 134, 129 and 130 nm (sample 1, 2 and 3, respectively) with unimodal narrow size distribution (SPAN factor 1.905, 1.743 and 1.782, respectively). By incorporation of RA-E into the aqueous phase, no significant differences in particle size of the prepared samples were observed and drug EE was found to quiet low (~ 17%). On the other hand, incorporation of RA-E into the lipid film resulted with increase in liposomes size (D_{50} 168, 136 and 161 nm for sample 1a, 2a and 3a, respectively), the particle distribution became broader and EE was 39.4-54.2%. From obtained data, it is possible to come to a conclusion that procedure of RA-E loading into liposomes seemed to have a significant effect on D_{50} and EE. Also, the differences in drug EE between the two different approaches could be related to the nature and physicochemical properties of the used herbal extract.

Therefore, sample 2a with D_{50} 136 nm and EE of 54.2% was selected for preparation of PEGylated liposomes. Blank PEGylated liposomes had a mean size of 141 nm (SPAN 3.845) The steric stabilization afforded by PEG incorporation into the lipid bilayer of RA-HE loaded liposomes affected the mean size of the vesicles. Namely, sample P2a had D_{50} of 163 nm (SPAN 2.06), which is known to be suitable for intravenous application achieving long plasma circulation time (Tanaka et al., 2004), and no significant difference in EE was observed. Further studies related to the influence of PEG quantity on the physicochemical and biopharmaceutical properties of liposomes should reveal the potential of designed system for efficient brain delivery of RA-E.

Conclusion

In this study, RA-E loaded conventional and PEGylated liposomes were successfully prepared. Considering the characteristics of the liposomes such as particle size, distribution and encapsulation efficiency, the delivery system seems suitable to overcome the drawbacks and to improve brain drug therapy after parenteral application.

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Macedonian pharmaceutical bulletin, **62** (suppl) 643 - 644 (2016)
ISSN 1409 - 8695
UDC: 582.688.3-119.2:543.544.5.068.7 (497.7)
Short communication

Determination of the arbutin content in wild growing populations of *Arctostaphylos uva-ursi* (L.) Spreng from Korab mountain

Viktorija Labroska *, Ivana Cvetkovikj, Gjoshe Stefkov

Institute of Pharmacognosy, Faculty of Pharmacy, "Ss. Cyril and Methodius" University, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Arctostaphylos uva-ursi (L.) Spreng. fam. Ericaceae, also known as bearberry or bear's ear, is a small procumbent woody groundcover shrub, widely distributed on a global level. The flowers are small, urn-shaped and grouped in terminal clusters that bloom in summer and the fruits are a red berries. The leaves are small, shiny and evergreen, remaining green for 1-3 years before falling.

The use of bearberry leaves for the first time was literally documented in the middle Ages in the Welsh "Physicians of Nyddfai" from the 13th century. From the beginning of 19th century, bearberry is in official use. It was used for treatment of different diseases such as hydrops, lithiasis, in diabetes, for the therapy of gonorrhoea, etc. Nowadays only the use as urinary tract antiseptic and diuretic remains due to the presence of arbutin and hydroquinone (Beaux et al., 1999; Jurica et al., 2015). Additionally, the herb contains tannins that have astringent effect and protect from early stage of infections (Dykes et al., 2003; Head, 2008; Pegg et al., 2008).

According to Ph.Eur.8.0 (2014), commercial forms that are used consist of whole or cut, dried leaf of *Arctostaphylos uva-ursi* that contains not less than 7.00% of anhydrous arbutin (Ph.Eur.8.0., 2014). The content of arbutin can be determined in plant extracts by many methods including: sprectrophotometry, capillary zone electrophoresis, and HPCL method, that was found to be the most suitable for arbutin separation and is prescribed within the Ph.Eur.8.0. (Amarowizh et al., 2009; Parejo et al., 2001; Ph.Eur.8.0, 2014).

In the Republic of Macedonia the natural populations of bearberry represent a unique source of leaves which are distributed in the following areas: Skopje Valley, mountains of Yakupitsa, Karadzitsa, Dautitsa, Shar Mountain, Osogovo Mountains and Mariovo. Bear's ear thrives on rocky ground in the light woods of black and white pine and in subalpic plant communities on limestone and dolomite ground. Although it is an indigenous species for this region there are no literature data for the content of the arbutin in bearberry populations.

Therefore, the aim of the present study was to quantify the amount of arbutin from seven different populations of *Arctostaphylos uva-ursi* from Korab Mountain with high performance liquid chromatography (HPLC).

Materials and methods

Plant Material

Leaves were collected from seven different natural populations of *A. uva-ursi* from seven different mountain areas of Korab, from R. Macedonia. Every area shows different geographical characteristics. Plant identity was verified and voucher specimens were deposited at the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia. Leaves were air dried, packed in paper bags, and kept in the dark and cold until analysis.

Extraction: The water extracts were isolated from dried leaves from bearberry, according to the method described in the European Pharmacopeia (Ph.Eur.8.0., 2014).

HPLC method

Chromatographic analyses were carried out using an HPLC system from the Agilent 1200 series, apply-

* viktorija.labroska312@gmail.com

ing the method described in the Ph.Eur.8.0 monograph (01/2008:1054), using a Zorbax Eclipse XDB RP C-18 column (150 mm × 4.6 mm, 5 µm, Agilent, Germany), protected with a guard column (4 mm × 4.6 mm, RP-18, 5 µm, Agilent, Germany). The eluents were: methanol R and water R, 10:90 (v/v). The flow rate was 1.2 mL/min and the injection volume was 20 µL. The column temperature was maintained at room temperature, and detection was carried out at 280 nm.

Quantification of the samples for arbutin was performed immediately after extraction to avoid possible chemical alterations using a 1 mg/mL stock of arbutin standard.

Results and discussion

The content of arbutin expressed as % of anhydrous arbutin, was determinate by high performance liquid chromatography from seven different populations of *A. uva ursi* from Macedonia and ranged from 7.41% to 10.46%. Among the analysed samples the highest content of arbutin was found in population 06 (P06) = 10.46% and the lowest was in the population 11 (P11) = 7.84%.

All seven sampled indigenous populations of bearberry leaf had arbutin content above 7.00%, which comply with the Ph.Eur.8.0 requirements.

Conclusion

Seven population of *Arctostaphylos uva-ursi* from Korab Mountain were evaluated regarding arbutin content according to the Ph.Eur.8.0 method. The content was determined by high performance liquid chromatography and the results were ranged from 7.84% to 10.46%. All populations complied with the requirements of the Ph.Eur.8.0 (not less than 7%).

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Macedonian pharmaceutical bulletin, **62** (suppl) 645 - 646 (2016)
ISSN 1409 - 8695
UDC: 615.273.53:616.132.2
616.132.2-085.273.53
Short communication

Influence of ABCB1 C3435T genotype on clinical cardiovascular outcomes in coronary artery disease patients on Clopidogrel treatment

Ana Vavlukis*, Lile Zdraveska*, Viktorija Nikolovska*, Aleksandra Kapedanovska Nestorovska, Zoran Sterjev, Aleksandra Grozdanova, Zorica Naumovska

Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Antiplatelet therapy is standard treatment in patients with acute coronary syndromes (ACS) and/or undergoing percutaneous coronary interventions (PCI). Clopidogrel, an adenosine diphosphate receptor (P2Y12) blocker, has been used as a gold standard (alone orinassociation with aspirin) to prevent vascular complications in atherothrombotic patients undergoing PCI, and as a long-term prevention of cardiovascular events (Campo et al., 2011). Clopidogrel is subject to efflux via P-glycoprotein (encoded by the ABCB1 gen, also known as MDR1). The clinical outcome of Clopidogrel may be influenced by both genetic and non-genetic factors. Among genetic factors, the ABCB1 polymorphisms, particularly C3435T, may be associated with altered drug metabolism, efficacy and clinical outcome (Mega et al., 2010). The aim of this study is to evaluate the influence of ABCB1 C3435T genetic polymorphism on clinical cardiovascular outcomes in coronary artery disease patients on Clopidogrel treatment.

Materials and methods

The study included a total of 227 subjects, of which 107 healthy volunteers (76 men and 31 women) and 120 patients diagnosed with coronary artery disease on treatment with Clopidogrel from the Special Hospital for Surgical Diseases "Filip II". The genotyping of both the control group and the patient group was performed with Real-Time PCR based on the allelic discrimination method using TaqMan SNP genotyping assay for C3435T (rs1045642 assay ID C_7586657_20) according to the guidelines of the manufacturer (Life Technologies, USA). Statistical analysis was performed using SPSS software (v. 22). The genotype distributions were assessed for the Hardy-Weinberg equilibrium (HWE) with $\chi 2$ test using an online calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).Odds ratios (OR) were calculated with 95% confidence interval limits (95% CI). The level of statistical significance was defined as $p \leq 0.05$.

Results and discussion

The distributions of the ABCB1 allele, genotype and haplotype frequencies of C1236T, G2677T/Aand C3435T genetic variations in ABCB1 gene for the Macedonian healthy population (control group) have been determined. According to our results, the frequency of the wild-type allele for C3435T (51%) in our study is similar to the general frequency reported for Caucasians of European descendant, but differs from that of Asian and African population. Allelic frequencies in exon 26 were 51.4% for C allele and 48.6% for mutant T allele, whereas the observed genotype frequencies were 25.2% for 3435CC, 52.3% for 3435CT and 22.5% for 3435TT (Naumovska et al., 2014). The determination of distribution of the ABCB1 allele, genotype and haplotype frequency of the C3435T genetic variation in ABCB1 gene in the patient group is in progress.

In recent years, there are vast numbers of published data that report on evidence for the influence of SNPs in the ABCB1 gene on P-gp function. These polymorphic

* ana.vavlukis@gmail.com

variants are potential determinants of inter-individual variability in drug response and potential for a negative clinical outcome. A meta-analysis of twelve studies (four involved platelet activity and ten involved clinical outcomes) indicated that the ABCB1 C3435T polymorphism might be a risk factor for early major adverse cardiovascular events (MACE) in patients on clopidogrel, and that TT homozygotes decreased the outcome of bleeding compared with CC homozygotes. The four platelet activity studies resulted in an association between high platelet activity and the ABCB1 C3435T polymorphism that was not statistically significant. The long-term MACE had no significant association with ABCB1 C3435T polymorphism. Three studies demonstrated that significant elevated risk of early MACE was associated with T allele, TT homozygote and CT + TT dominant genetic model. The ABCB1 C3435T polymorphism was unrelated to the rate of myocardial infarction, ischemic stroke and all-cause mortality. Seven cohort studies reported that stent thrombosis was not associated with ABCB1 C3435T polymorphism in all genotype models. The comparison of TT vs. CC was associated with a significant reduction in the outcome of bleeding in five cohort studies (Su et al., 2012). Another meta-analysis of six studies with 10.153 subjects failed to show an association between the ABCB1 C3435T polymorphism and the risk of long-term adverse clinical events in clopidogrel treated patients. The association of the C3435T polymorphism with risk of overall recurrent ischemic events and stent thrombosis in clopidogrel treated patients was not statistically significant for all genetic models. However, a significant association was identified between the TT homozygotes and risk of short-term recurrent ischemic events (Luo et al., 2012).Another study found no association of ABCB1 C3435T genotype with clopidogrel response or risk of stent thrombosis in patients undergoing coronary stenting. DNA samples from 1524 clopidogrel-treated patients undergoing PCI were genotyped, ADP – induced platelet aggregation was assessed and the clinical impact of the genetic variant was investigated by comparison of genotype frequencies in a registry of 66 cases with definite drug – eluting stent thrombosis versus an ST-free control cohort (n = 1408). The aggregation did not differ across genotype groups, and platelet aggregation values were similar and numerically lower in homozygous T-allele carriers compared to the remaining patients. The genotype distribution did not differ between case subjects and control subjects. Among the 66 ST case subjects, 19 were carriers of the ABCB1 3435TT genotype, which was not significantly different from the rate of TT carriers in the control group (Jaitner et al., 2012). In contrast to the previous, the Genetic Variants in ABCB1, CYP2C19 and Cardiovascular Outcomes Following Treatment with Clopidogrel and Prasugrel study showed that the C3435T genotype was significantly associated with risk of adverse cardiovascular events. 2932 patients with an acute coronary syndrome (ACS) in the TRITON-TIMI 38 treated with clopidogrel were genotyped. Among the ACS patients, ABCB1 C3435T genotype was significantly associated with risk of the primary endpoint of cardiovascular death, MI or stroke. TT homozygotes showed a 72% increased risk of the primary endpoint as compared with CT/CC individuals when evaluated through 15 months. The rates of stent thrombosis did not differ between the TT homozygotes and CT/CC patients (Mega et al., 2010).

Conclusion

ABCB1 C3435T SNP has been associated with an increased P-glycoprotein expression, which theoretically should lead to decreased absorption, lower platelet inhibition and an increased rate of ischemic event occurrence in patients treated with clopidogrel. However, controversy exists regarding whether the C3435T variant influences clopidogrel associated clinical outcomes. The ongoing ABCB1 C3435T genotyping study of patients with coronary artery disease is expected to determine the influence of this variant on clopidogrel associated clinical outcome in R. Macedonia.

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Macedonian pharmaceutical bulletin, **62** (suppl) 647 - 648 (2016)
ISSN 1409 - 8695
UDC: 615.272.4.074
Short communication

Formulation development of self-microemulsifying system containing Atorvastatin

Andrej Slavkovski*, Maja Simonoska Crcarevska, Marija Glavas Dodov

Institute of Pharmaceutical Technology, Faculty of Pharmacy, Ss. Cyril and Methodius University, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Atorvastatin (ATS) is an important and efficacious member of the statins drug class in treating dyslipidemia and coronary heart disease. It works by inhibiting 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase that reduces hepatocyte cholesterol levels which in turn causes up-regulation of low-density lipoprotein (LDL) receptors and increased clearance of LDL-cholesterol from the plasma. ATS is a poorly water-soluble BCS class II drug, which is highly lipophilic (partition coefficient [log P (octanol/water)] of 6.36) in nature (Oliveira et al., 2013). Its low oral bioavailability (~12%) is attributed to its poor aqueous solubility, pre-systemic metabolism in GI mucosa and efflux by P-gp (Khan et al., 2016).

To overcome these problems, various solubilization strategies have been proposed in the literature (Ali et al., 2013). One such more successful technique is formulation of spontaneous or self-emulsifying drug delivery system. Self-emulsifying drug delivery systems have been categorized as, self-microemulsifying (SMEDDS) and self-nanoemulsifying drug delivery systems (SNEDDS) depending upon the droplet size produced after dispersion.

The SMEDDS are preconcentrates of microemulsion, which form a microemulsion upon contacting water. These are anhydrous homogeneous mixtures of oil, surfactant and/or cosurfactant, and drug which when come in contact with aqueous medium (GI fluids) form a o/w (oil-in-water) microemulsion with little agitation (provided by peristaltic movement of GIT). This spontaneous formation of a fine emulsion in the GIT presents the drug in a solubilized form, and the small droplet size provides a large interfacial surface area for drug absorption (Kishore et al., 2015).

