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REVIEW

PLANT STEM CELLS AS INNOVATION IN COSMETICS

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Abstract: The stem cells thanks to their ability of unlimited division number or transformation into different cell types creating organs, are responsible for regeneration processes. Depending on the organism in which the stem cells exists, they divide to the plant or animal ones. The later group includes the stem cells existing in both embryo's and adult human's organs. It includes, among others, epidermal stem cells, located in the hair follicle relieves and also in its basal layers, and responsible for permanent regeneration of the epidermis. Temporary science looks for method suitable for stimulation of the epidermis stem cells, amongst the other by delivery of e.g., growth factors for proliferation that decrease with the age. One of the methods is the use of the plant cell culture technology, including a number of methods that should ensure growth of plant cells, issues or organs in the environment with the microorganism-free medium. It uses abilities of the different plant cells to dedifferentiation into stem cells and coming back to the pluripotent status. The extracts obtained this way from the plant stem cells are currently used for production of both common or professional care cosmetics. This work describes exactly impact of the plant stem cell extract, coming from one type of the common apple tree (*Uttwiler Spätlauber*) to human skin as one of the first plant sorts, which are used in cosmetology and esthetic dermatology.

Keywords: stem cells, plant stem cells, pluripotency, *Malus domestica*, *Uttwiler Spätlauber,* plant tissue culture

At the turn of XX and XXI centuries, one of the promising research directions in natural sciences became a regenerative medicine. Researches relating to the different multi-cell organisms abilities in regeneration of damaged organs or even body fragments are carried for years. In some animals, the regeneration processes are intensive and large body fragments may be regenerated (coelenterates, planarians, amphibia), while in others the regeneration processes may be limited only to regeneration of damaged tissue or organs (e.g., human being). In human being, together with the ageing process, which starts about the age of 25 years (chrono-ageing), increases the number of cells in tissues and organs that are subject to a degeneration, and their regeneration processes together with the age subject to slow-down. The stem cells are responsible for regeneration of damaged or wearing-out tissues or organs in human being and in other multi-cell animals (1, 2).

The organ, in which the ageing changes are best visible, is the skin inside which, together with the age the numerous superficial and then deep mimic wrinkles as well as teleangiectasies and melanosis appear. These changes occur both on the epidermal, dermal and subcutaneous tissue levels. Therefore, also the human skin must contain cells responsible for its regeneration (3).

Characteristics of stem cells

The stem cells are the ones able to potentially unlimited number of mitotic divisions and also differentiation to a specific cells. The stem cell division results in appearance the so called progenitor cells, i.e., the partially differentiated cells, which after sequent division shall become the differentiated cells only or they may be subjected to direct differentiation without any further cell division. Depending on what cells appear in result of the stem cell division, and namely if the division is

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symmetric or asymmetric, the role of the given division is defined. If the stem cell is divided symmetrically, and two descendant stem cells appear, then such division is aimed for enlarging the stem cells pool. If the asymmetric division results in appearance of two descendant stem cells that differ: the stem cell and the progenitor cell, then such division is aimed for creation of the line of cells that differ, with simultaneous maintenance of the stem cell line. It's also possible a symmetric stem cell division that however leads to appearing of two progenitor cells. Thus, such division shall result in increasing of the differentiating cells pool, without simultaneous reproduction of the stem line (1).

As mentioned above, the stem or progenitor cells may differentiate into specific cells that form a given tissue or organ. There are also different abilities of the stem cells differentiation (so called potential): from unlimited to limited. Therefore, the stem cells may be divided to (1):

a. Totipotential cells that may differentiate into any organ cell, and also in case of human being into the extra-embryonic structure cells such as fetal membranes. In human being, the totipotential character have the early embryo cells only, where there's no separated embryoblast and trophoblast yet.
b. Pluripotential cells are able to differentiate into the body building structures only, and they can create no extra-embryonic structures such as fetal membranes. In human being, the pluripotential character have, among others, the embryonic cells on the blastocyst stage, and exactly the embryoblast cell in the blastocyst. Thus, the pluripotential cells may differentiate into cells from all the three embryo germ layers: ecto-, endo- or mesoderm.
c. Multipotential cells may differentiate into cells coming, within the embryonic growth, from one germ layer only, thus, from the ecto-, endo- or mesoderm only.
d. Unipotential cells, in turn, are the ones with strongly limited differentiation abilities, thus, differentiating into one particular cell type. The unipotential cells exist in the adult human organs, e.g., in liver, where they are responsible for the given organ regeneration processes.

The other division criterion is the organism type, from which the cell originates. Thus, we can discern the plant or animal stem cells. The latter include the stem cells existing both in the adult human or embryo organisms.

Stem cells of epidermis

The epidermis, being the external body coating, is directly exposed to numerous external environment factors (e.g., mechanical traumas, temperature, pathogenic microorganisms, xenobiotics, UV radiation) that affect the homeostasis disorder not only of the skin, but also the entire organism. Therefore, mechanisms must be created enabling creation of the barrier against factors the action of which shall also speed-up the ageing process. One of such mechanisms is the keratinization process, i.e., keratosis of the epidermis external layers. The epidermis keratinocytes come from its basal layer through the prickle and granular layer (possibly also through the light layer) to the horny layer, inside which due to numerous changes they start to perform into korneocytes. Thus, keratinization means a series of biochemical and morphological changes, being the programmable processes that lead among others, to the cell proliferation ability loss, degeneration of the cell organelle parts (e.g., cell nucleus fragmentation and breakdown, partial breakdown of the endoplasmic reticulum cisterns) and appearing of the new ones (e.g., lamellar bodies, keratohyaline granules), changes in the cell membrane chemical compositions and also appearing of numerous proteins (e.g., involucrine, filagrine binding proteins) and lipids (sterols or phospholipids being the ceramides precursors) (4–6). Researches show that the epidermis granular layer keratinocytes degenerate by apoptosis (3, 7), while the corneocytes located on the horny layer, are subjected to peeling under action of proteolytic enzymes that degrade the corneodesmosomes, i.e., specialized inter-cellular links present in the epidermis horny layer. The ceramides, being the main inter-cellular cement component, are also subjected to changes: degradation to sphingosine and fat acids, which results in the intercellular cement liquefaction (4). Consequently, the corneocytes are mechanically removed from the epidermis surface.

The epidermis regeneration process is possible thanks to the presence of his stem cells in it. Their larger conglomeration is the hair follicle bulge, called the bulge area (3, 8). In this place, such cells intensively divide enlarging its pool, and then they migrate. Part of them migrates to the hair germinal matrix (where they participate in the hair appearing and growth), while the others go to the apical hair part, in order to home the epidermis basal layer. During the migration to the epidermis, the stem cells meet the area located just over the sebaceous gland, where there are concentrated stem cells responsible for production of sebum (8).

However, in order to enable the keratinization and epidermis regeneration process creating the protective layer for the entire organism, many factors must be controlled. Besides the many vitamins, calcium ions, or water level, attention should be paid to a keratinization control process by the keratinocytes themselves. They synthesize and emit many factors that control their process of proliferation and differentiation. They include, among others, EGF (*epidermal growth factor),* KGF (*keratinocyte growth factor)*, TGF-α (*transforming growth factor α*), TGF-β (*transforming growth factor β*) and interleukin 1 (IL-1) (2, 8). These factors, after their release from the cell to inter-cellular space, join the proper receptors on a neighbor (target) cell's membranes and stimulate their proliferation or differentiation (3, 8).

Plant stem cells

The plant stem cells are grouped into niches, called meristems. There are the primary and secondary meristems. The first ones are: apical meristem (stem and root growth cone), intercalary (insert) meristem and germ meristem. The secondary meristems are: lateral ones – cambium (smear) and phellogen and the traumatic (callus) one.

In the sprout top meristem the plant stem cells proliferation and differentiation are controlled by many factors, including the negative reversible loop process between the genes expression products, i.e., *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) proteins. The *WUS* protein is produced by the organization centre cells and it's a signal for the stem cells proliferation, while the *CLV3* protein is secreted by stem cells and it limits the *WUS* expression area. Excess of the stem cells leads to excess of the *CLV3*, which causes reduction of secretion of the *WUS* and it consequently reduces the stem cell proliferation signal. On the other hand, if the stem cell number is too low, then the *CLV3* deficit leads to an increase of the *WUS* protein synthesis, which in turn affects an increase of the stem cell number (9–13).