In light of above said facts, the aim of the present study was to establish the solubility of ATS in aqueous systems using self-microemulsification technique. ATS containing SMEDDS were developed using olive oil as oil phase, PEG 400 as surfactant and Tween 20 as co-surfactant screened from the solubility studies. Mixture simplex lattice design was applied for determination of the optimal formulation where mean droplet size, size distribution and optical clarity were followed as responses.

Materials and methods

Materials

Olive oil was supplied from Fluka Analytical, Spain. Tween 20 (polysorbate 20) was purchased from Merck, Germany. PEG 400 (polyethylene glycol 400) was obtained from Merck, Germany. Atorvastatin calcium (ATS; Form VII, D_{50} 1.4 μm) was kindly donated by Alkaloid, Macedonia. All the other chemicals and reagents were of the highest purity grade commercially available and used as received.

Solubility studies

The solubility determination of ATS in various liquid lipids (olive oil, mineral oil, peanut oil, sesame oil, oleic acid, castor oil), surfactants (Tween 20, Tween 60, Tween 80, Span 20, Span 80) and co-surfactants (PEG 200, PEG 400, propylene glycol, ethanol, isopropanol) was performed by the test tube method. Excess ATS (250 mg) was added into 1 g of each vehicle followed by vortex mixing for 30 s. Mixtures were equilibrated for 72 h at 37 ˚C on a controlled shaker water bath (Haake SWB 20, Germany). Afterwards, mixtures were centrifuged (4000 rpm, 45 min; Tehtnica, Centric 322B, Slovenia). Solubility of ATS in different vehicles was determined visually. Only sam-

* slavkovski.andrej@gmail.com

ples where ATS was completely dissolved were used for preparation of SMEDDS. The experiments were conducted in triplicate.

Preparation of SMEDDS

Based on the results obtained from solubility studies, olive oil, Tween 20 and PEG 400 were selected for preparation of ATS-SMEDDS (ATS 20 mg). Mixture simplex lattice design (Design-Expert V8 trial, Stat-Ease, Inc., Minneapolis, USA) was applied for determination of their optimal quantities. Mixture components in total of 100% (Smix = 0.5 g) were varied. Low and high levels of studied variables were: variable A, olive oil - 10 and 50%; variable B, Tween 20 – 10 and 50% and variable C, PEG 400 - 40 and 80%. A total of 13 experiments were carried out. Mean droplet size, size distribution and optical clarity were followed as responses. Optimization was carried out in terms of minimal values of followed responses. In brief, designed Smix`s with ATS were prepared in test tubes and stirred for 24 h (200 rpm, 37 ˚C; Variomag, USA). Afterwards, the prepared mixtures were added into 300 mL of 0.1 M HCl in a glass beakers maintained at 37 ˚C and stirred at 300 rpm until a transparent solution was formed.

Characterization of SMEDDS

Mean droplet size (D_{50}) and size distribution (expressed as SPAN factor value) of the obtained SMEDDS were assessed by laser diffractometry using Mastersizer 2000, Hydro 2000S, Malvern Instruments Ltd., UK.

Samples of freshly prepared SMEDD formulations were investigated for optical clarity spectrophotometrically (400 nm; UV/vis spectrophotometer, Perkin Elmer, Lambda 16, USA). The lower absorbance indicated higher optical clarity of the systems.

The ATS content present in prepared formulations was assessed by dissolving 1ml of the liquid SMEDD formulation in methanol. After suitable dilutions with methanol, drug content was assayed at 246 nm (UV/vis spectrophotometer, Perkin Elmer, Lambda 16, USA).

Results and discussion

Solubility studies were carried out in order to identify the suitable vehicles for the formulation of SMEDDS. Olive oil, Tween 20 and PEG 400 were selected as oil phase, surfactant and co-surfactant based on their highest solubility towards ATS.

Results from experimental design studies pointed that D_{50} was in range of 0.61 µm to 1.212 µm and SPAN factor from 1.072 to 2.958, with unimodal particle size distribution. The absorbance related to optical clarity of the prepared formulations was in range of 0.119 to 0.455.

A special cubic model for D_{50} and reduced cubic model for SPAN factor and optical clarity was applied. Mathematical models for influence of selected variables upon followed responses were created and significant variables were identified. Variables BC, AC, AB and ABC were significant for D_{50}. For SPAN factor value and optical clarity, variables BC, AC, AB and AC*(A-C) and BC*(B-C) and AC*(A-C) were significant factors, respectively.

Results from experimental design studies were used for determination of optimal formulation; sample prepared with 15.44% olive oil, 19.48% Tween 20 and 65.08% PEG 400 with predicted responses of 0.78 for D_{50}, 1.14 for SPAN factor and 0.18 for absorbance with the desirability value of 0.831. Cross validation of the model was carried out and satisfactory results for percent of relative error (less than 10%) of the predicted and experimental values were obtained.

The ATS content in prepared SMEDDS was found to be in the range of 94–99%. The data inferred good drug-loading with low standard deviation indicating the uniformity of drug content.

Optimized liquid formulation of ATS was further spray-dried in order to obtain solid SMEDDS.

Conclusion

Liquid SMEDDS of ATS were composed according to the solubility of drug in mixtures of oil phase, surfactant and co-surfactant systems. Optimal formulation was selected based on results from the experimental design studies. Further investigations will be focused on characterization of solid SMEDDS in order to confirm the potential of the designed system for solving the problem of formulating drugs with low aqueous solubility and poor systemic bioavailability.

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Macedonian pharmaceutical bulletin, **62** (suppl) 649 - 650 (2016)
ISSN 1409 - 8695
UDC: 615. 451.23
Short communication

A topical w/o/w multiple emulsions containing resveratrol: formulation and characterization

Radmila Stanojkovska*, Maja Simonoska Crcarevska, Marija Glavas Dodov

Institute of Pharmaceutical Technology, Faculty of pharmacy, "Ss. Cyril & Methodius" University, Majka Teresa 47, 1000 Skopje, Republic of Macedonia

Introduction

Multiple emulsions are complex polydispersed systems where both oil in water (o/w) and water in oil (w/o) emulsion exists simultaneously in a single system, stabilized by lipophillic and hydrophilic surfactants, respectively. The ratio of these surfactants is critical factor for achieving stable vesicular system (Kumar et al., 2012). Multiple emulsions have been formulated for controlled/sustained or targeted drug delivery, taste masking, enzyme immobilization, as well as cosmetic vehicles for skin care products.

Water/oil/water (w/o/w) multiple emulsions consist of dispersed oil globules containing smaller aqueous droplets; each inner aqueous droplet is separated from the outer aqueous phase by an oil phase layer which acts as a liquid membrane. Therefore, w/o/w multiple emulsions have many advantages over conventional emulsions such as: relatively high entrapment capacity for water-soluble agents, protection of substances that may undergo degradation, ability to introduce incompatible substances in two aqueous compartments of the same system and prolonged release rate of incorporated active agent (Mahmood and Akhtar, 2013). The release properties and stability of w/o/w multiple emulsions are influenced by different factors, such as surfactants type and concentration, method of preparation, and some physical properties of the designed vesicular system (globule size, viscosity, conductivity, phase volume ratio, etc) (Vasiljevic et al., 2009).

In our preliminary studies, different factors (surfactants concentration, phase volume ratio, preparation procedure) were investigated, and optimized formulation and process parameters were selected for further development of w/o/w multiple emulsion system suitable for topical delivery of resveratrol (RSV). RSV, an antioxidant polyphenol from red wine, has been the subject of intense interest in recent years due to a range of unique anti-aging properties (Baxter, 2008). Therefore, the purposes of this research were to develop w/o/w multiple emulsion formulation using Brij® 93 and Tween® 20 as surfactants with resveratrol as active agent and to evaluate the characteristic properties and stability of the designed system.

Materials and methods

Materials

Resveratrol (RSV) 2% aqueous solution was kindly donated from ACTIChem., France. Polyoxyethilene oleyl ether (Brij® 93) and polyoxyethilene sorbitan monolaurate (Tween® 20) were obtained from ICI, USA. Liquid paraffin was supplied from Merck KGaA, Germany. All other chemicals and reagent were of analytical grade and used as received.

Preparation of multiple emulsions

Multiple emulsions were prepared by a two-step emulsification process. Briefly, for primary emulsification, 3 g of aqueous phase and 1 g of oil phase (liquid paraffin and Brij 93, mass ratio = 1:0.25) were preheated at 38 ˚C. Afterwards, the aqueous phase was gradually added to the oil phase using high-shear rotor-stator homogenizer (2.5 min, 9500 rpm; Ultra-Turrax® T25, Ika-Werke, Germany). Homogenization was continued for additional 7.5 min. For secondary emulsification, prepared w/o emulsion was emulsified with 6 g of external aqueous phase containing 1% of Tween 20 under the same conditions. Three different formulations of RSV loaded (1.5%) w/o/w emulsions were prepared (sample 1 - RSV was incorporated into the inner aqueous phase (100%), sample 2 – 75% of RSV was

* stanojkovskaradmila@yahoo.com

incorporated into the inner vs. 25% in outer aqueous phase) and sample 3 – RSV was equally divided into two aqueous compartments (50:50%)). Blank sample was prepared for comparison.

Characterization of multiple emulsions with resveratrol

Microscopic analysis was conducted in order to gain information about the multiple structure of the prepared emulsions. An optical microscope (OPTIKA® B-352A, OPTIKA SRL, Italy) with camera (AIPTEK™ 1080p HD, Aiptek International GmbH, Germany) was used throughout the study. The droplet size was measured using Mastersizer 2000 (Malvern instruments, UK) equipped with Hydro 2000S, Malvern Instruments Ltd., UK.

In vitro RSV release studies were carried out using simple dissolution cells with semipermeable membrane (Visking tube from regenerated cellulose, thickness 0.9 nm, pores size 1.52-2 nm; Serva Feinbiochemica GmbH, Germany). Briefly, 2 g of each formulation was added to donor compartment and membrane was tied on the bottom end of the tube. The tube was dipped into vessel containing 40 ml of water and was stirred at 100 rpm on a magnetic stirrer and maintained at 32 ˚C. Aliquots of 5 ml were collected from receiving chamber at predetermined time intervals and the RSV content was determined UV spectophotometrically (UV/VIS Spectrophotometer, Perkin Elmer Lambda 16, USA) at 324 nm.

Stability studies

The emulsions were monitored for consistency, color, homogeneity, phase separation and particle size during the storage period of three months at 5 ± 3 °C. Conductometric analysis was performed after the preparation of the samples and after three months stability period. Conductivity of the emulsions was measured directly using conductivity meter SevenCompact™ Conductivity S230 (METTLER TOLEDO, Switzerland) at 25 °C ± 2 °C.

Results and discussion

Immediately after preparation, the w/o/w emulsions were apparently brownish and homogenous viscous liquids. The samples do not show any changes in appearance and homogeneity during the first 2 months of stability studied period. However, physical destabilization/flocculation was observed at the end of third month.

Microscopic analysis revealed the multiple character of the prepared samples with droplets containing a large number of small internal droplets (type A multiple emulsions) (Okushima et al., 2004). Blank sample had a mean size of 1.82 μm and SPAN factor of 1.45, with unimodal narrow size distribution. Mean droplet size of RSV loaded emulsions was 2.35, 1.76 and 2.38 μm and SPAN factor 1.45, 1.77 and 1.37 for samples 1, 2 and 3, respectively. No significant differences in particle size were observed during the first two months of stability studied period. After three months, mean droplet size was 0.21, 0.69 and 0.46 μm for sample 1, 2 and 3, respectively, with bimodal particle size distribution indicating physical destabilization of the prepared systems probably due to the osmotic flow of water from internal to the external phase thus leading to shrinkage of the internal water droplets. Electrical conductivity of the freshly prepared samples was 48.8 – 54.2 μS/cm. At the end of stability studied period only slight increase in the conductivity values was observed most likely due to coalescence of the internal and aqueous phases (Mahmood and Akhtar, 2013), but however no significant differences in conductivity were determined.

RSV release from the prepared samples was 50.13, 51.86 and 85.42% during the investigated period of 24 h for samples 1, 2 and 3, respectively. The rate of RSV release followed non-zero order kinetics ($R > 0.997$) with the release exponent ranging between 0.1 and 0.5.

Conclusion

Resveratrol (1.5%) loaded w/o/w multiple emulsions containing low concentration of surfactants were prepared and characterized with respect to their size, release properties and stability. Obtained results suggested that formulation prepared with equally divided amount of resveratrol into the two aqueous compartments could be explored as a skin rejuvenating candidate. Further studies will be focused on formulation modification in order to achieve long-term stability.

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Macedonian pharmaceutical bulletin, **62** (suppl) 651 - 652 (2016)
ISSN 1409 - 8695
UDC: 615.37
Short communication

Probiotics and immunological disorders

Spase Stojanov*, Katarina Smilkov

Faculty of Medical Sciences, Goce Delcev University, Štip, R. Macedonia,
"Krste Misirkov" no.10-A, 2000 Stip, R. Macedonia

Introduction

Probiotics are defined as live microorganisms that have positive health effects on the host. There are many strains of probiotics that can confer health benefits, but the most prominent species are *Lactobacillus* and *Bifidobacterium*. Numerous evidences indicate that selected probiotic strains can provide health benefits to the human host. Up to date, many clinical trials investigated the medical effects of these bacteria, particularly the positive effects to the gastrointestinal (GI) system. Moreover, studies have shown that probiotics exert beneficial effects to the immune system, the urogenital system, in diabetes, oral health etc. (Charalampopoulos and Rastall, 2009).

The immune system, being highly adaptable defense system, functions in preserving the integrity of the organism by eliminating all elements perceived as foreign. Several in vitro and in vivo studies have shown that specific strains of probiotics are able to modulate the functioning of the immune system, stimulate the immune function to protect against infectious diseases and different types of cancer and regulate over expressed immune responses associated with immune inflammatory disorders such as allergy (Gill and Guarner, 2004). In addition, studies presented beneficial effects in atopic diseases, including atopic eczema, allergic rhinitis and asthma.

The purpose of this review was to explore among the reported effects of different probiotic strains to the immune system, specifically in allergy.

Effects of probiotics in asthma and rhinitis

In one of the study with 41 asthmatic children (age 6-12 years) a decrease of the inflammatory cytokine, TNF-α and Th2 cytokine IL-10 and IL-13 was shown. Peripheral blood mononuclear cells (PBMCs) were collected from the patients assigned to the probiotic-treated groups prior to and following the treatment and were then incubated with phytohemagglutinin, *Dermatophagoides pteronyssinus*, supplemented with *Lactobacillus gasseri* for 72h. Production of the cytokines in the culture supernatants was assayed using enzyme-linked immune-sorbent assay (ELISA). The results of the study showed that the patients who took *L. gasseri* capsules for 8 weeks showed significant decrease in the cytokine level compared to the control group (Chen, et al., 2010).

Another study examined the effect of *Lactobacillus casei* in the treatment of asthma and allergic rhinitis. A group of 187 asthmatic children between the age 2 and 5 years were tested for period of 12 months. This study presented that the milk, fermented with *Lactobacillus casei* didn't show significant difference in the health status of asthmatic children compared to the control group. However, consumption of *Lactobacillus casei* fermented milk resulted in improvement in children with allergic rhinitis, i.e the number of rhinitis episodes was lower in the children treated with probiotics compared to the control group (Giovannini et al., 2007).

Effects of probiotics in allergy animal models

Animal studies concerning effects of probiotics in allergy also emerge, presenting a deeper understanding of the effects of probiotics. For example, one study examined the potential of 28 strains of probiotics in prophylaxis of peanut-induced allergy in mouse model. Groups of 8 mice were orally exposed to PBS (control) or peanut extract (PE) plus cholera toxin (CT). Oral exposure was performed on days 0, 1, 2, 10, 17 and 24. After a 6-week pro-

* spase.151134@student.ugd.edu.mk

biotic treatment with each strain and a 4-week oral exposure regimen to PBS or PE with CT, the IgE specific for PE was measured. The results showed that Lactobacillus plantarum increases the IgE in the serum, but *Lactobacillus salivarius* decreases the IgE for PE. In addition *Lactobacillus casei Shirota* didn't show any effect to the IgE for PE, but the measured IL-4 and IL-5 levels were lowered by just using these strains. Again, L. plantarum increases the IL-4 level but didn't show any effect on IL-5 and L. salivarius showed slightly decreased level of the both cytokines (Meijerink et al., 2012).

Another study in mouse model of asthma studied the effect of *Lactobacillus rhamnosus*, using clinical evaluations in vivo, bronchoalveolar lavage fluid analysis, serum IgG analysis, cytokine and lymphocyte proliferation assays. The mice were sensitized by intra peritoneal administration of 10 µg ovalbumin with alum mixture. Treg cells, isolated from the spleen were analyzed for $CD4^+$ $CD25^+$ $Foxp3^+$ expression using flow citometry. This study presented that *Lactobacillus rhamnosus* treatment led to a significant increase in $CD4^+CD25^+Foxp3^+$ Treg cells, compared to the percentage in positive control mice, suggesting that *Lactobacillus rhamnosus* induced attenuation of allergic responses in the mouse model of asthma is associated with an increased $CD4^+CD25^+Foxp3^+$ Treg cell population (Jang et al., 2012).

Conclusion

Many studies are trying to unveil the mechanisms of probiotic involvement and their beneficial effects to the health. Animal studies and clinical trials in different populations regarding different medical conditions, present effects that are somewhat controversial. In this context, as our literature research resulted, many of the evidences regarding immunological (allergic) diseases present weak or minimal effects.

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Macedonian pharmaceutical bulletin, **62** (suppl) 653 - 654 (2016)
ISSN 1409 - 8695
UDC: 616-008.83
Short communication

The functions of sialic acid and its polymers and associated diseases

Sofija Gicheva*, Marija Hiljadnikova Bajro, Tatjana Kadifkova Panovska

Faculty of Pharmacy, Institute of Applied Biochemistry, Department of Clinical Biochemistry, Ss. Cyril and Methodius University, Majka Tereza 47, Skopje, Republic of Macedonia

Introduction

The sialic acid is involved in many biological processes, related to the non-reducing terminal end of glycolipides, glycoproteins and other glycoconjugates. Most frequently, in glycoconjugate complexes, the sialic acid is found in a monomeric form.

Sialic acid is often found as a polymerized structure. Sialic monomers possess the ability to bond with one another and form dimers, oligomers and polysialic acids. In particular, polysialic acids are found to be involved in neural adhesion, more precisely, they regulate the activity of the molecules that control neural adhesion.

The group of sialic acids contains about 50 derivates of *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid and deaminoneuraminic acid. Methylation, sulfation, acetylation and interactions between monomers of these three substances form the large group of sialic acids.

Polysialic acids are mainly involved in changes of many functions of the nervous system. Neural cell adhesion molecule is the most studied polysialated protein in which the polysialic acid contains α2-8 bonded N-acetylneuraminic acid. This protein is connected to the cell membrane with glycosyl phosphatidylinositol or it can be present as a transmembrane protein involved in cell signalization. Polysialic acids are present in the adult brain in the hypocampus, thalamus, amygdale, subventricular zone and prefrontal cortex, to be precise, in the parts where neural plasticity, neural generation and remodeling of neural connections are ongoing. It is more abundant in embryonic brain tissue.

The processes that polysialic acids are involved include neural cell migration, axonal guidance, myelination, fasciculation and plasticity of the nervous system. The mechanism of these actions is the anti-adhesive effect of polysialic acids in cell-cell and cell-matrix interactions. The anti-adhesive effect on is a results of the polyanionic nature of these molecules, that generate a large negative electric field (Sato and Kijatima, 2013).

Clinical implication

Schizophrenia is a psychiatric disorder with multiple factors contributing to its pathogenesis. Some reports suggest that polysialic acids are involved in schizophrenia and other related psychiatric disorders. The background of this thesis lays in the gene that codes for an enzyme called alpha-2,8-sialyltransferase. Biochemical analysis suggests that the activity of this gene is decreased in schizophrenic patients, thus resulting with impairment of the quality and quantity of polysialic acids in the brain (Sato and Kijatima, 2013). The level of sialylation of the neural cell adhesion molecule is decreased as well.

Many cancer cells express polysialic acids on their cell surfaces. This results in attack to these cells by many molecules including anti-sialic antibodies (Sato and Kijatima, 2013). Furthermore, because of its anti-adhesive effect, polysialic acids stimulate metastasis of the disease.

Mono/di/oligosialic acids are considered as parts of other pathological changes in the human organism. Many bacterial toxins such as toxins from cholera, tetanus and pertussis, as well as various virus types bind to sialylated glycoconjugates. An example of this is the binding of influenza virus with sialic acid containing glycans. The mechanism of action is based on the activity of influenza's most important glycoproteins: hemagglutinine and neuraminidase. Both glycoproteins recognize and bind to the the sialylated glycans of the cell membrane. First, the virus replication occurs, and then the activity of neuraminidase begins stimulating dissociation of the sialic acids from the

* g.sofija28@gmail.com

glycan and subsequent increased permeability of the cell membrane for the virus (Gamblin and Skehel, 2010).

Sialic acids are also found to be involved in nonalcoholic fatty liver diease (Zhenya et al., 2016). The concentration of the sialic acids in the serum increases when nonalcoholic fatty liver disease occurs in non obese patients. Three possible mechanisms might underline the association between sialic acids and the disease. Firstly, nonalcoholic fatty liver disease patients are insulin resistant. The mechanisms that cause insulin resistance also causes elevated sialation of the glycolipides and glycoproteins in the human serum and secondly, the reason for a higher concentration of sialic acids in the serum of these patients can be the oxidative stress. Oxidative stress is an inevitable part of this disease, damaging the liver, and producing reactive oxygen species that interact with the terminal nonreducing parts of the serum glycoconjugates. This results with dissociation of the sialic acids in the human serum. Finally, sialic acids can be part of proteins produced by the liver as a response to the disease, called acute phase proteins. Sialic acids are involved in other inflammatory processes such as cardiovascular diseases.

Sialic acids are bioindicators of diabetes mellitus. Their presence is greater in type 2 diabetes patients suffering from diabetic nephropathy. Some of the sialylated glycoproteins in the human serum are called acute phase reactants, because their concentration increases during inflammation. In type two diabetes mellitus, there is a stage of cytokine induced-acute phase response which leads to elevated sialic acid concentration in the serum. In diabetic nephropathy, there is damage to the vascular endothelial cells of the kidney, which is accompanied with inflammation, resulting with elevated concentration of sialic acids in the serum. It is important to note that the concentration of these substances is greater when diabetic nephropathy occurs, in comparison to cases with uncomplicated diabetes (Varma et al., 2016).

Conclusion

Sialic acids play a great roll in the ethiopathology and the diagnosing of many pathological changes in the human organism. They are considered one of the indicators in diagnosing diabetic nephropathy in patients suffering from type 2 diabetes mellitus. Elevated levels of sialic acids in the human serum are associated with non obese fatty liver disease and indicate a potential risk for its development. This means that sialic acids play a role in the metabolic changes that result with this disease, and as such are used in its diagnosis. In infections on the other hand, sialic acids are used indirectly in therapy. Identification of the mechanisms of binding of the viruses to the cells, enables creation of antiviral medications for treatment of many infections caused by resistant viruses and bacteria. Various types of cancer show elevated concentration of polysialic acids, making the diagnosis of the disease easier. Finally, in vivo and in vitro analyses show that enzymatic activity of alpha 2,8-sialyltransferase is decreased in patients with schizophrenia, resulting with impairment of the quality and quantity of the polisyalic acids.

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Macedonian pharmaceutical bulletin, **62** (suppl) 655 - 656 (2016)
ISSN 1409 - 8695
UDC: 615.272
Short communication

Biosimilars in clinical use

Martina Keshkjec*, Martina Miloshevska*, Aleksandra Grozdanova

Faculty of Pharmacy, University "Ss Cyril and Methodius",
Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Biologic medicines are mainly protein-based medicines derived from living organisms or cells cultured in a laboratory. Those medicinal products are larger and more complex than conventional (non-biological) drugs. Biologicals can be hormones (growth hormones, insulin, erythropoietin), enzymes, monoclonal antibodies, blood products, immunological medicinal products (sera and vaccines) or advanced technology products such as gene and cell therapy (Consensus Information Paper, 2013). The structure–function relationships of biologicals are very sensitive, because modifications of tertiary or quaternary configuration may affect safety, purity and potency. They have a complex mechanism of action by targeting more selective to the cell or molecule that is responsible for the development of certain diseases.

The paradigm for biologicals "the process is the product" still is viable today. Their manufacturing is challenging process, where critical culturing and purification steps is required to produce a consistent, high quality active ingredient. Gene manipulation, fermentation and purification are used with high level of expertise in order to guarantee the safety and efficacy of the final product. Biologicals are usually more difficult to characterize than chemically derived medicinal products. Moreover, post-translational modifications such as glycosylation, oxidation and deamination can be significantly altered by changes (Geigert, 2013). Even minor changes in manufacturing process can cause significant changes in efficacy or immunogenicity.

The use of modern technologies like biotechnology, DNA recombinant techniques etc. results with the first recombinant biological medicine, human insulin (Humulin), approved for therapeutic use in 1982 from FDA. The era of recombinant monoclonal antibodies started in 1995. Since then, the number of biologicals approved for human use, including monoclonal antibodies (mAbs), has increased considerably.

These medicines have advanced the treatment of many chronic and life-threatening diseases. Even though biologicals are much more expensive than chemical entities, health care providers and payers considered that they are worth their cost - as long as the appropriate patients receive them and achieve the desired clinical outcomes. Patients for whom biologicals are a good choice include those who have failed on conventional therapies or for whom no other options exist. The predictions are that by 2017 global biological pharmaceutical market will amounts to about $220 billion (Dolinar and Reilly, 2013).

On the other hand, after the first biological medicines were launched, exclusivity rights on these drugs have begun to expire, and thus present an opportunity for pharmaceutical companies to consider developing biosimilar medicines of these products. In general, this means that the biological reference medicine must have been authorized for at least 10 years before a biosimilar can be made available by another company.

What are biosimilars?

Biosimilars are not generic versions of biologics. "A biosimilar is a biological medicinal product that contains a version of the active substance of an already authorized original biological medicinal product (called reference medicinal product). Similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise needs to be established." This includes physicochemical and biological characterization and requires knowledge on how to interpret any differences be-

* martinakeskec@hotmail.com
m.miloshevska@gmail.com

tween a biosimilar and its reference medicinal product (European Medicines Agency, 2014). Actually, the changing outlook for biosimilars comes at a time when the global pharmaceutical market is feeling the combined impact of two key events: a period of unprecedented patent expirations on many of the world's largest pharmaceutical products, and a financial crisis that has required healthcare systems to make significant and sustained cost reductions. Biosimilars may have the potential to increase patient access to potentially valuable therapies at a lower cost (IMS Health, 2011).

Approval and licensing pathways of biosimilars

In 2005, the EMA/ European Commission were the first to implement a well documented legal and regulatory pathway for the approval of biosimilar products that is distinct from the generic pathway. The approval of the first biosimilar product, somatropin (Omnitrope), following the EMA's new approach, was in 2006, followed by erythropoietin (2007) and filgrastim (2008). Five years later, in 2013 the first mAb biosimilar, infliximab, was approved in Europe. Market authorization for biosimilars is issued on "step by step, case by case" basis to demonstrate similarity with respect to structural and functional characterization, in vitro biological assays and pharmacokinetic and pharmacodynamics evaluation, as well as safety, efficacy and immunogenicity studies.

Substitution and interchangeability

Substitution of generic drugs for reference drugs is uncontroversial because the two will be identical if they have demonstrated bioequivalence. Since biological drugs can never be exact copies, the question whether they can be substitutes of original biologics remains unclear (Ebbers and Chamberlain, 2014). These medicinal products are not directly interchangeable. In EU these terms, interchangeability and substitution, are not used as synonyms. (Espin et al., 2011). Interchangeability refers to the prescription of biosimilar in place of the reference product by prescribers, while substitution means that pharmacists are allowed to dispense a biosimilar. EMA does not guarantee interchangeability and established that these aspects are beyond its competence. The decisions on the interchangeability of biosimilars and innovator products rest with the Member States in the EU. The European Generic Association (EGA) reported that more than 12 countries across the EU have introduced rules to avert automatic substitution of innovator biological with biosimilars.

The increased use of this medicine have arisen many questions and there remains an need to continued guidance for substitution from regulatory agencies with regards to safety issues, as well as those for pharmacovigilance and risk- mitigation activities. This is anticipated and will be critical to the further therapeutic use of biosimilars.

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Macedonian pharmaceutical bulletin, **62** (suppl) 657 - 658 (2016)
ISSN 1409 - 8695
UDC: 615.918:582.282.123.4
Short communication

The toxicology of aflatoxins and public awareness

Elisaveta Durolojkova*, Marija Hiljadnikova Bajro and Tatjana Kadifkova Panovska

Faculty of Pharmacy, Institute for Applied Biochemistry, Department of Toxicology, "Ss. Cyril and Methodius" University, Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

The aflatoxins are toxic substances produced as secondary metabolites by the fungus *Aspergillus flavus* and *Aspergillus parasiticus*. Foods in which aflatoxins are commonly found include rice, corn, peanuts, tree nuts, cocoa beans. The aflatoxins are potent toxic, carcinogenic, mutagenic and immunosuppressive agents. Today, there are approximately 16 known types of aflatoxins including: aflatoxin B1, aflatoxin B2, aflatoxin B3, aflatoxin G1, aflatoxin G2, aflatoxin G2a, aflatoxin M1, aflatoxin M2, aflatoxin P and aflatoxin T2. The four major aflatoxins that cause illness in humans and can be found in food are: aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2. The International Agency for Research on Cancer has classified aflatoxin B1 as a group 1 carcinogen (carcinogenic to humans) and aflatoxin M1 as a group 2b carcinogen (carcinogenic to laboratory animals and possibly carcinogenic to humans, respectively). The exposure to aflatoxins and the resulting aflatoxicosis can vary from acute to chronic, and the illness can range from mild to severe. Acute exposure to high doses of aflatoxins can result in aflatoxicosis, having the liver as a target organ, which can lead to serious liver damage. Chronic exposure to sublethal doses of aflatoxins can result in liver cancer, impaired protein formation, impaired blood coagulation and toxic hepatitis.