The root growth cone, on the example of *Arabidopsis* sp., consists of quiescent centre, the cells of which aren't mitotically active. The centre is surrounded with the stem cells producing the distal (cap), lateral (lateral cap cells, epidermis) and proximal (endoderm, primary cortex, axis cylinder) root cells. After a division, one of the cells, directly adjoining the immobile centre, remains the stem cell, while to other loses its connection with the centre and it starts to differentiate. In the roof top meristem, the stem cells proliferation and differentiation are controlled by plant hormones – auxins and probably by some transcription factors, however, these processes aren't fully recognized yet. In the immobile centre, the *WOX5* protein (homologous to *WUS* in the stem growth cone organization centre) is produced, responsible for proper differentiation of the cap cells (9–15).

The traumatic meristem – callus appears in the plant hurting place, it differentiates most frequently with a cambium, however, other tissues are also able to produce it. The phenomenon of callus creation from the differentiated adult plant cells was for first time described in 1902, by the Austrian botanist, Gottlieb Heberlandt. He suggested that the individual plant cell is able to regenerate the entire plant. This experiment was demonstrated in 1958 by cloning of a carrot from the *in vitro* cultivated carrot cells. From this time, many articles appeared dedicated to regeneration of the entire plant from the cultivated cells and/or tissues. The callus creation process is one stage of the somatic embryogenesis (no-fertilization formation of a zygote) – the plant cells are subjected to dedifferentiation and become again the stem cells able to produce a new tissue or even entire organ. The *WUS* protein is responsible for turning back the somatic cells into the stem cells. The researches show that the cytokines are responsible for production of stems from a callus, while auxins are responsible for production of roots (11, 12, 14, 15).

Ability of the differentiated plant stem cells for dedifferentiation back to the pluripotential status are currently used, among others, in elimination of the human skin ageing symptoms, i.e., in production of care preparations or cosmetic procedures.

Plant cell culture technology

The plant cell culture technology consists of many and complicated methods that should ensure growth of plant cells, tissues or organs in the environment with a microbe-free nutrient. The plant cell culture allows synthesis of the biologically active substances that exist in plants, but aren't commonly available in natural environment or are difficult to obtain by chemical synthesis. Such cultures allow access to plant material free from environmental pollutions, microorganisms or toxins, available in every season, with uniform composition, and, first of all, with almost identical content of the active substances in each batch. These aspects were exposed by FAO (United Nation's Food & Agriculture Organization), which as early as in 1994 did propose the plant cell or tissue culture technology as the biotechnological process for pro-

duction of diet supplements. Despite that fact, this technology is still not popular yet and there are few compounds used in cosmetic or pharmaceutical industries, produced using this method, e.g., arbutin obtained from the rose periwinkle (*Catharanthus roseus* L.) used as a whitening agent, safflower and saflorin obtained from coloring safflower (*Carthamus tinctorius* L.) used as a pigment or taxol (paclitaxel) obtained from the western yew (*Taxus brevifolia* Nutt.) being the antitumor medicine (16–19).

The biological bases inside all the above plants are the reservoir of pluripotential stem cells, and also ability of the differentiated cells for dedifferentiation back to the pluripotential status. Any mechanical damage in a plant shall induce appearing of a callus, which consists of the grouped non-differentiated stem cells. In the presence of a proper nutrient the callus may grow in a culture, and using a proper hormonal stimulation it may be stimulated for regeneration of adult plants (so called micro-reproduction technique) (17, 18).

First step in generating of a new plant cell line is to select proper plant material. Next, very important step is sterilization of a tissue, because all the microbes (bacteria, fungi and molds), which may hold or slow-down the culture development, must be eliminated causing no irreversible damage in the meristem cells, necessary for creation of the new cell line. The sterilized plant tissue is reduced to microscopic fragments (called „explants") and placed on the Petrie scale pans including a solid nutrient.

The produced callus is regularly transferred to a proper nutrient including all the substrates necessary for cell metabolism. The culture occurs at dark and therefore the culture looses its photosynthesis ability and it becomes behaving as a heterotrophic organism. At this stage, the culture is supplied with a substance being a source of organic carbon and energy (most frequently a saccharose) and plant hormones (auxin and cytokine), vitamins and also micro- and macroelements. Thanks to the nutrient composition variations we can obtain the cell lines with different properties, from which later on the cell line is selected with the best biochemical and metabolic characteristics (most productive cells with shortest division time). Repeated transfers are made till the moment of obtaining the cell line with the stable and uniform characteristics. It's worthy to point out that the obtained cell line isn't genetically modified, and its selection bases totally on morphological, biochemical characteristics and on the callus tissue growth ability. Fermentation is controlled by a routine measurement of a.o. sugar contents, pH level, cell viability and volume of the produced biomass (16–18).

The essential element of the production process transferred to the industrial scale is adaptation of the selected cell lines for growth in a liquid nutrient that allows significant enlargement of a biomass volume. The suspension cultures require gradual adaptation for growth inside a cone flask (volume ca. 200 mL) and then to growth inside bioreactors that ensure more possibilities (volume up to 100 L). This process is called "scaling". Cultures carried in bioreactors must have ensured a constant temperature and they must be mixed in order to ensure proper gas exchange level required for cell metabolism. The increasing biomass is monitored by measurement of: sugar contents, conductance, pH level, optical density, cell vitality and contents of secondary metabolites such as e.g., ursolic acid (16–18).

Final stage of the fermentation cycle is a biomass processing dependend on the product type and target designation. The first method means a mixing of the culture content in a suspension including liposomes, phenoxyethanol (preservative) and antioxidant (ascorbic acid or tocopherols). Next, the mixture is subjected to a high-pressure homogenization, during which the stem cell walls are demolished, the included components are released and simultaneously the lipophilic components are closed inside liposomes, while the hydrophilic components are dissolved in a water phase. The obtained product is a yellowish and amber color liquid (16). This solution was elaborated and described by a Swiss company Mibelle AG Biochemistry, which did name their technology PhytoCellTec™ (PCT), and the products made by it – PCT™ Malus Domestica, PCT™ Solar Vitis or PCT™ Alp Rose. The other way is a biomass homogenization allowing release of the cell-encapsulated secondary metabolites, and then their extraction and densification. The final product is a powder colored from yellowish to amber and characterizing with a defined active substances quantity. The last way is spreading of the whole stem cells (together with the produced secondary metabolites, polysaccharides complex, phytosteroles and amino acids) in a solid glycerin. The obtained preparation is a liquid with characteristic smell and amber color (16, 19–21). The two solutions are used by an Italian company Instituto di Ricerche Biotecnologiche (IRB) in the technology called High Tech Nature™ (HTN). The preparations with

the exactly defined active substance contents are Teoside™ 10, Dermasyr™, Echigena PluS™ & Echigena 25™. The whole stem cell extracts in a glycerin are named: Echinacea angustifolia stems G™, Leontopodium alpinum stems G™ or Buddleja davidii stems G™.

Uttwiler Spätlauber stem cells

The beneficial apple properties are known for centuries. Apples are cultivated today only for their taste, but earlier the main criterion of the type selection was first of all fruits viability after their picking. One of such apple-tree types is *Uttwiler Spätlauber* growing in Switzerland till today. This is a type cultivated solely due to a possible long-time storage of fruits, which remain fresh even for several months. Some trees come from the quicksets planted in the middle of XVIII century.

Swiss company Mibelle Biochemistry did create the innovative product named PhytoCellTec™ Malus Domestica including the stem cells from the *Uttwiler Spätlauber* apple tree. The tissue, from which the stem cell culture was initiated in external environment, was taken from their fruits (apples). The extract, obtained in a biotechnological process described above, passed a series of tests and researches within the range of anti-ageing action to a human skin and hair (22, 23).

Characteristics of the PhytoCellTec™ Malus Domestica preparation

The *Malus domestica* Borkh. preparation is assigned for use in cosmetics for protection of a face or total body skin. The recommended concentration is 2–5%. The preparation should be entered into the cosmetic water phase by mixing at the temperature not exceeding 40°C. The preparation thermal stability allows its short-time processing at the temperature up to 60°C.

Series of the carried laboratory tests did confirm the preparation efficiency in the range of the human stem cells protection, fibroblast ageing inversion, retardation of the insulated hair follicles and anti-wrinkle action in the "crow's feet" areas. The idea of the PhytoCellTec™ Malus domestica creators was to invent a preparation, which would protect the skin's stem cells vitality, delay their ageing and decrease their effects, and also maintain a healthy skin look and vitality (23).