People who have aflatoxicosis might exhibit the following characteristics:

- Liver damage may be confirmed by jaundice and characteristic yellowing of tissues
- Gall bladder may become swollen
- Immunosuppression may provide an opportunity for secondary infections
- Vitamin K functions may decrease
- High level of aflatoxin B1 – albumin adducts may be present in plasma.

Clinical implication

The most commonly used human samples for aflatoxin analysis are blood and urine. Aflatoxin exposure can be monitored by using biomarkers that detect the presence of aflatoxin metabolites, excreted DNA adducts and blood protein adducts. Aflatoxin - albumin adducts are found in peripheral blood after exposure to aflatoxin B1. Aflatoxin M1 and aflatoxin B1 – DNA adduct (aflatoxin B1 – guanine adduct) can be detected in the urine of people consuming sufficient amounts of aflatoxin B1. Increased levels of serum alkaline phosphatase are a biochemical indicator for aflatoxins toxicity.

Detection methods for aflatoxins in human body samples are radioimmunoassay (RIA), enzyme – linked immunosorbent assay (ELISA), high – performance liquid chromatographic fluorescence detection (HPLCf).

Albumin samples are digested with proteinase K in a phosphate - buffered saline (PBS, pH 7.4) for 15 h at 37°C. Following hydrolysis, bovine serum albumin (BSA) is added to improve precipitation, followed by cold ethanol addition. Samples are kept at -20°C for 2 h, centrifuged at 1500xg for 15 min and the supernatant is diluted with 6% ethanol in PBS. The samples are then loaded into an activated Sep – pak CIS cartridge (Waters, Milford, MA), washed with water, 5% methanol, and the aflatoxins are eluted with 80% methanol. Eluates are dried, reconstituted in PBS containing 1% fetal calf serum (to saturate residual proteinase K activity), and tested with ELISA using aflatoxin – lysine standard (Wild et al., 1990).

A study conducted in sub – Saharan Africa proved that chronic hepatomegaly is common among school – age children in sub – Saharan Africa. This study examined the

* elisaveta.durolojkova@hotmail.com

role of aflatoxin in chronic hepatomegaly. Plasma samples were collected in 2002 and 2004 from 218 children attending two schools in neighboring villages (Mantagini and Yumbuni). Plasma samples were assayed for aflatoxin exposure using the aflatoxin – albumin adduct biomarker (AF – alb). The blood AF – alb levels were determined using ELISA. One negative and three positive controls were analyzed alongside each batch of samples. The limit detection for AF – alb was 3 pg/mg albumin. AF – alb levels were high in children from both schools, but the geometric mean value (95 % confidence interval) in 2002 was significantly higher in Matangini (206.5 pg/mg albumin) than in Yumbuni (73.2 pg/mg). AF – alb levels were also higher in children with firm hepatomegaly (176.6 pg/mg) compared to the control group (79.9 pg/mg). In 2004, AF – alb levels were higher than in 2002 (539.7 vs. 114.5pg/mg), but with no significant difference between the villages or between hepatomegaly and control groups (539.7 vs. 512.6 pg/mg). Exposure to aflatoxin was associated with childhood chronic hepatomegaly in 2002 (Gong et al., 2012).

Aflatoxin is known to cross the placental barrier and exposures *in utero* could influence genomic programming, fetal growth and development. In a study, pregnant Gambian women were examined for aflatoxin exposure at two stages of their pregnancy (early - < 16 weeks; and later - 16 weeks onward), during the rainy (June to October 2009) or dry (November to May 2010) season, using aflatoxin albumin adducts (AF – alb). Plasma samples were analyzed for AF – alb using a competitive ELISA. One negative and three positive controls were analyzed alongside each batch of samples. The limit of detection for AF –alb was 0.6 pg/mg albumin. Mean AF – alb was higher during the dry season than in the rainy season, in both early and later pregnancy, although the difference was strongest in later pregnancy. There was a modest increase in AF – alb in later compared to early pregnancy (geometric mean 41.8 vs. 34.5 pg/mg), but this was restricted to the dry season when exposures were generally higher. The study confirmed that Gambian pregnant women were exposed to aflatoxin throughout the pregnancy, with higher levels in the dry season. Overall, the stage of pregnancy was not significantly associated with AF-alb but season was (Castelino et al., 2014).

Prevention

Considering the danger of human contamination with aflatoxins, prevention is an imperative, as the safest and most economical protection method. The most promising strategy currently being used to reduce contamination of crops with aflatoxin is to introduce non – aflatoxin (biological control) *A. flavus*. The method involves spreading non – aflatoxigenic spores onto the field. It is assumed that addition of the non – aflatoxin producing strain would then allow it to out – compete the wild – type populations and thereby displace the wild – type fungus. This strategy is called a "displacement" strategy. Assuming this is efficient, the resulting treated fields should never have to be treated again, because, then, only the non – aflatoxigenic population of fungi would be present in the fields. Whether or not introduction of biological control strains into the fields is enough to reduce aflatoxin contamination to level required for acceptance of the contaminated food as fit for consumption is still unknown. There is no doubt that biological control strains are able to reduce the size of the populations of aflatoxin – producing strains, but the available data suggest that at most only a four – to five – fold reduction in aflatoxin contamination is achieved (Ehrlich, 2014).

Conclusion

Aflatoxins are mycotoxins contaminating large proportion of the food all around the world. Knowing that aflatoxins are carcinogenic, teratogenic and mutagenic agents and cause different forms of toxicosis (aflatoxicosis), emphasizes the fact that serious efforts are needed towards researching and discovering appropriate strategies for prevention and treatment. The prevention and treatment should be directed towards finding strategies to protect the grains and discovering agents which will be used to protect the human body from the toxic effect of aflatoxins. Awareness should be raised in governments to assemble groups of healthcare workers which will contribute to lowering the aflatoxins exposure, and hopefully their elimination.

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Macedonian pharmaceutical bulletin, **62** (suppl) 659 - 660 (2016)
ISSN 1409 - 8695
UDC: 614.27:006.73
Short communication

World Health Organization standards for ethical and efficient promotion of over-the-counter pharmaceuticals

Aleksandar Derlis*, Rosana Bozhinovska, Elena Todorovska, Monika Mitreska, Renata Slaveska Raicki

Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Advertising and promotion of medicines are among key regulatory functions of the medicines chain. The World Health Organization (WHO) defines promotion as: "all informational and persuasive activities by manufacturers and distributors, the effect of which is to induce the prescription, supply, purchase and/or use of medicinal drugs". In 1989, WHO established ethical criteria for medicines promotion that are still relevant today (WHO, 1988). According to the principles of good practice for promoting medicines to the general public, this function is permitted for pharmaceuticals classified as nonprescription medicines (over-the-counter, OTC), but should not be directed at children as target audience. However, the manufacturers of prescription only medicines are in restrictive position in providing information to patients since the advertising of prescription only medicines to the general public is prohibited. As acknowledge, OTC medicines as part of health coverage are medicines which are approved for use without a medical prescription. These medicines and health-related products from general sale list can be purchased by patients and consumers through pharmacies, and in many countries (including our country), from non-pharmacy retail outlets. In this context, as no healthcare professional is necessarily involved in OTC medicines use it is evident that the nature of their promotion to the general public have a special importance for supporting their appropriate and responsible use by patients and consumers. From all these underlying reasons associated with advertising and promotion of medicines, it is generally accepted that this pharmaceutical function in each country must comply with national health policies and regulations, as well as with standards, guiding principles and ethical criteria. Different mechanisms can be used for regulation of advertising of OTC medicines.

The aim of our study is to present the elements of efficient and ethical promotion of OTC medicines according to the WHO standards, and to demonstrate the special role and benefit of OTC medicines promotion in the public health context. Moreover, the preliminary results of conducted analysis in the domain of OTC medicines promotion on domestic market are also discussed in this context.

Materials and methods

The varieties of OTC medicines promotional materials available on the domestic market for the provision of patient and consumer information are also analyzed. In fact, approximately 30 OTC medicines from retail environment for pain relievers, laxatives, cough and cold from different manufacturer has been included in the study.

Results and discussion

In general, advertising and promotional materials intended to patient and consumer can be available in a variety of forms, including: 1) printed material for example leaflets, posters then, materials in newspapers and magazines or direct mail materials etc.; 2) electronic media advertising form such as websites and on-line material, 3) audio and audiovisual advertising forms (e.g. television, videos or radio commercials); 4) ''other'' forms (e.g. outdoor advertising, promotional text messages, and so on).

WHO describes the purpose of advertising as: "Attract attention, offer choices, and provide limited general information to mass audiences of consumers. It must stimulate

* der.aleksandar@hotmail.com

the interest of prospective buyers in a… product; inform them of what it may do for them… Therefore, advertising should not be overloaded with information to the point that the individual prospective buyer may fail to comprehend it or may even ignore it". Overall, the OTC medicines promotional materials aimed to raise patient and consumer awareness about their condition and, in particular about the possibility for suitable self-medication and self-care. Moreover, these materials should also advice patients and consumer about new products and/or attract their attention on the new indications for use of old one OTC medicines. Another positive benefit of OTC medicines promotional material, but not less important, is that they can lead to reinforce of other forms of communication for example, with health professionals and as a consequence, they should promote and facilitate the rational use of OTC medicines. The obvious example is the warning information on the promotional material stating: "if symptoms persist, consult a health care professional". It is well known that the OTC medicines promotional materials play also important role in patients and consumers decision making process towards selection of medicines, where there is a multiplicity of choice and also direct them to medicines labeling important for safe and appropriate use. From the practical point of view, OTC medicines promotional material may also have a positive effect on patient's compliance. The OTC medicines manufacturers are competing with each other to encourage patients and consumers to use their products.

Basic ethical standards for OTC promotion have to ensure that the information conveyed to patients and consumers is truthful and not misleading. The concept of promotion is not to force people to buy and use an OTC medicine they do not want or need. General ethical criteria for OTC medicine advertising are highlighted in WHO assessment document (WHO, 2000): 1)"While advertisements to the general public should take account of people's legitimate desire for information regarding their health, they should not take undue advantage of people's concern for their health"; 2)"While health education aimed at children is highly desirable, drug advertisements should not be directed at children"; 3)"Advertisements may claim that a drug can cure, prevent, or relieve an ailment only if this can be substantiated"; 4)"They should also indicate, where applicable, appropriate limitations to the use of drugs"; 5) "When lay language is used, the information should be consistent with the approved scientific data sheet or other legally determined scientific basis for approval. Language which brings about fear or distress should not be used".

These criteria constitute general principles for ethical standards which could be adapted by governments to national circumstances as appropriate to their political, economic, cultural, social, educational, scientific and technical situation, laws and regulations, and the level of their health system development. It is worthy to mention that in our country these criteria are being fully met.

Other important dimension of OTC medicines promotion is the mechanism for its regulation. In line with this, it is highly recommended that two systems should be put in place, such as pre-marketing release and post-marketing surveillance system. Actually, the first system formally approved promotional material while, the second one would secure enforcement mechanism.

Patients have a legitimate right to get good quality information, in a well-regulated way, from the reliable source for each OTC medicines.

Conclusion

Patients have a legitimate right to get good quality information, in a well-regulated way, from the reliable source for each OTC medicines. In continuum, they are being conveyed with increasing amounts of OTC medicines promotional materials from different parties and through multiple channels. It is obvious, that some information might be of varying quality and accuracy. The high standards should be applied in OTC medicines promotion. Apart from ethical and effective promotion standards of OTC medicines this regulatory function, alone is not providing detailed and comprehensive information on OTC medicines, but it can convey needed information for responsible self-medication by patients.

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Macedonian pharmaceutical bulletin, **62** (suppl) 661 - 662 (2016)
ISSN 1409 - 8695
UDC: 582.998.16:633.88-156.32
Short communication

The impact factors during proper chamomile drying

Petar Davcev[1]*, Ile Canev[2]

[1]Faculty of Medicine, Department of pharmacy, University "Goce Delcev", St. Marko Cepenkov no.9 2-16, 1200 Stip, Republic of Macedonia
[2]Faculty of Agricultural Science and Food, University of "Ss. Cyril & Methodius, blvd. Aleksandar Makedonski bb, 1000 Skopje, Republic of Macedonia

Introduction

During the harvest of the chamomile, which can be done as monophase, or as dual phase, that in other words means picking up only the flower that has its own purpose or to harvesting completely the stem and the flower together? In the Republic of Macedonia the right conditions are in the second half of May and beginning of June, depending on soil-climate conditions and on larger parcels it is done by machines with special constructed combine harvester or it is done manually.

After the harvest, the chamomile is being dried in special driers which are various, with different capacities for drying and are mainly divided into:

1. Driers on solar energy
2. Driers on solid fuel
3. Driers on oil derivates
4. Driers on electric current
5. Combined driers

1. Principle of work and driers' role in the chamomile drying process

The chamomile as culture is very specific in the drying process and the harvest depends on the drying process itself. It is harvested the whole and is haymaking, or the flower is collected by combine harvester, depending on the market demands it is intended to. The chamomile contains active and hidden moisture, mainly in the harvest phase. When the flower is collected, it contains about 65% moisture that is active and should be lowered to 12% during the drying process, percentage of moisture that allows the chamomile to be stored in various packages, where the drying process continues, and does not harm while is stored, kept, transported or used.

2. Techniques and technologies for chamomile drying

Chamomile can be dried as flower or as whole plant with the stem and the flower together. From thermal aspect, and mechanical laws, especially thermodynamics, the drying is done by thermal energy along with cold and hot air circulation. That is the essence of chamomile drying as a culture, because chamomile contains active and hidden moisture and needs strictly defined principle of regulation by temperature amplitudes and air circulation amplitudes, depending on that, time duration of the drying and the consumed energy we get the price of cost of this working process, and the quality as general (Dimov, 2014).

The driers are placed in Ltd. "Koro Company" in Skopje which is a company main activity that deals with cultivation and processing of medical and aromatic plants. The first drier was with smaller dimensions, 5.8 meters in length, 1.55 meters width and 2 m height, 18 m^3 cover 12 000 m^3 air. The heaters' energy (capacity) was 60 calories. The second drier was with the following dimensions: 5.8 meters in length, 3.4 meters width and 2 m height. The fan was with 26000 m^3 capacity and the heaters had maximum capacity of 160 kg calories.

There is proportion of the dimensions of the drier with thermodynamic laws and there was quality drying necessary for balance of the inflow, entrance air circulation for time unit and entrance energy which are 2 essential factors for drying, and if the driers have different dimensions, also it is necessary to modify the values of the heaters and the fan that are basic parameters.

Materials and methods

Methods - Machines with special constructed combine harvester or it is done manually.

Materials - The term Chamomile actually refers to a

* petar_davcev@hotmail.com

range of different daisy-like plants, which are a member of the Asteraceae family. There are many different species of chamomile, the two most commonly being German chamomile (Marticaria recutita) and Roman chamomile (Chamaemelum nobile). They have been used since Ancient times for their calming and anti-inflammatory properties and each offer their own additional health benefits (Dimov, 2014).

The chamomile stem is procumbent, and the leaves are alternate, bipinnate, finely dissected, and downy to glabrous. The chamomile flowers are held in solitary, terminal flowerheads, rising 8-12 inches above the ground. Chamomile flowers consist of prominent yellow disk flowers and silver-white ray flowers. The flowers are arranged in conical centre (having 18 white rays), receptacle, on which the yellow, tubular florets are placed. Chamomile flowers bloom during the months of June and July. The Chamomile is from from Northeastern Region of Macedonia in Municipality of Rankovce.

Results and discussion

From the obtained results, it can be noticed that different volume of the driers 1 and 2, receive different green mass, which in our case is in the center of attention, the factor of height green mass (layer thickness) that are average 45 centimeters.

During the measurements it was noticed that chamomile layer of 40 cm with three switches had duration for drying of about 24 hours with constantly turned on heaters, and in the case with layer bigger than 45 cm, drier of 2 meters height, the process of drying shall be prolonged to 30 hours, with 4 or 5 switches.

Only the flower was dried which has structure like this: flower is 70% green mass, and 30% flower lattices. We noticed that by each switching, the green mass was damaged and quality lowered. While measuring in both driers, we came to a conclusion that the layer of spread mass should not exceed 40 cm spread on the floor, without any shelves. That conclusion is due to the fact that each harvest has to be done in appropriate weather conditions on temperature above 20 °C with average air humidity of 40%, which are ideal climate conditions for chamomile harvesting.

That was the reason for such measurements and monitoring of this issue and we gained wide knowledge for quality of chamomile production and regime of drying and that the quality itself and economy of the final product depends on it.

Conclusion

Our results are contribution in understanding of this medical plant and its process of drying. Obtained results have a contribution in the pharmaceutical industry and give opportunity for future research. It will be worthwhile to perform further studies in order to confirm these results.

Acknowledgments

The authors would like to acknowledge “Koro” Company Ltd., Blvd. Boris Trajkovski bb, 1000 Skopje

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Macedonian pharmaceutical bulletin, **62** (suppl) 663 - 664 (2016)
ISSN 1409 - 8695
UDC: 615.014.22
Short communication

Development of microsponges as drug delivery carriers: Optimization of formulation variables using sequential experimental strategy

Elena Markova*, Monika Kostovska*, Tanja Kjurkchieva Olumcheva, Marija Glavas Dodov, Maja Simonoska Crcarevska

Institute of Pharmaceutical Technology and Center of pharmaceutical nanotechnology, Faculty of Pharmacy, University of "Ss. Cyril & Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

Quality by design (QbD) principles are implemented into the design and development of microsponges as drug delivery carriers (MDDC) with potential to be incorporated into gel as semi-solid dosage form for topical application. As a stand for identification and optimization of critical material attributes (CMA) and critical process parameters (CPP), MDDC particle size was used as it is critical for their encapsulation efficiency, drug loading capacity and drug release profile (properties related to drug product efficacy and safety). In the previous work of Crcarevska et al. (2015) CMA and CPP were identified using Quality Risk Management (QRM) analysis and hence CPP were further optimized using design of experiments studies. Optimal process conditions for minimal particle size (D10, D50, D90) and minimal distribution (Span factor) were determined to be rotation speed of 24000 rpm and rotor–stator homogenizer as type of stirrer used for evaporation of organic phase for MDDC preparation by double emulsion-solvent-diffusion technique. Amount of chitosan (CTS), ethylcelulose (EC), span 80 (S80), tween 80 (T80), acetone (ACT), dichlormethane (DHM) and internal water phase (W1) were identified as potentially significant CMA. In this work factorial design of experiments (DoE) was applied for rationalization of previously identified potentially significant formulation variables. Afterwards formulation optimization of MDDC loaded with clindamycin hydrochloride was carried out using Central Composite (CC) Response Surface Method (RSM) DoE.

Materials and methods

Materials

EC was purchased from Sigma, USA. CTS (high viscous) was supplied from Aldrich, Island. T80, S80 and DHM were obtained from Merck, Germany; while ACT was purchased from Alkaloid, Macedonia. Clindamycin hydrochloride (CM-HCl) was kindly donated by Higija Farm, Bitola, Macedonia. All other used chemicals and reagents were with analytical grade and were used without any modifications.

Methods

Determination of statistical significant influence of previously identified CMA (Crcarevska et al., 2015) on particle size (D50) and particle size distribution (Span factor) was carried out using 2_{IV}^{7-3} factorial DoE (Design-Expert® V8 trial, Stat-Ease, Inc., Minneapolis, USA). All formulation variables were varied on two levels, with 8 central points. The total of 24 experiments was carried out. Low and high levels of actual values for studied variables were as follows: for factor A: CTS (0.8%) - 0.1 to 0.5 ml, factor B: W1 - 1.5 to 3.5 ml, factor C: ACT - 0.2 to 1 ml, factor D: S80 - 0.03 to 0.13 g, factor E: EC - 0.03 to 0.13 g, factor F: DHM - 6 to 12.5 ml and factor G: T80 - 0.15 to 0.45 g.

In order to optimize formulation variables for preparation of MDDC with desired particle size and distribution, as well as drug content and encapsulation efficiency, formulation factors identified with factorial DoE, were varied in the relevant design space. For this purpose a to-

* elena.salvatore9@yahoo.com,
moni-kostovska@hotmail.com

tal of 40 experiments were created using face centered CC type Min Run Res V - RSM DoE (Design-Expert® V8 trial, Stat-Ease, Inc., Minneapolis, USA). The design matrix was constructed in one block where each studied factor was varied at 5 levels (low and high level of factorial and axial points and center point). Used low and high actual values of studied factors were as follows: factor A1 (W1) - 1.5 to 3.5 ml, factor B1 (ACT) - 0.2 to 1 ml, factor C1 (S80) - 0.03 to 0.13 g, factor D1 (DHM) - 6 to 12.5 ml and factor F1 (CM-HCl) - 0.03 to 0.13 g. CTS (0.8%) and T80 amounts were fixed at 0.1 ml and 0.45 g respectively.

MDDC were prepared using previously optimized double emulsion-solvent diffusion technique and characterized in means of particle size (D10, D50, D90) and particle size distribution (Span factor) (Crcarevska et al., 2015).

Results and discussion

MDDC were prepared by double emulsion solvent diffusion technique using rotor–stator homogenization technique. In the previous work of Crcarevska et al. (2015) formulation and process variables that might have critical influence upon drug product quality were identified. Having in mind number of variables identified a sequential strategy in the further development of MDDC was selected. Process variables were optimized, and hence the current work is related to detailed characterization of influence of formulation variables on MDDC properties. Results from factorial design studies enabled identification of variables with statically significant influence on D50 and Span factor. D50 of prepared MDDC was in a range of 15.255 ± 0.37 μm to 220.816 ± 5.23 μm, while Span factor ranged from 2.612 ± 0.28 to 16.092 ± 0.45. Mathematical models of inverse function of D50 square root and inverse function of Span factor and studied variables were established. One way ANOVA pointed that factors B (W1), C (ACT), D (S80), E (EC), F (DHM) and factors interaction AF (W1*DHM) and AG (CTS*T80) are significant model terms in case of D50. W1 was determined to be statistically significant in case of Span factor.

Based on the obtained results for further studies related to optimization of formulation of MDDC CM-HCl loaded, variables W1, ACT, S80, EC and DHM were selected. Also, amount of CM-HCl was considered as formulation variable that might influence MDDC properties. Results from CC-RSM studies permitted detailed characterization of influence of studied variables on particle size (D10, D50, D90) and particle size distribution (Span factor). D10 of MDDC CM-HCl loaded was in range of 1.375 ± 0. 64 to 64.538 ± 3.22 μm. Mathematical modeling of $\log_{10}$ (D10) resulted with linear model. One way ANOVA pointed that S80 and EC are statistically significant model terms. D50 for MDDC CM-HCl loaded was in range of 6.582 ± 0.85 μm to 338.146 ± 8.72 μm. Reduced quadratic model for square root of D50 was established. Variables W1, S80, EC, S80*EC, $(EC)^2$ and $(CM\text{-}HCl)^2$ were found to be statistically significant in case of D50. D90 was in a range of 53.104 ± 2.35 μm to 1235.82 ± 15.46 μm. Influence of investigated variables on D90 of MDDC CM-HCl loaded could be described by reduced quadratic model. Statically significant model terms were DHM, W1*ACT, W1*DHM, ACT*EC, ACT*CM-HCl, S80*EC, S80*DHM and $(ACT)^2$. Span factor ranged from 1.546 ± 0.37 to 13.841 ± 2.62. Inverse function of square root of Span factor was also modeled by reduced quadratic model. Variables ACT, EC, CM-HCl and interaction terms W1*S0, EC*DHM, DHM*CM-HCl as well as $(S80)^2$ were determined as statistically significant model terms in case of Span factor.

Conclusion

A sequential experimental strategy was applied in the development of MDDC-CM-HCl loaded. Screening DoE (2_{IV}^{7-3} factorial design) was employed for identification and rationalization of significant formulation variables. Detailed characterization of variable influence upon parameters of interest was performed using Central Composite (type Min Run Res V) - Response Surface Method DoE. In the current study results related to particle size and particle size distribution were presented. MDDC-CM-HCl loaded will be further characterized in means of drug content (DC) (mg CM-HCl/g EC ± SD, and encapsulation efficiency (EE%) (percentages of mass of entrapped CM-HCl compared to used CM-HCl for preparation of MDDC (%) ± SD). Obtained results will be used for formulation optimization.

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Macedonian pharmaceutical bulletin, **62** (suppl) 665 - 666 (2016)
ISSN 1409 - 8695
UDC: 614.27
Short communication

Pharmacovigilance practice in community pharmacies

Aleksandar Spirov*, Aleksandra Kapedanovska Nestorvska, Aleksandra Grozdanova, Zoran Sterjev, Zorica Naumovska

Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Pharmacovigilance is the science that allows systematic detecting, gathering, monitoring and evaluation of new data on drug safety and risk/benefit ratio in their use. It is in continuous upswing and it is represented as a control tool which is vital for providing safe, effective and high quality drugs for human use. The specific targets for pharmacovigilance include better patient care and increased safety in the use of drugs, better public health, giving its contribution in the assessment of the benefit, hazard, risk and the effectiveness of the use of medicines, encouraging their safe, rational and efficient use and providing education and clinical practice for pharmacovigilance for the public.

The new pharmacovigilance legislation came into effect in July 2012 and it was the biggest change to the regulation of human medicines in the European Union since 1995. This new legislative had significant implications for applicants, as well as for patients, healthcare professionals and regulators.

The National center for pharmacovigilance in Republic of Macedonia is founded in 1997 in the Institute for preclinical and clinical pharmacology, Medicinal Faculty, Skopje. Since 2015 National PV center is transferred in Macedonian agency for Drugs and Medical Devices (MALMED). This center works in accordance with the National regulative for pharmacovigilance, which has adopted main recommendations from European Union legislation. Since 2002 Macedonia is full member in the Uppsala monitoring center. Although the National center for pharmacovigilance exists for almost twenty years, the awareness for reporting adverse drug reactions (ADR) by the healthcare professionals, especially the community pharmacists is on a minimal level. This problem is even bigger with respect to biosimilars because they are relatively new drugs and very different from the non – biosimilars in terms of generics.

For these reasons we are established a questionnaire that evaluates the main PV questions and accesses the awareness, the knowledge and the methods of application of pharmacovigilance among pharmacists in the community pharmacies (Meher et al., 2015).

Materials and methods

Study design

This is a questionnaire based study.

The study setting

This study is conducted in over 20 community pharmacies in Skopje, Republic of Macedonia.

The study population

This is a non – interventional, but required and useful study which is done among the post graduated pharmacists who work in the community pharmacies. Those who are not willing to participate and those who will not return the questionnaires, are going to be excluded from this study.

The study instrument

The study instrument is validated and predesigned questionnaire which is structured by following other questionnaires from similar studies. This study questionnaire is designed to evaluate the awareness, knowledge and the methods of application of pharmacovigilance in the community pharmacies.

* arspirovbt@gmail.com

- The term 'awareness' mean the perception of a situation or a fact.
- 'Knowledge' mean the theoretical or practical application of pharmacovigilance.
- 'Method of application' is the practical application of pharmacovigilance.

This questionnaire is comprised of 20 questions (awareness – 7, knowledge – 7, methods of application – 6) (Hema et al., 2012)

Discussion

The main aims of pharmacovigilance are the early detection of the adverse reactions and interactions, monitoring their frequency and identification of the risk factors for the adverse reactions of the drugs and especially of the biosimilars. Because of this, active involvement by the community pharmacists is needed in coordination with other healthcare professionals and medical institutions (Hema et al., 2012).

With this study we access the awareness, knowledge and the methods of application of pharmacovigilance among the postgraduate pharmacists and pharmacy technicians who work in community pharmacies. We consider that with this study we can provide information which will be significant for the proper education and training for the pharmacists in the future, as well we will gather information about the real gap between the knowledge and the real experience for reporting adverse drug reactions, especially for biosimilars (Gupta et al., 2015). Also we will determine the factors responsible for under reporting the adverse drug reactions, although we think that a proper education is the most essential factor. For this, the most appropriate time to do so is during the undergraduate and postgraduate training of the pharmacists.

To facilitate the activity of phamacovigilance, a culture of learning about it should start early in the professional training of the pharmacy students and postgraduated pharmacists. This will enable the pharmacists to realize that all medicines, including biosimilars can cause adverse drug reactions and learn about the procedures for proper reporting (Hema et al., 2012).

Conclusion

Nowadays, in the well developed countries the need for an efficient pharmacovigilance system has been realized more than ever to ensure the safe use of medicines, but that is not the case with the non – developed countries like ours. For that, after this study there will be an educational intervention with lectures and workshops on pharmacovigilance to understand the importance of pharmacovigilance and the necessity of reporting adverse drug reactions.

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Macedonian pharmaceutical bulletin, **62** (suppl) 667 - 668 (2016)
ISSN 1409 - 8695
UDC: 543.07:061.64
Short communication

Quality assurance of volumetric glassware in analytical laboratory

Blagoj Achevski*, Vasil Karcev, Katerina Brezovska

Institute of Applied Chemistry and Pharmaceutical Analysis Faculty of Pharmacy, University "Ss. Cyril and Methodious", Majka Tereza 47, 1000 Skopje, Macedonia

Background

According to ISO/IEC 17025:2005 any analytical equipment used in a testing laboratory should comply to required specifications and perform suitably for its intended purpose to be capable of achieving the required accuracy for the tests performed, including volumetric glassware if it's use has significant influence on the test result, or the volumetric accuracy is critical to the performance of the method.

Volumetric glassware used in analytical laboratory may either deliver volume (burettes and bulb burettes, single volume pipettes, one-mark pipettes, bulb burettes, graduated pipettes, including blow-out pipettes) or contain a stated volume (one-mark volumetric flasks, graduated measuring cylinders). Most items of volumetric glassware are available commercially in two classes, Class A or AS and Class B. The distinction between the two Classes is based principally on the tolerance limits of the nominal volume of the glassware as specified in the relevant Standards. Normally, for a given volume, the tolerance for Class B is twice that for Class A. When the laboratory acquires class A and AS volumetric glassware, it is supplied with a batch calibration certificate, where the tolerance and the error of the material is traceable to an international standard. If the item has been calibrated in-house, the laboratory shall have a documented record of the calibration data showing traceability to national standards (UKAS, 2009).