Protection of the human stem cells

The stem cell extract from *Uttwiler Spätlauber* has been tested in the aspect of ability to maintain alive the stem cells taken from a cord blood. Two test types were carried-out. First experiment was aimed for check of the extract impact to the human stem cell proliferation activity. It have shown that only just 0.1% extract concentration can stimulate growth of the cells multiplication by 80%.

Second experiment was the UV irradiation of the human stem cells. In the preparation, where the human stem cells were placed on the basis without extract, the apoptosis, i.e., programmed cell death was found in more than 40% of cells, while on the basis with 0.1% concentration extract the cell's death was below 10% (22, 23).

Reversal of fibroblasts ageing symptoms

Each cell has a defined life length, which is determined by its divisions number. After such period the cell naturally starts to grow old and it looses its ability to subject sequent divisions. However, the cell ageing may occur earlier if its DNA is damaged. The premature ageing is especially negative if occurs in the stem cells necessary for regeneration of tissues.

The company Mibelle AG Biochemistry carried out an experiment on fibroblast cells, which were subjected to action of H_2O_2. After 2 h exposition, the proper skin fibroblasts did show typical ageing symptoms. After this time, one portion of cells was placed in the 2% extract of the *Uttwiler Spätlauber*, apple tree stem cells, while the other one was placed in the neutral environment and used as a control sample for results comparison. Expression of genes responsible for a cell proliferation and cell growth stimulation was analyzed in two groups with special attention paid to the cell growth and the ageing process with its symptoms. In the control sample the genes were damaged, while the incubation with 2% stem cell extract from *Uttwiler Spätlauber* not only reversed this process, but also stimulated expression of the valid antioxidant enzyme – heme oxygenase 1 (22, 23).

Retarding of the insulated hair follicle ageing

Human hair follicles, being at the anagenic phase, are insulated from skin fragments after a face lifting. Next, they are placed on the growth nutrient, on which they are able to grow in length for more 14 days. After this period, due to no blood flow, the insulated hair follicles are no longer able to grow, because their cells grow old or they pass the apoptosis. Thus, they became a perfect model, on which the company Mibelle Biochemistry shows ability of the *Uttwiler Spätlauber* stem cells for retarding of the tissue atrophy process.

The experiment consisted of location of the one insulated hair follicle portion on the growth nutrient (control sample), while the other on the nutrition with added 0.2% of the stem cell extract. It appeared that the follicles from the control sample become shrink after 14 days of experiment, while the ones located on the nutrient with the plant stem cell extract did grow till 18-th day from their location on the nutrient (22).

Anti-wrinkle action at the „crow's feet" area

The Mibelle AG Biochemistry company carried-out clinical tests lasting 4 week on the group of 20 women aged from 37 to 64 years. The tests were aimed for showing the *in vivo* action of the PhytoCellTec™ Malus domestica preparation included in the cream being the O/W type (oil in water) emulsion. The preparation content in the cream was 2%.

The test included application of the cream, twice a day, on the „crow's feet" area. Depth of wrinkles was measured using the PRIMOS system (optical device for 3D skin surface display), on the test start and after two and four weeks from it. The research showed that the wrinkles became shallow by 8% after two weeks and by 15% after four weeks (22).

Summary

The *Malus domestica* Borkh. apple tree is one of many plant types, on which currently researches are carried enabling use of the plant stem cells in cosmetic preparations. Besides the apple trees, currently they are testing impacts of preparations created using, among others: grapes (*Gamay Teinturier Fréaux)*, alpine rhododendron (*Rhododendron ferrugineum* L.), jasmine gardenia (*Gardenia jasminoides* J. Ellis, *Gardenia augusta* Merr.), common horehound (*Marrubium vulgare* L.), stolonate ground-pine (*Ajuga reptans* L.), lilac (*Syringa vulgaris* L.), alpine edelweiss (*Leontopodium alpinum* Cass.), David's buddleia (*Buddleja davidii* Franch.), narrow-leaved purple coneflower (*Echinacea angustifolia* DC.) or blue cornflower (*Centaurea cyanus* L.). The plant stem cell extracts are invaluable sources of precious active substances. The plant stem cells culture technology, despite possible production of large volumes of active substances, is absolutely environmental friendly and its application enables obtainment of precious compounds, even from the endangered or hardly available plants, causing no unbalance in their natural ecosystem.

The preparations using the plant stem cell extracts may be applied in both cosmetics for everyday care and in the face-mask, serums or procedures carried in beauty salons. The producers define no age limit, however, due to the products properties they shall be rather assigned for complexions with decreased firmness, elasticity and humidity, susceptible ones or for physically active people. The requirements towards contemporary cosmetics and/or cosmeceutics are first of all safety, but also actions giving visible results. The important elements are also the product innovativeness and ecologic advantages. The preparations that include the plant stem cell extracts meet each of these requirements and they perfectly satisfy the XXI-century cosmetology market needs.

REFERENCES

1. Li L., Xie T.: Annu. Rev. Cell Dev. Biol. 21, 605 (2005).
2. Chen Y., Shao J.Z., Xiang L.X., Dong X.J., Zhang G.R.: Int. J. Biochem. Cell Biol. 40, 815 (2008).
3. Lavker R.M., Sun T.-T.: Proc. Natl. Acad. Sci. USA 97, 13473 (2000).
4. Harding C.R., Watkinson A., Rawlings A.V., Scott I.R.: Int. J. Cosmet. Sci. 22, 21 (2000).
5. Arct J., Pytkowska K.: Wiadomości PTK 6, 2 (2003) (Polish).
6. Kacalak-Rzepka A., Bielecka-Grzela S., Klimowicz A., Wesołowska J., Maleszka R.: Ann. Acad. Med. Stetin 54, 54 (2008) (Polish).
7. Gojniczek K., Jurzak M., Boryka M., Garncarczyk A.: Pol. J. Cosmetol. 10, 146 (2007) (Polish).
8. Alonso A., Fuchs E.: Proc. Natl. Acad. Sci. USA 30, 11830 (2003).
9. Byrne M.E., Kidner C.A., Martienssen R.A.: Curr. Opin. Gen. Dev. 13, 551 (2003).
10. Laux T.: Cell 113, 281 (2003).
11. Singh M.B., Bhalla P.L.: Trends Plant Sci. 11, 241 (2006).
12. Tucker M.R., Laux T.: Trends Cell Biol. 17, 403 (2007).
13. Dinneny J.R., Benfey P.N.: Cell 132, 553 (2008).
14. Stahl Y., Simon R.: Int. J. Dev. Biol. 49, 479 (2005).
15. Sablowski R.: Proc. Natl. Acad. Sci. USA 106, 16016 (2009).
16. Schürch C., Blum P., Zülli F.: Phytochem. Rev. 7, 599 (2008).

17. Dal Toso R., Melandri F.: Nutrafoods 8, 29 (2009).
18. Dal Toso R., Melandri F.: Personal Care 35 (2010).
19. Dal Toso R.: Household Personal Care Today 1, 54 (2011).
20. Dal Toso R., Melandri F.: Nutrafoods 10, 19 (2011).
21. Schürch P., Blum C., Zülli F.: Phytochem. Rev. 7, 599 (2008).
22. Schmid D., Schürch C., Blum P., Belser E., Zülli F.: SOFW Journal 5, 30 (2008).
23. Schmid D.: Household Personal Care Today 1, 26 (2009).

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ANALYSIS

HPLC METHOD FOR IDENTIFICATION AND QUANTIFICATION OF THREE ACTIVE SUBSTANCES IN A DERMATOLOGICAL PREPARATION – VIOSEPT OINTMENT

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Abstract: The study was aimed at developing a HPLC method to identify and quantify domiphen bromide, tripelennamine hydrochloride and clioquinol in Viosept ointment. The tested substances were successfully separated using Inertsil ODS-3 (250 × 4.6 mm, 5 μm) as a stationary phase and a gradient elution. Detection at 310 nm wavelength was applied for tripelennamine hydrochloride and clioquinol, and at 215 nm wavelength for domiphen bromide. Methods of extraction of the tested substances were developed: domiphen bromide and clioquinol were extracted with acetone from heated solutions, and tripelennamine hydrochloride was extracted in a hexane-water system. Validation procedure confirmed the method to be sufficiently selective, precise and accurate. Correlation coefficients of calibration curves pointed out that they were linear within the examined concentration range.