The laboratory should identify the needs for calibration and/or verification and develop a policy for management of the appropriate volumetric glassware. Before being placed in use, laboratory glassware should be calibrated or checked to establish that it meets the laboratory's requirements and complies with the relevant standard specification. Intermediate checks may be needed to maintain the confidence in the calibration status of the glassware, which should be carried out according to a defined procedure (ISO/IEC 17025:2005).

Marking of volumetric glassware

Commercially available volumetric glassware may be manufactured either from sodalime glass or borosilicate glass and should be marked in accordance with the national/international standard to which it is purchased, with regards to the tolerance (Class A or B), capacity in volume unit, reference temperature (i.e. calibration temperature), time of flow/delivery time (for verification or certification purposes for legal metrology) and identification number (Class A). The volumetric glassware should bear also colour code (pipettes complying with BS 700 and BS 1583 may be colour coded, in which case the coding shall comply with BS 3996), manufacturers and/or vendor's name or mark (UKAS, 2009).

Cleaning of volumetric glassware

The volume contained in or delivered from volumetric glassware depends on cleanliness of the entire internal surface to ensure uniform wetting and performing a well shaped meniscus Cleaning procedures, storage and segregation of volumetric equipment may be critical, particularly for trace analyses where leaching and adsorption can be significant. The laboratory should establish procedures for the cleaning of the glassware (for hand washing or washing with the washing machine), including cleaning validation protocol considering the possibility of the poor/deficient washing or the cross contamination with cleaning agents, like detergents (ISO 4787, 2010).

* blagojachevski@gmail.com

According to ISO 4787 it is recommended that volumetric instruments should not be heated to a temperature considerably above 180 °C. Washing and drying the glass material for volumetric purposes should be made to a temperature below 90°C; above 150°C may be significant changes in borosilicate glass volumetric capacity of the material and above 90°C for the soda-lime glass.

Qualification of volumetric glassware

Qualification of equipment refers to actions of proving and documenting that any analytical equipment complies with the required specifications and performs suitably for its intended purpose. The performance of volumetric equipment should be verified at appropriate intervals according to a plan and procedures established by the laboratory, taking into account the type of equipment, the extent of use and supplier's recommendations (WHO, 2010).

The initial level of the qualification of the volumetric glassware should include selection of the volumetric glassware that is manufactured according to the requirements of the corresponding ISO standard (ISO 385, ISO 648, ISO 1042 and ISO 4787).

The second level of the qualification of the volumetric glassware should include establishment that it meets the laboratory's requirements and complies with the relevant standard specification, by performing visual inspection of the glassware (compliance with marking requirements), checking the batch calibration certificate of the supplied equipment, or in the cases where certificate is not available, calibration of the volumetric glassware should be performed prior use.

The need for the calibration of the laboratory glassware before first use depends of the volumetric accuracy that is critical to the performance of the method, for which the glassware is intended to be used. The criteria for performing the calibration of the volumetric glassware, regarding the maximum permissible overall error in the testing method and maximum specified tolerance for the volumetric glassware should be established by each laboratory in accordance to the requirements of the corresponding ISO standard (ISO 385, ISO 648, ISO 1042 and ISO 4787).

Calibration of the laboratory glassware may be performed internally by the authorized (experienced and trained) personnel, according to the suitably documented procedure, developed by the laboratory, or externally by the accredited institution.

According to ISO 4787, the general procedure for calibration of laboratory glassware is based upon a gravimetric method, for determination of volume of water, either contained in or delivered by the volumetric instrument. This volume of water is based upon knowledge of its mass under consideration of buoyancy and its tabulated density.

The third level of the qualification of the volumetric glassware should include verification of the calibration status, by performing calibration on the glassware, at regular intervals, depending on the extent and the nature of usage. The initial calibration intervals should be at least once a year. If justified, these intervals may be extended.

The fourth level of the qualification of the volumetric glassware should include visual inspection of the glassware before each use (glass surface shall be free from obvious damage, the graduations and inscriptions shall be clearly readable and especially with instruments adjusted to deliver the jet shall be free from damage and allow an unrestricted outflow of liquid). Careful visual inspection of the material in use is made to check for signs of deterioration or attack, such as, scratches, blur glass, broken glass, chipped glass, scale or calibration mark not visible; the deteriorated material must be rejected (if it would compromise the safety of personnel or the purposes for which it intended), or segregated and used for qualitative work or tasks which are compatible with their status. In the case of aggressive uses of this material, such as the use of hydrofluoric acid or other corrosive substances, thermal shock, mechanical shock, visual inspection should be performed after cleaning and before storing the glassware.

Conclusion

Analytical laboratories should confirm the traceability of volumetric measurements used in support of testing and calibration activities, by establishing procedures for calibration and check of the laboratory glassware used in volumetric measurements. Different national accreditation bodies (NAB) establish different minimum requirements for the volumetric glassware used in a laboratory, with regards to the validity of the calibration and the frequency of the verification of the suitability of the volumetric glassware. The intervals, methods and the specification criteria for qualification of volumetric glassware are established by each laboratory, taking into account the type of equipment in accordance with the relevant standard requirements.

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Macedonian pharmaceutical bulletin, **62** (suppl) 669 - 670 (2016)
ISSN 1409 - 8695
UDC: 613 292:543.4/.5
Short communication

Application of AAS vs ICP-OES in determination of macro and microelements in dietary supplements

Rosana Trpeska*, Matilda Petrova, Liljana Bogdanovska, Aneta Dimitrovska, Rumenka Petkovska

Institute of Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University of "Ss. Cyril and Methodius", Majka Tereza 47, 1000 Skopje, Macedonia

Background

Dietary supplements are complex products intended to supplement human diet with nutrients such as vitamins, minerals, herbs, amino acids, extracts or combination of the mentioned ingredients (Sander et al., 2006). The minerals are inorganic nutrients which are divided in two categories: macro-elements (Na, K, Ca, Mg, P and Cl) and microelements which are also classified as "trace elements" because of their essentiality at low quantities in humans (Cu, Fe, Ni, Zn, Mn, Se, Co, Cr, Mo, V, F and I) (Gupta and Gupta, 2012). Since the human body cannot synthesize them, macro and microelements must be supplied by food or dietary supplements (Lesniewiecz et al., 2006).

One of the most important characteristics of a dietary supplement, from a nutritional point of view, is "how much" macro and microelements are present in the product. They can be regarded as essential or harmful depending on the concentration, speciation (the distribution among the different chemical compounds) and the presence or absence of other elements (Korfali et al., 2013). Additionally, dietary supplements are regulated as food and in many countries they are not subjected to quality control in the same manner as pharmaceutical dosage forms, which is a potential threat to public health (Tumir et al., 2010; Marerro et al., 2013). The increased consumption of these products, especially in developed countries requires monitoring of elemental concentration with emphasis on harmful or potentially harmful elements for human health. Therefore, a multi-element analysis method applicable to various types of dietary supplements should be used for determination of element content. This review covers the application of atomic absorption (AA) techniques vs. ICP-OES for determination of macro and microelements in dietary supplements.

Comparative assessment of the application of AAS vs. ICP-OES in the analysis of macro and microelements in dietary supplements

The determination of macro and microelements in dietary supplements has been made utilizing different methods such as X-ray fluorescence, capillary zone electrophoresis, flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS). The choice of the method mainly depends on the type of dietary supplement and the components in the matrix. However, the applied methods must have sufficient selectivity and sensitivity, therefore analysts may find that GFAAS, ICP-OES or ICP-MS are the most suitable (Abernety et al., 2010).

GFAAS is a frequently used technique for determination of macro and microelements as well as trace metals in biological, clinical, food, environmental or geological matrices. It is capable of detecting low concentrations (parts per billion, ppb, w/w) in microliter quantities of the sample (Lewen, 2011). Although this technique is relatively inexpensive, it can be used only for single-element analysis. Also, non-metals cannot be determined because their atomic absorption wavelengths are in far UV range which is not suitable for analysis due to absorption of air components (Baysal et al., 2013).

Plasma-based techniques have gradually gained importance in verifying whether food specimens, dietary supplements and pharmaceutical products comply with health requirements and/or national or international regulations. These techniques can be applied to all possible matrices

* Rosana_Trpeska@hotmail.com

and analytes. They are characterized by extended dynamic concentration range (several orders of magnitude) and are multi-elemental in nature, thus they possess high sensitivity and suitable detection power (Marrero et al., 2013).

Inductively coupled plasma-optical emission spectrometry (ICP-OES) is a multi-element technique that allows the determination of major, minor and trace elements in complex samples. This technique offers high sample through output enabling analysis of large batches, simultaneous determination of many elements in a single sample, large dynamic liner range and low chemical and matrix interferences (Gupta and Gupta, 2012). For the majority of analyses for ICP-OES it is necessary to convert solid samples into liquid solution by acid digestion. For this purpose, the closed –vessel microwave oven has been used, due to short heating times, reduced risk of contamination and low volume reagent requirements. The utilization of closed-vessel microwave oven improves limits of detection of ICP-OES at the same time reducing reagent quantities and cost of analysis. Given all the advantages of ICP-OES as well as the characteristics of the dietary supplements (complex matrix, presence of various elements in wide range of concentrations), it is slowly but surely becoming the method of choice for determination of macro and microelements in different types of dietary supplements (Barbosa et al., 2015; Castro et al., 2009; Marrero et al., 2013; Pytlakovska et al., 2012).

Conclusion

The data clearly demonstrates the effectiveness and productivity of ICP-OES as compared to more traditional methods such as atomic absorption spectrometry. The ICP-OES can make measurements across a wide range of concentrations, thus allowing the analysis of a single solution per sample for all the elements of interest. In future, it seems more likely that sensitive methods such as ICP-OES will begin to play even greater role in the analysis of macro and microelements in different types of dietary supplements. The performances of modern ICP-OES instruments make it possible to meet the challenges of the complicated dietary supplement matrix and low limits of detection in order to address both product safety as well as product quality.

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Macedonian pharmaceutical bulletin, **62** (suppl) 671 - 672 (2016)
ISSN 1409 - 8695
UDC: 631.416:582.688.3
Short communication

Mineral composition of soil substrate of *Arctostaphylos uva-ursi* (L.) Spreng. fam. Ericaceae

Elena Petrova[1]*, Mile Markoski[2], Gjoshe Stefkov[1]

[1]*Institute of Pharmacognosy, Faculty of Pharmacy, University "Ss Cyril and Methodius", Majka Tereza 47,1000 Skopje, Republic of Macedonia*
[2]*Faculty of Agricultural Sciences and Food, Department of Soil Sciences, University "Ss Cyril and Methodius", Blvd. Aleksandar Makedonski bb, 1000 Skopje, Republic of Macedonia*

Introduction

Arctostaphylos uva-ursi (L.) Spreng. fam. Ericaceae, also known as red bearberry, bearberry or bear's ear, is a trailing, evergreen woody ground cover shrub with paddle-shaped leaves on flexible branches. It has thick, leathery leaves, rolled under the edges, which are yellow-green in spring, dark green in summer and reddish-purple in the fall, remaining on the bearberry for 1-3 years before falling. The flowers are small, white or pink, bell-shaped and grouped in nodding clusters that bloom in summer. The fruits are white-red berries and they persist into winter. The bearberry is widely distributed on a global level.

The use of bearberry leaves for the first time was literally documented in the middle Ages in the Welsh "Physicians of Nyddfai" from the 13th century. From the beginning of 19th century, bearberry is in official use. It was used for treatment of different diseases such as hydrops, lithiasis, in diabetes, for the therapy of gonorrhoea, etc. Nowadays only the use as urinary tract antiseptic and diuretic remains due to the presence of arbutin and hydroquinone (Jurica et. al., 2015). The herb also contains tannins that have a powerful astringent effect and protect from early stage of infections (Head, 2008). The usual form of administration is as infusion and it is of great value in diseases of the bladder and kidneys, it strengthens and tones the urinary passages.

In Republic of Macedonia the natural populations of bearberry represent a unique source of leaves which are distributed in the following areas: Skopje Valley, mountains of Jakupica, Karadzica, Dautica, Shar Mountain, Osogovo Mountains and Mariovo. The knowledge of the texture of these soils has a great importance, since these soils are formed only on certain substrates (pure and compact limestones and dolomites), where all physical, physical – mechanical, chemical and biological properties greatly depend on the parent material. The mechanical composition of these soils varies extensively and depends on the mechanical composition of the residuum from which the mineral part of the soil is composed, on the character of the limestone and the dolomite (the degree of weathering and silicification), on the deposition of nearby materials (from the higher fields) and on the degree of erosion (Filipovski, 1997).

Bearberry thrives on rocky ground in the light woods of black and white pine and in subalpic plant communities on limestone and dolomite ground. The content of bearberry is tightly connected to the environmental conditions. Basic precondition for normal growth are the soils which is important to be rich in $CaCO_3$. According to the field surveys the bearberry mostly grows on two soil types: calcomelanosol, regosol (WRB, 2006). Here in Republic of Macedonia there are soils formed upon lime stones and dolomites (Markoski et. al., 2013). Also important conditions for this plant are the climate conditions and sea level. Bearberry usually lives at sea level over 1500 m on soils with high humidity, it cannot be seen in urban areas. Although it is an indigenous species for this region there is no literature data for the composition of the soils that bearberry thrives on.

Therefore the aim of this study was to analyze the mineral composition of the soils that bearberry grows on.

* petrova_elena@live.com

Materials and methods

Soil samples S2, S3, S4 and S6 collected from four locations on Mountain Korab, where bearberry populations are growing.

Soil samples were collected from 10cm, 20cm and 30cm depth and used in ruined condition for the laboratory analyzes. The mean values are given in the results.

The following methods were used for the analyses of the soils:

- pH (reaction) of the soil solution was determined electrometrically with a glass electrode in a water suspension and a suspension of 1M HCl (Mitrikeski andMitkova, 2006),
- Content of $CaCO_3$ determineted by Schebler calcimetar (Mitrikeski and Mitkova, 2006),
- Content of organic matter was determined according to total Carbon (Orlov et. al.. 1981),
- Total N was determined by calculation (Filipovski, 1974),
- Easily available forms of Phosphorus and Potassium were determined by Al-method (Pelivanovska, 2011),
- Determination of cations (Al, Fe, Mg, Mn, Cr, Cu, Ni, Pb, Zn) was done on Agilent Technologies 700 Series ICP – OES.

Results and discussion

Common chemical characteristics of the soil (pH, $CaCO_3$, organic matter, total N, P_2O_5, K_2O) were evaluated. The obtained data showed pH=7.7 for S2, pH=8 for S3, pH=7.9 for S4 and pH=7.6 for S6 in a water soil suspension. The values of the pH reaction of the soil solution were closely correlated with the content of $CaCO_3$. Highest value for $CaCO_3$ was found in S3 (60.81%) and lowest in S2 (44.16%). Regarding the content of easily accessible forms of Phosphorus and Potassium, these soils are poor in P_2O_5 and little to middle supplied with K_2O.

The presence of target elements (CaO, Al, Fe, Mg, Mn, Cr, Cu, Ni, Pb, Zn) was analyzed with ICP OES. CaO was most present in S3 and least in S6, Al was most present in S2 and least in S3, Fe was most present in S2 and least in S3, Mg was most present in S6 and least in S3, Mn was most present in S6 and least in S3, Cr was most present in S2 and least in S4, Cu was most present in S2 and least in S3, Ni was most present in S2 and least in S4, Pb was most present in S4 and least in S3, Zn was most present in S4 and least in S3.

Conclusion

Bearberry population viability and the content of bioactive compounds in bearberry are tightly connected to the environmental conditions. pH value of analyzed samples varied from 7.6 to 7.9. Precondition for normal growth of this plant that lives on limestone and dolomite ground are the soils which is important to be rich in $CaCO_3$ and highest value of $CaCO_3$ (60.81%) found in S3 and lowest (44.16%) found in S2 confirms that. CaO was most present in S3 and least in S6. These soils are poor in P2O5 and little to middle supplied with K_2O. Regarding cations, when the analyzed soil samples were compared between them self, S2 was richest in Fe, Cr, Ni and Cu and S6 in Mg and Mn; S3 was poorest with Al, Fe, Mg, Mn, Cu, Pb and Zn.

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Macedonian pharmaceutical bulletin, **62** (suppl) 673 - 674 (2016)
ISSN 1409 - 8695
UDC: 547.918:543.544.5.068.7
Short communication

HPLC determination of amygdalin in different plant material

Cvetanka Petrevska*, Ivana Cvetkovikj, Gjose Stefkov

Institute of Pharmacognosy, Faculty of Pharmacy, "Ss Cyril and Methodius" University, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Amygdalin (D-mandelonitrile-β-Dgentiobioside) is a cyanogenic glycoside derived from the aromatic amino acid phenylalanine. Amygdalin is hydrolysed in the presence of the enzyme amygdalase and water, involving a two-stage process to produce glucose along with an aglycone made up of benzaldehyde and odourless hydrocyanic acid. Natural amygdalin has a dextrorotatory (*R*) configuration that is considered to be the active form. Neo-amygdalin is its inactive (*S*) isomer and does not occur in nature. Isoamygdalin is the name of the mixture of the epimers R-amygdalin and S-amygdalin. The term 'laetrile' (D-mandelonitrile-ß-glucuronide) is an acronym from laevorotatory and mandelonitrile, used to describe a purified form of amygdalin (Fenselau et al., 1977). The occurrence of cyanogenic glycosides is widespread. Amygdalin is very common among plants of the Rosaceae, particularly the Prunus genus. This includes not only the bitter almond but also the kernels of apricots, peaches and plums (Pengelly, 2004). Hydrocyanic acid, which is the product of the hydrolysis of amygdalin reflexively stimulates the respiratory center and produces antitussive and antiasthmatic effects (Lv et al., 2005).

Amygdalin is supposed to be useful in tretman of cancer, but still there is no reliable clinical evidence for this indication (Blahata et al., 2016). Many hypotheses have been proposed to explain the anticancer effects of amygdalin, among them, amygdalin enhances the functions of pancreatic enzymes, which may prevent transformation of cancer primordial germ cells or has the capacity to restores the vitamin deficiency that could lead to metabolic disorders in cancer patients (Chang et al., 2006). Moreover, treatment with high concentrations of amygdalin on the human DU145 and LNCaP prostate cancer cells can induce apoptotic cell death (Chang et al., 2006). On the other hand, Chang et al. (2005) reported that Armeniacae semen containing abundant amygdalin exhibits analgesic and anti-inflammatory effects, showing that low doses of amygdalin may relieve pain.

Amygdalin is water and methanol soluble. Traditionally, the extraction method of the chemical ingredients in the plant materials is decoction in boiling water. Other methods used are soxhlet extraction and reflux extraction (Lv et al., 2005). Ultrasonic extraction with boiling methanol can be also applied to extract amygdalin from plant kernels. The last method is promising, because some amygdalin is decomposed into benzaldehyde, HCN, and glucose by emulsion (a hydrolysis enzyme present in kernels), and some are converted into its epimers, neoamygdalin (L-mandelonitrile-β-D-gentiobioside) during the process of decoction in water. Other way for inhibiting the conversion of amygdalin to neoamygdalin is by changing the pH (adding citric acid in the medium for extraction).

The determination and quantification of amygdalin was performed mainly by high-performance liquid chromatography (HPLC) (Wasserkrug et al., 1997).

Therefore, the aim of this study was to determine the amygdalin content in kernels of Prunus cerasifera, Prunus armeniaca and Prunus domestica, as well as in commercial available food supplement using HPLC method.

Materials and methods

Plant material

Kernels of Prunus armeniaca, Prunus cerasifera and Prunus domestica were purchased from a green market, while the food supplement containing extract of Prunus armeniaca kernels was purchased from pharmacy store.

* cvetanka.petrevska@outlook.com

Extraction

The seeds from the kernels were pulverized by means of a blender. 0.5 g of each sample (including the content from the capsule) was extracted with 25 mL boiling methanol with ultrasonic bath under reflux for 30 min. The temperature of system was the boiling point of methanol. After extraction, the extracts were filtered through a filter paper in 25 cm^3 flask. Furthermore, 1.5 mL of the extracts were additionally filtered through disposable Econofilter 25/0.45 μm RC pore size and injected in the HPLC system for analysis.

Quantification of amygdalin was performed using UV/VIS DAD at 215 nm with calibration standard of amygdalin prepared as 0.1 mg/mL stock in methanol.

HPLC method

Chromatographic analyses were performed on an Agilent 1200 HPLC/DAD system equipped with quaternary pump G1311A, column thermostat G1316A TCC, thermostatted autosampler G1329A TCC, and controlled by LC 3D software (Wilmington DE). Separation was achieved using a Zorbax Eclipse XDB C-18 column (150 mm × 4.6 mm, 5 μm, Agilent, Germany), protected with a guard column (4 mm × 4.6 mm, RP-18, 5 μm, Agilent, Germany). The mobile phase consisted of acetonitrile and water of HPLC grade in ratio 15:85 (v/v). The flow rate was 1 mL/min and the injection volume was 20 μL.

Results and discussion

With HPLC analysis amygdalin was identified in the extracts of the kernels from the samples of Prunus armeniaca, Prunus cerasifera and according to Rt and UV/VIS spectra corresponding to the Rt and UV/VIS spectra of the peak in the HPLC chromatogram of amygdalin standard. We were unable to identify amygdalin in the sample of Prunus domestica. In the Prunus armeniaca kernels 4.89% of amygdalin was determined, while in Prunus cerasifera kernels amygdalin content was 2.64%. On the other hand, in the commercial food supplement preparation 17.86% of amygdalin was quantified that complied with the declared amount of amygdalin.

Conclusion

An extraction procedure and high performance liquid chromatography method were successfully developed and applied for determination of amygdalin in plant material as well in pharmaceutical preparation/extract that can be further utilized for amygdalin content determination in various natural and pharmaceutical samples. Using, HPLC the amygdalin was quantified in kernels of Prunus cerasifera (26.4 mg/g DW), and Prunus armeniaca (48.9 mg/g DW) and these data can serve as a starting point for further investigations and assessment of other natural sources of amygdalin.

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Macedonian pharmaceutical bulletin, **62** (suppl) 675 - 676 (2016)
ISSN 1409 - 8695
UDC: 663,51:543.544.3
Short communication

GC determination of potential phytoestrogenic compounds in alcoholic beverages

Marjan Gjurcheski*, Gjoshe Stefkov, Ivana Cvetkovikj

Institute of Pharmacognosy, Faculty of Pharmacy, "Ss. Cyril and Methodius" University, Majka Tereza 47, 1000 Skopje, Macedonia

Introduction

Pimpinella anisum (Fam. Apiaceae) - aniseed and *Foeniculum vulgare* (Fam. Apiaceae) - sweet fennel are traditionally and commercially used in preparing of alcoholic beverages like OUZO, MASTIKA and YENI RAKI which are usually consumed at the Balkan's countries. These kinds of drinks contain an extracts of *Foeniculum vulgare* or seldom *Pimpinella anisum*. Sometimes estrogenic effects can be observed when they are consumed by female human population. These estrogenic effects are manifested with stimulation of the lactic secretion, stimulation of menstruation or facilitate birth. In man population can relieves the symptoms of climacterium (Albert-Puleo, 1980). One of the components which are responsible for estrogenic activity is dianethol, which is formed after the fusion of two molecules of *cis*-anethole, but there is information that *trans*-anethole has also estrogenic effects (Kulevanova, 2004).

There are also some studies on laboratory animals assessing the bioactivity of *trans*-anethole. When administered orally to immature female rats at 80 mg/kg b.w. for 3 days significantly increased uterine weight to 2 g/kg compared to 0.5 g/kg in controls and 3 g/kg in animals given estradiol valerate subcutaneously at 0.1 μg/rat/day ($p < 0.001$). The results confirmed that *trans*-anethole has estrogenic activity. On the other hand, some other experiments showed that it has no anti-estrogenic, progestational, antiprogestational, androgenic or anti-androgenic activity. Estrogenic activity of *trans*-anethole at high concentrations has been determined by a sensitive and specific bioassay using recombinant yeast cells expressing the human estrogen receptor (Howes et al., 2002).

The aim of the research was to analyze potential estrogenic components like *trans*-anethole and *cis*-anethole in alcoholic beverages OUZO, MASTIKA and YENI RAKI using GC-FID-MS technique.

Materials and methods

Samples: Alcoholic beverages: commercially purchased alcoholic spirit – OUZO; commercially purchased alcoholic spirit – YENI RAKI; commercially purchased alcoholic spirit – MASTIKA and traditionally homemade MASTIKA.

Sample preparation: 100 μL of each beverage were diluted to 1000 μL with methanol and analyzed on GC/FID/MS.

GC/FID/MS analysis: Samples were analysed with an Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C mass spectrometer. For this purpose, an HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm) was used. Adams' analytical conditions were as follows: oven temperature at 60 °C (0 min), 3 °C/min to 240 °C (1 min) and at the end increased to 280 °C at a rate of 10 °C/min (1 min) (Adams, 2007); helium, as carrier gas, at a flow rate of 1 mL/min (at the end of analysis we eluted 10 more minutes with helium with aim to separate some of the components in extract enough well); injector temperature 220 °C and that of the FID detector 270 °C. One μL of each EO was injected at split ratio 1:1. The mass spectrometry conditions were: ionisation voltage 70 eV, ion source temperature 230 °C, transfer line temperature 280 °C and mass range from 50 - 550 Da. The MS was operated in scan mode.

Identification of the components: The components were identified according to the literature (Adams, 2007), and Kovat′s (retention) indices determined using a homologous mixture of normal alkanes (C_9-C_{25}) analysed under Automated Mass Spectral Deconvolution and Identi-

* marjangjurceski@yahoo.com

fication System (AMDIS) conditions (AMDIS ver.2.1.). Confirmation was by comparing the mass spectra of components present in the extracts with reference spectra obtained from NIST, Wiley and Adams' mass spectra libraries. Quantification of extracts components was performed using the normalisation method of the GC/FID peak areas with no correction factors (Adams, 2007).

Results and discussion

Using GS/FID/MS analysis five different components were identified in OUZO sample (2-butanol (5.92%), 1-buten-3-ol (7.65%), anis aldehyde (9.50%), *trans*-anethole (23.98%), 1(1'-azetinidyl)-2,6-dimetil-1-cyclohexan (8.06%) and 4-metoxyphenyl ((2-methylenecyclohexyl)methanol), four different components in a sample of homemade MASTIKA (estragol – 7.42%), *cis*-anethole (42.38%), *trans*-anethole (35.09%), 3-metoxy-2methyl-2H-pyrazolo[4,3,3-E][1,2,4]triazin – 8.52%) and a single component of *trans*-anethole in the YENI RAKI and commercial purchase MASTIKA (100% *trans*-anethole). This indicates that in traditionally homemade MASTIKA and commercial OUZO an extract of aniseed or sweet fennel seed was added or plant seeds were used in the process of production or distillation. On the other hand single peak of *trans*-anethole in the YENI RAKI and the commercial available MASTIKA indicates that probably a pure compound of *trans*-anethole was added into the production process. The amount of *trans*-anethole was highest in the sample of YENI RAKI, almost twice higher compared with the OUZO sample, two and half times higher than commercial MASTIKA and approximately 5x higher than in the sample of traditionally homemade MASTIKA. *cis*-Anethole was identified only in the sample of traditionally homemade MASTIKA and was present in amount even higher than *trans*-anethole. This can be explained with the fact that *trans*-anethole has been converted into *cis*-anethole during the four years period of storage, consequently possessing probably higher estrogenic potency than the other analyzed samples.

Conclusion

For the purposes of this research, a method of GC/MS/FID was applied for determination of the target components, particularly *cis*- and *trans*- isomers of anethole, in alcoholic beverages. YENI RAKI has highest content of *trans*-anethole, followed by OUZO and MASTIKA, commercial and homemade. Further research is needed in order to evaluate the relationship between the content of these compounds and their respective estrogenic potential.

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Macedonian pharmaceutical bulletin, **62** (suppl) 677 - 678 (2016)
ISSN 1409 - 8695
UDC: 582.684.1-113.55
Short communication

Essential oil composition of St. John's wort (*Hypericum perforatum* L.)

Veronika Angelovska*, Marija Karapandzova

Institute of Pharmacognosy, Faculty of Pharmacy, University "Ss. Cyril and Methodius", Majka Tereza 47, 1000 Skopje, R. Macedonia

Introduction

The genus Hypericum consists of about 470 species of flowering plants that occur in all temperate parts of the world (Robson, 2003). Well known medicinally valuable species Hypericum perforatum (common St. John's Wort) is native to parts of Europe and Asia but has been spread worldwide as a cosmopolitan invasive weed including temperate regions of Canada, United States and Africa, as well.

Chemical investigations of Hypericum perforatum have detected several groups of medicinally active compounds. The most common classes include naphthodianthrones (hypericin, pseudohypericin, hyperforin, adhyperforin), phloroglucinols, catechins and proanthocyanidins, flavonoids and biflavonoids (hyperoside, quercitrin, isoquercitrin, rutin, biapigenin, amentoflavone), xantones (noratbyrol), phenolic acids and other phenolic compounds, antraquinone derivatives, as well as essential oils. The phytochemical investigation of Hypericum perforatum essential oil mainly began with the determination of the essential oil content. According to some authors, it is a highly variable value and ranges from 0.1% to 1% (Klemow et al., 2011; Robson, 2003) hence this species is considered as essential oil poor plant. Concerning the literature data, there have been numerous investigations of the chemical composition of Hypericum perforatum essential oil. The major constituents were found to be monoterpenes (α-pinene, β-pinene, limonene, myrcene, geraniol and α-terpineol), sesquiterpenes (β-caryophyllene and caryophyllene oxide) as well as aliphatic (2-methyloctan, n-nonan, n-undecan, n-octanal and n-decanal) and saturated hydrocarbons (dodecanole, 3-methylnonane, isoundecane and undecane) that could be present in appreciable concentrations (Bertoli et al., 2011; Crockett, 2010; Robson, 2003).