Keywords: domiphen bromide, tripelennamine hydrochloride, clioquinol, HPLC method

Human body is covered with skin, which provides main protection against external factors. Skin is exposed to mechanical and thermal damage as well as to adverse effects of radiation, chemicals, bacteria, viruses or fungi. These factors can cause a variety of diseases, many of which require long-term and complex treatment. A topical therapy with multi-component extended-spectrum preparations, as the Viosept ointment, typically provides sufficient therapeutic response. The drug contains three active substances: domiphen bromide, tripelennamine hydrochloride, and clioquinol. Structural formulas of the substances are shown in Figure 1.

Tripelennamine hydrochloride is an antihistamine and topical anesthetic agent, whereas clioquinol (quinoline compound) and domiphen bromide (quaternary ammonium compound from a group of cationic emulsifiers) have antibacterial and antifungal properties

Qualitative requirements and methods of quantification for domiphen bromide, tripelennamine hydrochloride, and clioquinol are described in pharmacopoeial monographs. Clioquinol monograph is included in European Pharmacopoeia (Ph. Eur.) and United States Pharmacopoeia (USP). British Pharmacopoeia (BP) contains a monograph for domiphen bromide, and USP features a tripelennamine hydrochloride monograph.

According to USP, purity and assay of clioquinol substance can be tested using gas chromatography (GC). USP also provides a detailed description of selected pharmaceutical forms containing clioquinol, i.e.: cream, suspension and skin powder containing clioquinol along with zinc oxide, lactic acid and lactose. Clioquinol concentrations in ointment and suspension have been determined with gas chromatography after transforming the substance into a silanol derivative, whereas clioquinol concentration in skin powder with a spectrophotometric method. An isocratic HPLC method for purity test of clioquinol is described in Ph. Eur.

Tripelennamine hydrochloride monograph in USP contains a detailed specification of purity and concentration tests using HPLC method with ion pair chromatography. HPLC has also been used in

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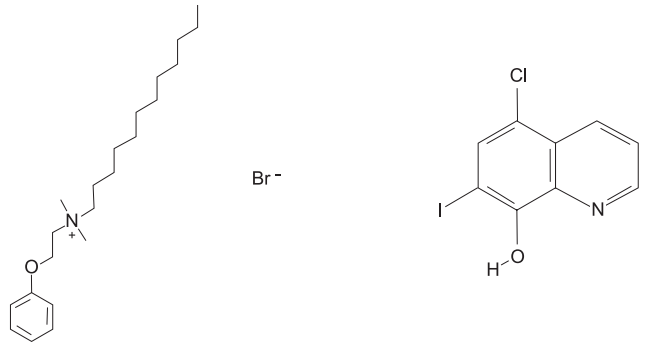
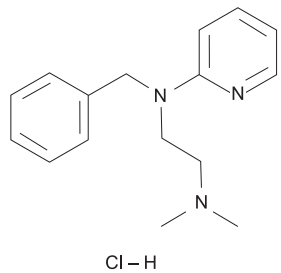


DOMIPHEN BROMIDE CLIOQUINOL



TRIPELENNAMINE HYDROCHLORIDE

Figure 1. Structures of the studied compounds

determining the drug substance concentration in solutions for injections.

In a monograph of tablets containing tripelennamine, a spectrophotometric method intended for testing the concentration of active substance and its quantities released in dissolution tests is also laid down.

According to a domiphen bromide monograph in BP, its concentration in active substances can be tested with a titration method. No pharmaceutical forms containing domiphen bromide are mentioned in any pharmacopoeia.

The present study was aimed at developing a HPLC method to identify and assay domiphen bromide, tripelennamine hydrochloride and clioquinol in the Viosept ointment.

Analysis of multi-component drugs such as ointments and creams containing active substances exhibiting different physicochemical properties presents some difficulties, e.g., isolation of individual substances.

Many papers refer to tripelennamine tested in pharmaceutical preparations (1–10) and biological materials (11–15). Different procedures were developed: chromatographic method such as liquid chromatography (HPLC) (1–6, 12), capillary electrophoresis (CE) (7, 8, 15), gas chromatography (GC) (11, 13). Other methods were also adopted like potentiometric methods (9), photometric (10) and spectrophotometric method (14). In almost all cases HPLC, GC, CE were used for the simultaneous determinations of antihistamine drug substances such as diphenhydramine, carbinoxamine, azatadine, chlorpheniramine, pheniramine, brompheniramine and others.

The available literature mentions a few reports related to the quantification of clioquinol in drugs. Determination of clioquinol in creams was carried out by spectrophotometric methods (16, 17). Liquid chromatography after precolumn derivatization was used for determination of clioquinol in the presence of metronidazole and tolnaftate (18). HPLC method

with electrochemical detection was developed for determination of clioquinol in plasma and tissues (19). Only one paper reporting an analysis of domiphen bromide by a HPLC method as a surfactant in bioprocess intermediates was found (20).

EXPERIMENTAL

Materials

Drug product: Viosept ointment containing tripelennamine hydrochloride 20 mg/g + clioquinol 20 mg/g + domiphen bromide 0.5 mg/g (batch number 10203; manufactured by: "Jelfa" S.A.)

Reference standards: tripelennamine hydrochloride (USP), clioquinol (CRS Ph. Eur.), domiphen bromide (RBH Ltd. UK), impurities A, B, C originating from clioquinol (CRS Ph. Eur.).

Reagents and instruments

Reagents: acetonitrile was HPLC grade, all other chemicals were of analytical grade.

Computer-controlled liquid chromatograph (Shimadzu) was fitted with UV-VIS SPD-10AV$_{VP}$ detector, DAD SPD-M10AV$_{VP}$, LC-10AT$_{VP}$ pump system, DGU-14A sample degassing unit, SCL-10A$_{VP}$ controller, and SIL-10AD$_{VP}$ automatic sample feeder.

Chromatographic column: Inertsil ODS-3, 250 × 4.6 mm, 5 mm, by HICHROM.

Standard solutions: clioquinol solution at 120 µg/mL concentration in acetone, domiphen bromide solution at 3 µg/mL concentration in acetone, tripelennamine hydrochloride solution at 120 µg/mL concentration in water obtained by extraction in the following procedure: 5.0 mL of aqueous solution of tripelennamine hydrochloride at 1.2 mg/mL concentration was transferred to a separator, with 30 mL of hexane and 15 mL of water added, and handled as described in the sample preparation procedure for concentration determination of tripelennamine hydrochloride. (see: content determination)

Development of the method

Chromatographic conditions

First, suitable wavelengths were determined based on the recorded spectra of the substances in acetone in the 200–350 nm range using DAD detector. The selected wavelengths were: λ = = 215 nm for domiphen bromide assay, and λ = 310 nm for tripelennamine hydrochloride and clioquinol. Next, a specific HPLC system has been sought, which would provide identification and sufficient separation of the three substances characterized by significantly different polarity.

Therefore, a number of chromatographic reversed-phase HPLC systems were tested, i.e., columns of various polarities (YMC-Pack C4, 5 µm, 150 × 4.6 mm by YMC Co. Ltd.; Spherimage-80 C6, 5 µm, 125 × 4.0 mm by Knauer; Symmetry C8, 5 µm, 250 × 4.6 mm by Waters; Supelcosil ABZ+PLUS, 5 µm, 250 × 4.6 mm by SUPELCO; CPS-2 Hypersil, 5 µm, 250 × 4.6 mm by Thermo; Inertsil ODS-3, 5 µm, 250 × 4.6 mm by HiChrom) and mobile phases of different compositions, containing the most popular solvents: methanol, acetonitrile and tetrahydrofuran. No system with isocratic elution was found, which would allow to separate and identify the three active substances within a relatively short time period. So, different variants of the gradient elution were tested.

Low polarity columns (C18) were found eligible to obtain a satisfactory separation of the tested substances with a suitable gradient elution.

The following chromatographic system was finally selected: Inertsil ODS-3 chromatographic column, 250 × 4.6 mm, 5 µm; temperature: 40°C; mobile phase A: 1.0 mL H_3PO_4 in 1.0 mL of water; mobile phase B: acetonitrile

Gradient elution:

time (min)	mobile phase B (%)
0 – 5	10
5 – 12	10 → 85
12 – 20	85
20 – 22	85 → 10
22 – 25	10

Mobile phase flow rate of 1.5 mL/minute and injection volume 50 µL were applied.

Sample solutions

The following solvents were tried for an efficient isolation of drug substances from the ointment: methanol, ethanol, acetonitrile, tetrahydrofuran, dimethylformamide and acetone.

All active substances: tripelennamine hydrochloride, domiphen bromide and clioquinol were well soluble in acetone, which was used to extract them as follows:

– in a one step without heating, or with heating up to 60°C, or with heating up to 80°C.