The widespread popularity of Hypericum perforatum usage as an herbal remedy results from its efficacy in a treatment of a variety of diseases. This plant has been used over thousands of years to treat cuts, abrasions, bruises, sunburns and other wounds as well as to treat anxiety, depression and rheumatic pain. Also, this plant is thought to possess antiseptic, antiviral, anti-inflammatory and gastroprotective effects (Crockett, 2010).

Despite the numerous literature data about the chemical composition of the Hypericum perforatum essential oil, still there is no officially available data about the chemistry of essential oil isolated from Macedonian species, therefore the principal aim of our further research study will be assessment of the chemical composition of essential oil obtained from the aerial parts of this plant harvested from its natural habitats in R. Macedonia.

Materials and methods

The plant material of investigated Hypericum perforatum populations is generally consisted of air-dried aerial parts collected during the flowering stage, packed in paper bags and kept in a dark and cold place until analysis.

The essential oil isolation is usually made from dried and properly minced plant material by steam distillation in all-glass Clevenger apparatus according to standard method described in European Pharmacopeia.

The common method of choice for the investigation of chemical composition of the essential oil is gas chromatography tandem mass spectrometry (Bardhi et al., 2015).

Results and discussion

Data analysis of the chemical composition of Hypericum perforatum essential oil isolated from species origi-

* veronikaangelovska@gmail.com

nating from southern Albania revealed six different classes of components: monoterpene hydrocarbons, oxygen-containing monoterpenes, sesquiterpene hydrocarbons, oxygen-containing sesquiterpenes, diterpenes and non-terpene components. Generally, monoterpene (1.94-47.21%) and sesquiterpene (6.67-50.88%) hydrocarbons were dominant fractions in all tested samples. Beside terpene compounds, these essential oils contain high levels of other non-terpene components (11.68-29.25%) mainly consisted of aliphatic chains. GC/FID/MS analyses of the examed essential oils revealed a total of 126 compounds from which seven compounds were found in amounts higher than 3.00%: α-pinene (2.03–36.74%), β-pinene (0.36–6.89%), 2-methyl-decane (0.82–3.14%), carvacrol (0.14–5.60%), trans-(E)-caryophyllene (0.5–19.27%), β-selinene (1.34–13.86%) and caryophyllene oxide (1.15–12.35%) (Bardhi et al., 2015). The main fraction of compounds in Hypericum perforatum essential oils obtained from plant material collected in southeastern Serbia were found to be sesquiterpenes (57.7%), followed by monoterpenes (22.4%), both mostly consisted of hydrocarbons. Other components present in this oil were non-terpene compounds (18.1%); alkanes, isoalkanes, n-alkanes, anteiso-alkanes and fatty acids and their derivatives. The main components were found to be germacrene D (18.6%), (E)-caryophyllene (11.2%), 2-methyloctane (9.5%), α-pinene (6.5%), bicyclogermacrene (5.0%) and (E)-β-ocimene (4.6%) (Đorđević, 2015). The essential oil of Hypericum perforatum collected from different localities in Lithuania was rich in β-caryophyllene (5.1-19.1%) and caryophyllene oxide (6.1-35.8%), followed by germacrene D (4.5-31.5%), spathulenol (3.9-8.5%), β-farnezene (0.6-8.2%), α-muurolene-14-ol (1.6-9.1%) and α-cadinol (2.2-6.2%). In this study, all tested samples, the amount of the most abundant fractions, sesquiterpene hydrocarbons and oxygenated sesquiterpenes, comprised 62.0-81.8% of the essential oils (Mockutė et al., 2003). Chemical investigation of the essential oil composition of Hypericum perforatum was also made on plant material collected from Turkey. GC/MS analysis has shown that the main constituents were monoterpenes: α-pinene, β-pinene and β-myrcene, followed by sesquiterpenes: α-copaene, (E)-caryophyllene and α-selinene. Hydrocarbons (nonane, decane, 2, 6-dimethyl decane and undecane) and aldehydes (hexanal) were also identified in the essential oil (Derun et al., 2013).

Conclusion

Concerning the literature data, some similarity in the chemical composition of essential oil obtained from the aerial parts of Hypericum perforatum collected from Albania and Serbia could be found, probably due to the influence of appropriate environmental conditions. In our further research study, we can expect similar chemistry in essential oil isolated from Hypericum perforatum harvested from its natural habitats in R. Macedonia which can help to assess the potential of this plant for its further sustainable exploitation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 679 - 680 (2016)
ISSN 1409 - 8695
UDC: 615.014.2:544.351-145.82]:615.074:615.1
Short communication

Comparison of pharmacopoeial methods for analysis of residual solvents

Marija Brezovska*, Ana Ivcevska, Ana Poceva Panovska

Institute for Applied chemistry and Pharmaceutical analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Introduction

Organic solvents are widely used in the manufacturing process of pharmaceutical products. Their application in the synthesis of active pharmaceutical ingredients is of exceptional importance since the use of specific solvents can determine the physicochemical characteristics, influence the yield of the synthetic reaction and purity of the product. Organic solvents are also routinely applied in the manufacturing of excipients and during the drug product formulation. Using standard manufacturing processes organic solvents are generally removed to the extent possible from the final product. Small amounts that remain in the final drug product are commonly referred as residual solvents (RS). Their presence, even at low levels, may influence the efficacy, safety and stability of the pharmaceutical products (Grodowska and Parczewski, 2010).

Regulation on residual solvents testing

ICH regulation

The International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, under ICH Topic Q3C (published in December 1997), require for "testing to be performed for residual solvents when production or purification processes are known to result in the presence of such solvents".

ICH categorizes commonly used RS into three different classes based on their toxicity: Class 1 (solvents to be avoided), Class 2 (solvents to be limited), Class 3 (solvents with low toxic potential). The European Pharmacopoeia (Ph. Eur.) and The United states Pharmacopoeia (USP) limit the amount of RS in pharmaceuticals, considering the ICH guidelines for RS. The control limits for RS are formulated based on the potential risks when the drug is administered orally. Class 1 contains known solvent that are human carcinogens, compounds strongly suspected of being human carcinogens, and environmental hazard. The limits for Class 1 solvents are between 2-8 ppm except for 1,1,1-trichloroethane 1500 ppm, due to its environmental hazard. Class 2 solvents are non-genotoxic animal carcinogens or possible causative agents of irreversible toxicity and their concentration limits vary between 50 and 3880 ppm. Class 3 solvents have low toxic potential and have been found less toxic in acute or short-term studies and negative in genotoxicity studies. Their limit of 5000 ppm is acceptable without justification. A higher amount may also be acceptable with proper justification.

ICH Q3C (R5) guideline for residual solvents also sets criteria for analytical methods used to identify and quantify these residual solvents as well provide acceptable concentration limits. Ph. Eur (3rd edition) and USP (USP 28,) have adopted this guideline and have revised their general methods to reflect it.

European Pharmacopoeia

In general chapter of Ph. Eur, *Identification and control of residual solvents*, two different methods for qualitative and quantitative analysis of RS: System-A and System-B are described. Gas chromatography (GC) with headspace (HS) injection is proposed in two systems (A and B). System A utilize fused-silica capillary wide-bore column (30m x 0.32mm or 0.53mm i.d) coated with cross-linked 6 % polycyanopropylphenylsiloxane and 94 % polydimethylsiloxane while System B uses fused-silica capillary wide-bore column (30 m x 0.32 mm or 0.53 mm i.d.) coated with macrogol 20000R. System A is preferred whilst

* marijabrezovska@gmail.com

System B is employed for confirmation of identity.

Three different sample preparation procedures are proposed based on the sample solubility—I: water for the water soluble samples; II: DMF (N,N-dimethylformamide) for the water insoluble samples; III: DMI (1,3-dimethyl-2-imidazolidinone) for the control of DMF and DMA (N,N-dimethylacetamide) in water insoluble samples. In the case of water soluble samples where water insoluble RS are present, the reference RS solutions in water are prepared using DMSO (dimethylsulfoxide) as bridging solvent. Moreover, the Ph. Eur. takes into account the possibility of using, apart from FID, a mass spectrometer (MS) or electron capture detector (ECD). MS and ECD detectors are proposed as alternatives for FID in the analysis of chlorinated RS of Class-1 due to the poor sensitivity of FID towards chlorinated solvents.

United States Pharmacopoeia

Current official methods for residual solvent determination are described in chapter Organic Volatile Impurities. USP chapter <467> suggests analysis of residual solvents using a gas chromatograph (GC) equipped with a flame ionization detector (FID) and an automated headspace sampler (HS). The three testing procedures are used to screen and identify (Procedure A), confirm (Procedure B) and quantitatively determine (Procedure C) the residual solvents in the sample. When the user has information about the specific solvents utilized during the manufacturing of the article, only Procedure C needs to be performed. If the solvents used are unknown, all three procedures are needed for identification and quantization. These three procedures (A, B, C) differ among themselves in column type (film-coatings G43 A or G16 and dimensions) and in chromatographic conditions. Procedure A in the USP <467> General Chapter has the same chromatographic and headspace conditions as in System A. Similarly, Procedure B finds its equivalent in Ph. Eur. in the system configuration of System B.

Sample preparation is different for water-soluble and water-insoluble articles.

Three different headspace conditions are available although USP does not specify which HS conditions should be chosen. These are dependent on the solvent that was chosen for preparation of the sample (water or *N,N*-dimethylformamide), residual solvents under analysis (high or low boiling) and analyzed material (thermally stable or unstable).

According to USP, determination of class 3 RS can be also done by loss on drying (USP <731> Chapter), as long as the total loss on drying is less than the maximum acceptable limit for class 3 residual solvents (5000 ppm).

USP general procedures do not relate to specific solvents, but they try to compromise chromatographic and headspace conditions, in order to analyze all or the majority of organic solvents mentioned in chapter <467>.

Conclusion

Methods for analysis of residual solvents described in pharmacopoeias are gas chromatographic methods that differ in procedures for sample preparation and headspace parameters. Proposed stationary phases for chromatographic columns and their intended used are the same. In Ph. Eur. mass spectrometer or electron capture detectors have been additionally taken into account (apart from FID). Generally, all methods for quantitative determination of residual solvents taken from pharmacopoeias need validation.

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Macedonian pharmaceutical bulletin, **62** (suppl) 681 - 682 (2016)
ISSN 1409 - 8695
UDC: 615.2:343.52
Short communication

Comparative analysis of EU and USA falsified medicine legislation

Filip Cvetanovski, Belma Asanova, Katerina Brezovska

Institute for Applied Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, Republic of Macedonia

Background

Falsification of medicines is a global problem that should be addressed with deep attention and interest. This problem represents a threat to the well-being, health and even the life of the patients, and cause a great damage on the economy and society.

A medicine can be falsified regarding its identity, including its packaging and everything that is visible on it: the marking, the ingredients, the quantity of ingredients; its source (the manufacturer, the land of origin, the country where it is manufactured, the marketing authorization holder) and the documents regarding the channels of distribution. Falsified medicines may contain different quantity of the active ingredient, wrong active ingredient or no active ingredient, wrong excipients and are produced in sub-standard manufacturing conditions. Therefore the use of falsified medicine may result in no therapeutic effect or even negative effect on patient's health. Additionally, falsified medicines undermine the credibility of the health care systems, leading to a reduction in patient trust in the legal supply chain of medicines and spend valuable human and financial resources. Innovative pharmaceutical companies invest in pharmaceutical development to assure the quality, safety, efficacy of their respective products order to provide maximal therapeutic effect with minimum side effects. In contrary, the main goal of the manufacturers of falsified medicines is easy money. Falsified medicines may also cause loss of the credibility and reputation of the pharmaceutical companies (Council of Europe, 2013a; WHO, 2008).

The "life-cycle" of falsified product involves unauthorized manufacturers, brokers, illegal/unregulated supplier, wholesalers, and unregulated internet. Patients receive falsified medicines easily without them knowing that the product is not what it is declared to be, not being aware of the consequences, the effect of these products, the internet, and the manufacturers are encouraged to enter this business because it is extremely profitable and in many countries unregulated (MHRA, 2012).

Taking into the account the threat and the extent of the phenomenon with the falsified medicines, a serious multilateral approach is necessary which includes taking measures to combat the falsification of medicines at both the national and global level.

Legislation in EU

The European Union (EU) has a strong legal framework for the licensing, manufacturing and distribution of medicines, centered around the Directive on falsified medicines for human use (21. 07. 2011 - EU Directive 2011/62 of the European Parliament and of the Council amending Directive 2001/83/EC) as regards the prevention of the entry into the legal supply chain of medicinal products which are falsified in relation to their identity, history or source, so that only licensed pharmacies and approved retailers are allowed to offer medicines for sale, including legitimate sale via the internet (Council of Europe, 2011). According to EU Directive 2011/62, falsified medicine is defined as any medicinal product with a false representation of: its identity, including its packaging and labeling, its name or its composition as regards any of the ingredients including excipients and the strength of those ingredients; its source, including its manufacturer, its country of manufacturing, its country of origin or its marketing authorization holder; or its history, including the records and documents relating to the distribution channels used. This definition does not include unintentional quality defects and is without prejudice to infringements of intellectual prop-

* filip_cvetanovski@rocketmail.com

erty rights. According to EMA and the Directive, there are going to be different penalties and allegations for falsifying and counterfeiting medicines. This Directive aims to prevent falsified medicines entering the legal supply chain and reaching patients. It introduces harmonized safety and strengthened control measures across Europe by applying new measures, which can be grouped into four main pillars: safety features of medicines, supply chain and good distribution practice, active substances and excipients and internet sales.

The Medicrime convention is the tool which EMA uses to criminalize the falsification of medicines. It gives directions on strategic approach for development the legal framework in the field of medicinal products, both nationally and internationally in order to better protect public health and national healthcare systems, through cooperation between sectors in public administration, measures for national control, preventative measures etc. (Council of Europe, 2013b).

Legislation in USA

EMAs equivalent in the USA is the FDA. It is a federal agency and is a part of the U.S. Department of Health and Human Services. It is responsible for taking care of the general health, among other things, and therefore provides legislation about falsified medicines. What the DIRECTIVE 2011/62 is for EU, FDASIA is for USA, Congress of USA (Title VII of the Food and Drug Administration Safety and Innovation Act (FDASIA, 2012). In this act through so called "sections" are described the ways to protect the distributive chain and criminalize the falsification of medicines.

Legislation in the Republic of Macedonia

In R. Macedonia there is no specific law concerning falsification of medicines. The problem with falsification of medicines is covered partially by the Criminal Code of R.M., the Law of Medicines and Medical Devices and the Law of Customs measures and Intellectual Property Rights.

Conclusion

Falsification of medicinal products is a crime carried out using deception and other techniques typical of organized crime and represents a public health problem and a problem of the trade competition as an intellectual property right infringement. An essential starting point in the fight against falsified medicines in R. Macedonia is the establishment of an adequate national legal framework and regulatory system, based on the EU and USA legislation concerning this issue. Additionally, development of mechanisms for effective collaboration and communication between health authorities, police, customs, the judiciary, manufacturers, wholesalers, retailers, health professionals and patients on national and international level is necessary aspect that should be considered for the success in this fight.

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