The ointment was weighted and transferred to a 50 mL volumetric flask, approximately 40 mL of acetone was added, then agitated on mechanical shaker for approximately 30 min or heated in a water bath for approximately 30 min. The heater temperature was 60 or 80°C. The solutions were cooled to room temperature, made up to 50 mL volume and filtered through cellulose filter (84 g/m^2).

– A triple procedure with heating up to 40°.

Table 1. Effect of applied extraction methods on determination of active substances.

Substance determined [declared content]	Solvent: acetone			
	Single extraction without heating	Triple extraction 30 min/ 40°C	Single extraction 30 min/ 60°	Single extraction 30 min/ 80°
	Found amount of active substance [mg/g]	Found amount of active substance [mg/g]	Found amount of active substance [mg/g]	Found amount of active substance [mg/g]
Tripelennamine hydrochloride [20 mg/g]	13.14 16.74 16.81 16.00	18.44 17.84 17.54 18.33	18.64 19.08 17.69 17.23	15.28 16.44 19.26 18.87
	Mean = 15.67 mg	Mean = 18.04 mg	Mean = 18.16 mg	Mean = 17.46 mg
	RSD = 11.02%	RSD = 2.34%	RSD = 4.68%	RSD = 10.98%
Clioquinol [20 mg/g]	19.61 20.68 19.39 19.90	18.89 19.18 19.30 19.22	19.35 19.78 19.44 19.77	19.68 19.35 19.23 19.77
	Mean = 19.90 mg	Mean = 19.15 mg	Mean = 19.59 mg	Mean = 19.51 mg
	RSD = 2.83%	*Mean* = 0.93%	RSD = 1.14%	RSD = 1.33%
Domiphen bromide [0.5 mg/g]	0.48 0.44 0.42 0.42	0.49 0.47 0.47 0.46	0.48 0.46 0.43 0.47	0.49 0.50 0.48 0.49
	Mean = 0.44 mg	Mean = 0.47 mg	Mean = 0.46 mg	Mean = 0.49 mg
	RSD = 6.43%	RSD = 2.41%	RSD = 4.70%	RSD = 2.04%

The ointment was weighted and transferred to a conical flask, approximately 15 mL of acetone was added and heated in a water bath at 40°C for 30 min. The solution was cooled during a few minutes in refrigerator until excipients were precipitated; after that the supernatant was filtered to a 50 mL volumetric flask through cellulose filter (84 g/m^2). The operation was repeated twice and then the volumetric flask was made up to 50 mL with acetone.

RESULTS AND DISCUSSION

The results shown in Table 1 proved that acetone was a suitable solvent for extraction of clioquinol and domiphen bromide from the Viosept ointment, preferably at 80°C. However, the results for tripelennamine hydrochloride assay after extraction in acetone were unrepeatable and too low (Table 1). Therefore, acetone was not suitable for the content determination of tripelennamine. For this compound a different method was needed. An extraction hexane-water was found to be the most suitable one (see: content determination). The obtained results are shown in Table 2.

Method validation

Specificity

To verify the specificity of the method, the solvent (acetone), the solutions of standard active substances, and of potential impurities originating from clioquinol (A, B, C acc. to Ph. Eur.) were injected onto the column. Figure 2 shows a chromatogram of the standards' mixture in acetone (approximately 0.1 mg/mL) at 215 nm and 310 nm. The peaks of the solvent (acetone), impurities and placebo do not interfere with the peaks of the actives substances.

Linearity

Linearity was tested within a concentration range: 5–160 μg/ml for clioquinol (in acetone, λ = 310 nm); 0.4–6 μg/mL for domiphen bromide (in acetone, λ = 215 nm), 2–200 μg/mL for tripelennamine hydrochloride (in water, λ = 310 nm). Table

Table 2. Effect of applied extraction method on determination of tripelennamine hydrochloride (triple extraction; hexane-water).

Substance determined	Declared content [mg/g]	Found amount of active substance [mg/g]	Mean of found amount of active substance [mg/g]	RSD [%]
Tripelennamine hydrochloride	20.0	20.83 21.19 20.78 21.13	20.98	0.99

Table 3. Calibration curve parameters.

	λ = 310 nm		λ = 215 nm
	Clioquinol	Tripelennamine hydrochloride	Domiphen bromide
Line equation (n = 6)	y =36436.3x + 69143.5	y = 95918.7x - 2536.5	y = 63212.2x - 8610.6
Standard error of the estimate S_y	17381	19614	2322
Correlation coefficient (r)	0.9999	0.9999	0.9996
Limit of detection (LOD)	1.6 μg/mL	0.7 μg/mL	0.1 μg/mL
Limit of quantitation (LOQ)	4.8 μg/mL	2.0 μg/mL	0.4 μg/mL

3 shows the linear regression parameters with correlation coefficients, which are very close to unity.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were established based on the calibration curve parameters: LOD = $3.3 \cdot S_y/a$ and LOQ = $10 \cdot S_y/a$, where S_y = standard error of estimation, a = slope of a straight line. Results are presented in Table 3.

Validation of the chromatographic system

Precision

Standard solutions: acetone solution of domiphen bromide (3 μg/mL), clioquinol (120 μg/mL) and aqueous solution of tripelennamine hydrochloride (120 μg/mL) were injected onto the column six times. The RSD of the peak areas ranged from 0.2% (tripelennamine), 0.4% (clioquinol) to 1.5% (domiphen bromide). RSD ≤ 2.0% was chosen as an acceptance criterion.

Accuracy

Weighted portions of standards of the active substances (80, 100 and 120% of the declared content) were added to approx. 0.3 g of placebo. The samples were analyzed for content determination. Recovery of 98.0–102.0% (Table 4) confirmed accuracy of the method.

Robustness

The effect of the mobile phase flow ratio (± 0.2 mL/min), column temperature (± 5°C), composition of the mobile phase A (± 0.2 mL H_3PO_4) and of the mobile phase B (ACN or ACN : H_2O 9 : 1, v/v), and column type on retention times, symmetry factor (A_S) and resolution (R_S) were evaluated for the standard mix solutions (approximately 0.1 mg/mL). The results are presented in Table 5.

Stability of solutions

Stability studies were performed for tripelennamine hydrochloride and clioquinol standard solu-

Table 4. Results of recovery in the Viosept ointment.

Substance determined	Amount added [declared content]	Recovery [%]	Mean value of recovery [%]	RSD [%]
Tripelennamine hydrochloride	96 μg/mL 80%	99.32 98.75 99.02	99.03	0.29
	120 μg/mL 100%	99.48 98.72 101.12	99.77	1.23
	144 μg/mL 120%	99.22 98.56 100.10	99.63	0.45
Clioquinol	96 μg/mL 80%	100.70 100.33 100.30	100.44	0.22
	120 μg/mL 100%	100.19 100.63 100.91	100.58	0.36
	144 μg/mL 120%	99.80 100.48 101.09	100.46	0.65
Domiphen bromide	2.4 μg/mL 80%	98.29 100.84 99.43	99.52	1.28
	3 μg/mL 100%	102.00 99.30 102.00	101.10	1.54
	3.6 μg/mL 120%	102.00 99.30 102.00	100.87	1.37

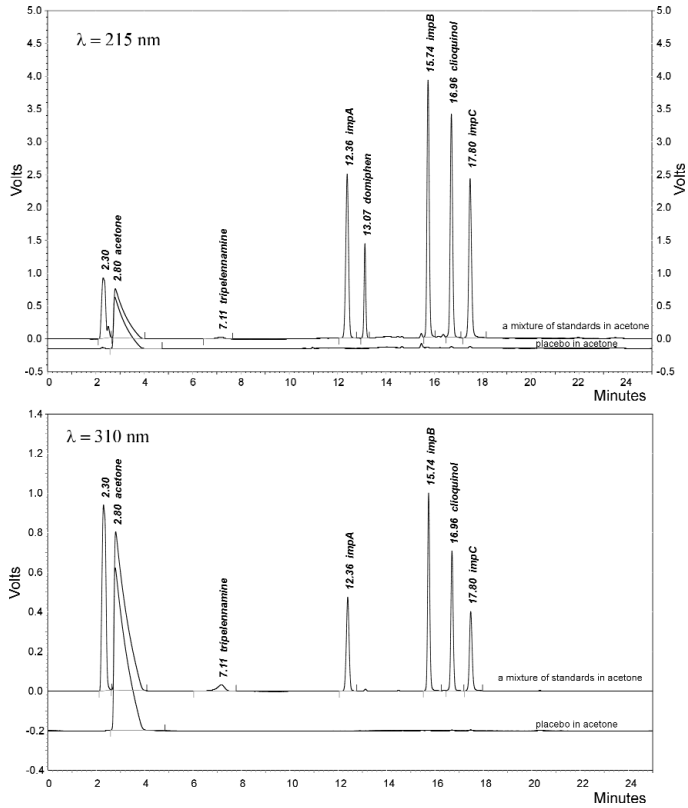


Figure 2. Chromatograms of a mixture of standards: tripelennamine, clioquinol, domiphen, impurities originating from clioquinol (approximately 0.1 mg/mL) and placebo in acetone, at λ = 215 nm and λ = 310 nm

tions. Determination of impurities originating from domiphen was omitted due to its stability and low dose in the product (0.5 mg/g).The aqueous and acetone solutions of tripelennamine hydrochloride (120 µg/mL) stored at ambient temperature were assayed after 12 and 48 h. The RSD of the assay of tripelennamine were within 1% (n = 6). The stability experiments showed that no significant changes in the content of impurities were observed in acetone or aqueous solution either. In both cases the total impurities percent was lower than the trace level (< 0.05%). This confirms that the standard aqueous solutions of tripelennamine used during the assay were stable for at least 48 h at room temperature.

The effect of the heating process of acetone solutions (up to 80°C for 30 min in a water bath) was also analyzed to make sure that the potential degradation products of tripelennamine did not interfere with determination of the two other actives substances. Similar results (< 0.05% of total impurities) were observed.

Standard solutions of clioquinol (120 µg/mL) in acetone and acetone heated up to 80°C for 30 min in a water bath were also tested after 12 and 48 h. The solutions were protected from light and stored at ambient temperature. The following results, for the solutions heated, or not were obtained: impurity A 1.0%, impurity B 0.6%, impurity C 0.5%, and other impurities in trace quantities. Additionally, stability of clioquinol solutions in methanol, which is used in a method for purity described in the monograph of clioquinol in Ph. Eur., were investigated. No significant effect on the quantity of degradation products was observed.

Content determination

Clioquinol and domiphen bromide

The solutions were prepared in brown volumetric flasks. Approx. 0.3 g of the ointment was weighted and transferred to a 50 mL volumetric flask, then 40 mL of acetone was added and heated in a water bath up to 80°C for approximately 30 min. The solution was cooled to room temperature, made

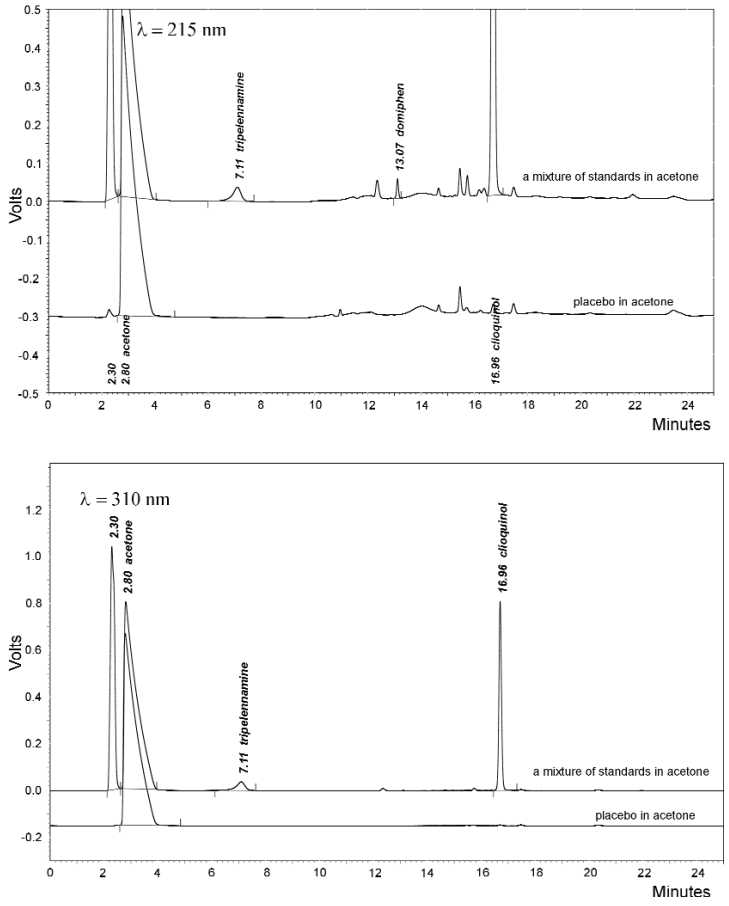


Figure 3. Chromatograms of a Viosept ointment and placebo extracted to acetone at ë = 215 nm (for determination of domiphen) and λ = 310 nm (for determination of clioquinol)

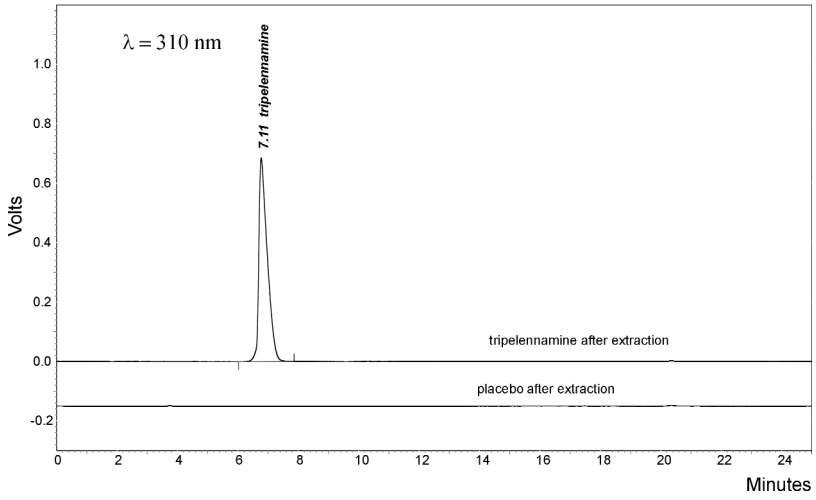


Figure 4. Chromatograms of a Viosept ointment and placebo after extraction with hexane-water, at λ = 310 nm (for determination of tripelennamine)

Table 5. Robustness of the proposed HPLC method.

		Tripelennamine	**Imp. A**	**Domiphen**	**R_S imp. A / domiphen**	**Imp. B**	**Clioquinol**	**Imp. C**	**R_S clioquinol /imp. C**
		RT [min] (A_S)	**RT** [min] (A_S)	**RT** [min] (A_S)		**RT** [min] (A_S)	**RT** [min] (A_S)	**RT** [min] (A_S)	
Flow rate	1.7 mL/min	**6.38** (1.42)	**11.66** (1.10)	**12.67** (1.11)	**5.16**	**15.12** (1.06)	**16.14** (1.09)	**16.98** (1.42)	**3.51**
	1.5 mL/min	**7.11** (1.39)	**12.36** (1.07)	**13.07** (1.08)	**4.89**	**15.74** (1.06)	**16.96** (1.07)	**17.80** (1.42)	**4.08**
	1.3 mL/min	**8.32** (1.48)	**13.13** (1.08)	**13.59** (1.10)	**3.44**	**16.51** (1.06)	**17.75** (1.07)	**18.79** (1.42)	**3.90**
Temperature	45°C	**7.33** (1.43)	**12.40** (1.07)	**13.04** (1.10)	**4.64**	**15.62** (1.08)	**16.67** (1.05)	**17.54** (1.34)	**3.85**
	40°C	**7.11** (1.39)	**12.36** (1.07)	**13.07** (1.08)	**4.89**	**15.74** (1.06)	**16.96** (1.07)	**17.80** (1.42)	**4.08**
	35°C	**6.99** (1.40)	**12.25** (1.10)	**13.10** (1.10)	**5.88**	**15.84** (1.05)	**17.03** (1.06)	**18.02** (1.24)	**3.89**
Mobile phase A	1.2 mL H_3PO_4	**7.06** (1.42)	**12.06** (1.07)	**13.22** (1.08)	**6.48**	**15.72** (1.07)	**16.86** (1.05)	**17.80** (1.34)	**4.38**
	1.0 mL H_3PO_4	**7.11** (1.39)	**12.36** (1.07)	**13.07** (1.08)	**4.89**	**15.74** (1.06)	**16.96** (1.07)	**17.80** (1.39)	**4.08**
	0.8 mL H_3PO_4	**7.84** (1.40)	**12.66** (1.11)	**13.05** (1.11)	**2.78**	**15.78** (1.08)	**16.89** (1.07)	**17.84** (1.34)	**3.82**
Mobile phase B	**ACN**	**7.11** (1.39)	**12.36** (1.07)	**13.07** (1.08)	**4.89**	**15.74** (1.06)	**16.96** (1.07)	**17.80** (1.39)	**4.08**
	ACN:H_2O (9 : 1)	**7.76** (1.40)	**12.59** (1.09]	**13.33** (1.08)	**5.09**	**16.23** (1.13)	**17.67** (1.11)	**18.91** (1.44)	**4.06**
Columns	**s: 2850**	**7.11** (1.39)	**12.36** (1.07)	**13.07** (1.08)	**4.89**	**15.74** (1.06)	**16.96** (1.07)	**17.80** (1.42)	**4.08**
	s:17212722	**6.32** (1.44)	**12.08** (1.19)	**12.94** (1.09)	**6.09**	**15.59** (1.13)	**16.66** (1.16)	**17.55** (1.60)	**3.38**

RT – retention time; **A_s –** symmetry factor; **R_s** – resolution

Table 6. Results and statistic evaluation of assay in the Viosept ointment.

Active substances	Declared amount of active substances [mg/g]	Found amount of active substances [mg/g]	
		Mean X ± ΔX (PU = 95%, n = 6)	RSD [%]
Tripelennamine hydrochloride	20.0	21.04 ± 0.22	0.99
Clioquinol	20.0	19.55 ± 0.27	1.33
Domiphen bromide	0.5	0.46 ± 0.008	1.66

up to 50 mL volume and filtered through a cellulose filter (84 g/m^2).

Aliquots of 50 µL of the prepared standard and sample solutions were injected onto the column. Chromatograms were recorded at two different wavelengths: 310 nm for determination of clioquinol and 215 nm for determination of domiphen. Figure 3 shows chromatograms of sample solutions and placebo in acetone. Table 6 shows the results of determination.

Tripelennamine hydrochloride

Approx. 0.3 g of the ointment was weighted and transferred to a separatory funnel; 30 mL of hexane was added and agitated for approx. 2 min until the base of ointment was dissolved. Next, 15 mL of water was added. The funnel was then agitated for approximately 2 min and left until the layers have separated. The water layer was transferred to a 50 mL volumetric flask. The extraction was then repeated twice, and the water layers were collected in the flask, made up to volume and filtered through a cellulose filter (84 g/m^2).

Aliquots of 50 µL of the prepared standard and sample solutions were injected onto the column. Figure 4 shows chromatograms of the sample and placebo solutions after extraction with hexane-water. Table 6 shows the results of determination.

CONCLUSIONS

A simple HPLC method for the quantitative determination of three active substances: domiphen bromide, tripelennamine hydrochloride, and clioquinol in the Viosept ointment was optimized and validated. A C18 reversed-phase column and a gradient elution were used.

The developed method was selective, precise and accurate, and complied with the acceptance criteria, including symmetry factor ($A_S < 1.5$) and resolution ($R_S > 2.0$) between all peaks. The specificity was confirmed as the peaks originating from the solvent, placebo, impurities and drug substances did not interfere. Calibration curves of the three active substances were linear ($r \geq 0.999$). Values of recovery of the substances from the ointment were within the range 98.0–102.0%.

REFERENCES

1. Kelly M.T., Smyth M.R., Dadgar D.: Analyst 114, 1377 (1989).
2. Dolan J.W, Snyder L.R., Djordjevic N.M., Hill D.W., Waeghe T.J.: J. Chromatogr. A 857, 1 (1999).
3. Gil-Agusti M., Monferrer-Pons L., Esteve-Romero J., Garcia-Alvarez-Coque M.C.: J. AOAC Int. 84, 1687 (2001).
4. Capella-Peiro M.E., Gil-Agusti M., Monferrer-Pons L., Esteve-Romero J.: Anal. Chim. Acta 454 125 (2002).
5. Bose D., Durgbanshi A., B Capella-Peiro M.E., Gil-Agusti M., Carda-Broch S., Esteve-Romero. J.: Indian J. Chem. 43, 9 (2004).
6. Martínez-Algaba C., Bermúdez-Saldaña J.M., Villanueva-Camañas R.M., Sagrado S., Medina-Hernández M.J.: J. Pharm. Biomed. Anal. 40, 312 (2006).
7. Leung G.N.W., Tang H.P.O., Tso T.S.C., Wan T.S.M.: J. Chromatogr. A 738, 141 (1996).
8. Rambla-Alegre M., Paris-Vincente J., Esteve-Romero J., Capella Peiró M.E., Bose D.: Anal. Chim. Acta 666, 102 (2010).
9. Lima J.L.F.C., Montenegro M.C.B.S.M., Sales M.G.F.: J. Pharm. Biomed. Anal. 14, 931 (1996).
10. San Andres M.P., Sicilia D., Rubio S., Perez Bendito D.: Analyst 114, 1079 (1989).

11. Mackell M.A., Poklis A.: J. Chromatogr. 235, 445 (1982).
12. Thomson H.C. Jr., Holder C.L., Althaus J.R.: Talanta 30, 251 (1983).
13. Chen X.H., Franke J.P., Wijsbeek J., De Zeeuw R.A.: J. Anal. Toxicol. 18, 150 (1994).
14. Ragno G., Ioele G., Risoli A.: Anal. Chim. Acta 512, 173 (2004).
15. Martínez-Gómez M.A., Carril-Avilés M.M., Sagrado S., Vilanueva-Camañas R.M., Medina Hernández M.J.: J. Chromatogr. A 1147, 261 (2007).
16. Gimenez-Izquierdo J., Guiteras J., Izquierdo A, Prat M.D.: Fresenius J. Anal. Chem. 341, 638 (1991).
17. Van De Vaart F.J., Hulshoff A., Indemans A.W.M.: J. Pharm. Biomed. Anal. 1, 507 (1983).
18. Rizk M., Belal F., Ibrahim S., Ahmed S., Sheribah Z.A.: J. Pharm. Biomed. Anal. 27, 813 (2002).
19. Bondiolotti G.P.,Pollera C., Pirola R., Bareggi S.R.: J. Chromatogr. B 837, 87 (2006).
20. Chen L.H., Price C.E., Goerke A., Lee A.L., DePhillips P.E.: J. Pharm. Biomed. Anal. 40, 964 (2006).

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FAST DETERMINATION OF DIPHENHYDRAMINE HYDROCHLORIDE IN RECONSTITUTABLE SYRUPS BY CWT, PLS AND PCR METHODS

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Abstract: Diphenhydramine hydrochloride (DPH), a histamine H1-receptor antagonist, is widely used as antiallergic, antiemetic and antitussive drug found in many pharmaceutical preparations. In this study, a new reconstitutable syrup formulation of DPH was prepared because it is more stable in solid form than that in liquid form. The quantitative estimation of the DPH content of a reconstitutable syrup formulation in the presence of pharmaceutical excipients, D-sorbitol, sodium citrate, sodium benzoate and sodium EDTA is not possible by the direct absorbance measurement. Therefore, a signal processing approach based on continuous wavelet transform was used to determine the DPH in the reconstitutable syrup formulations and to eliminate the effect of excipients on the analysis. The absorption spectra of DPH in the range of 5.0–40.0 µg/mL were recorded between 200–300 nm. Various wavelet families were tested and Biorthogonal1.1 continuous wavelet transform (BIOR1.1-CWT) was found to be optimal signal processing family to get fast and desirable determination results and to overcome excipient interference effects. For a comparison of the experimental results obtained by partial least squares (PLS) and principal component regression (PCR) methods were applied to the quantitative prediction of DPH in the mentioned samples. The validity of the proposed BIOR1.1-CWT, PLS and PCR methods were achieved analyzing the prepared samples containing the mentioned excipients and using standard addition technique. It was observed that the proposed graphical and numerical approaches are suitable for the quantitative analysis of DPH in samples including excipients.

Keywords: diphenhydramine hydrochloride, reconstitutable syrup formulation, continuous wavelet transform, quantitative analysis

Most of the population have not preferred the use of solid oral dosage forms due to their swallowing problem. In this reason, oral pharmaceutical syrup is one of the most favorable dosage forms for pediatric patients or patients unable to tolerate solid dosage forms. The liquid form has been preferred because it is useful for swallowing and flexibility in the administration of doses (1, 2).

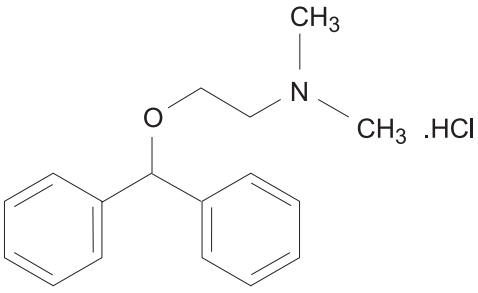

Figure 1. Molecular structure of diphenhydramine hydrochloride (DPH)

Diphenhydramine hydrochloride (DPH) named as 2-(diphenylmethoxy)-*N*,*N*-dimethylethylamine hydrochloride (Fig. 1), which is a first generation antihistamine, has been mainly used for the treatment of allergies and itchiness, insomnia, motion sickness, and extrapyramidal symptoms. Additionally, DPH has significant antitussive activity. The syrups containing DPH have been used as a cough suppressant for the control of cough due to colds or allergy (3). Recently, the use of DPH in combination with other drugs has been reported as antiemetic for the prevention of cisplatin-induced emesis in chemotherapy treatment. Furthermore, it has been used as sedative in dentistry for children and in local anesthesia (4–6). DPH oral syrups or elixirs are available commercially. However, previous studies described that DPH is more stable in solid form than

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that in liquid form (7). Therefore, we focused mainly on the preparation of a reconstitutable syrup formulation of DPH. DPH was distributed in dry mixture and reconstituted with required volume of water before administration to form oral liquid syrup.

Several methods including capillary electrophoresis (8–10), atomic absorption spectrometry (11), fluorometry (12), flow injection analysis (13, 14) and spectrophotometry (15–17) have been proposed for the determination of DPH in pharmaceutical preparations. Many chromatographic methods such as gas chromatography (18), liquid chromatography (19, 20) and high performance liquid chromatography (HPLC) (7, 21, 22) have been used for the analysis of DPH in samples.

In the existing interferences of pharmaceutical excipients, traditional spectrophotometric methods are not suitable for the quantitative resolution of single component and multicomponent formulations. In this case, the use of a separation method like HPLC is an answer in order to solve the mentioned problem. However, in some cases, HPLC approach may not give expected results due to similar chemical and physical properties of analytes with excipients. In addition, this separation procedure requires long period of analysis time, high cost and tedious procedures. It is clear that there is a need of new analytical powerful method for resolving these problems. Recently, wavelet transform methods have been used for the signal processing tools in many branches due to versatile and flexible properties (23–26).

In previous studies, some applications of the continuous wavelet transform (CWT) to the resolution of overlapped spectra for the quantitative determination of multicomponent mixtures were reported (27–32).

Chemometric calibration methods, partial least squares (PLS) and principal component regression (PCR) are popular methods for the quantitative resolution of the multicomponent mixture system. Recently, these chemometric methods have been used as alternative methods for several analytical problems from qualitative analysis to quantitative analysis in analytical chemistry and related branches (33–36).

The purposes of this study were to prepare a new pharmaceutical formulation and to improve new analytical methods for the quantification of DPH in its samples containing pharmaceutical excipients. The first aim is the preparation of reconstitutable syrup formulation of DPH. The second aim is the quantitative analysis of DPH in the prepared reconstitutable syrup formulation in the presence of pharmaceutical excipients, D-sorbitol, sodium citrate, sodium benzoate and sodium EDTA, by using CWT signal processing, PLS and PCR methods without requiring any separation step. We observed that the improved CWT, PLS and PCR methods gave satisfactory results for the quantitative estimation of the DPH content in a reconstitutable syrup formulation in spite of the spectral interferences of the pharmaceutical excipients.

EXPERIMENTAL

Reagents and equipment

Reagents were: diphenhydramine hydrochloride (DPH) (Bilim Pharmaceuticals, Turkey), D-sorbitol and sodium citrate (Merck, Germany), sodium benzoate and sodium EDTA (Sigma-Aldrich, Germany). All other chemicals were of analytical grade and distilled water was used for all experiments.

Equipment used: double-beam UV-visible spectrophotometer (Shimadzu UV-2550, Japan).

METHODS

Preparation of the dry mixtures for reconstitutable syrup formulation

In order to produce dry mixture for reconstitution, all the powder components were reduced to more or less the same particle size. To obtain a homogeneous mixture, ingredients were mixed based on

Table 1. Composition of the dry mixture for syrup formulation.

Component	g/100 mL
Diphenhydramine HCl (DPH)	0.250
D-sorbitol	24.29
Sodium citrate	1.27
Sodium benzoate	0.2
Sodium EDTA	0.0074

Table 2. Training (concentration) set of DPH in the presence of excipients.

No.	DPH	µg/mL			
		Sodium benzoate	Sodium citrate	Sodium EDTA	D-sorbitol
1	5	10	10	10	10
2	10	20	20	20	20
3	15	30	30	30	30
4	20	40	40	40	40
5	25	40	40	40	40
6	30	30	30	30	30
7	35	20	20	20	20
8	40	10	10	10	10

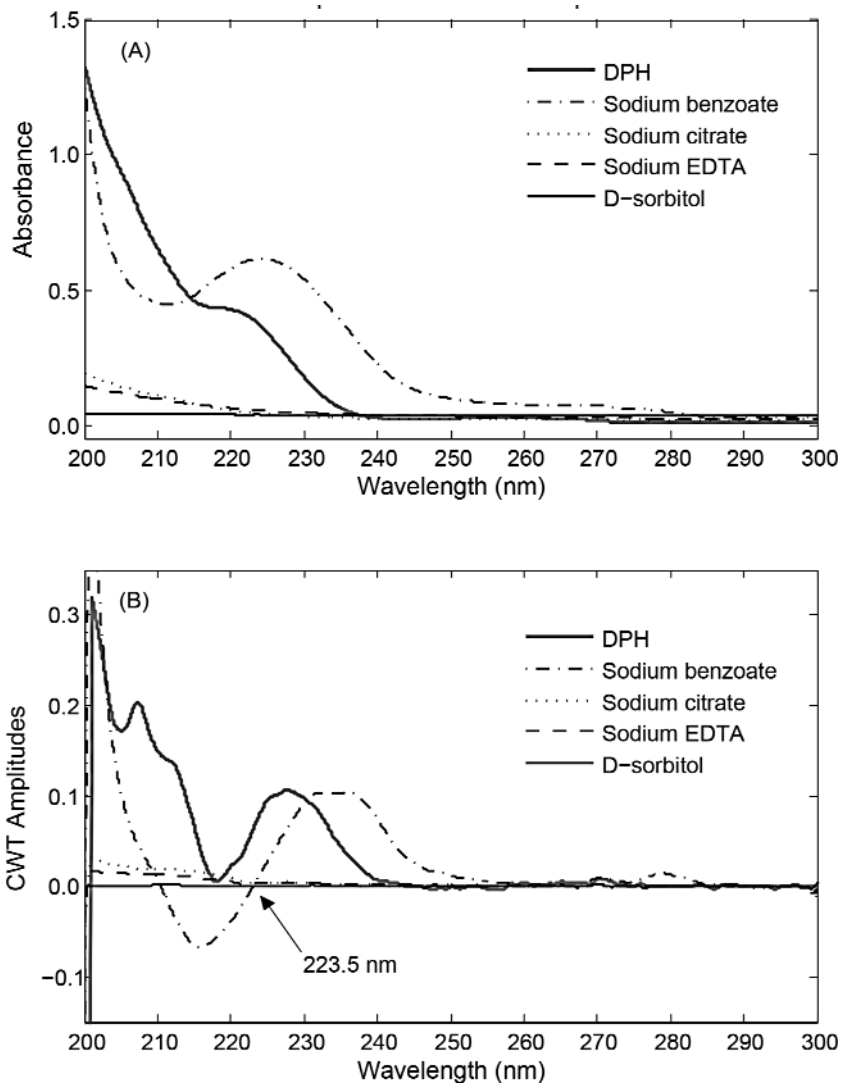

Figure 2. (A) Absorption spectra and (B) CWT spectra of DPH (——), sodium benzoate (– · –), sodium citrate (·····), sodium EDTA (– – –) and D-sorbitol (——) in distilled water

geometric dilution, which is a method used in mixing two or more ingredients of unequal quantities. Briefly, mixing process was started with the smallest quantity (sodium EDTA) and the equal of the other of the larger amount (sodium benzoate and DPH) were added. The same procedure was followed for sodium citrate and D-sorbitol until all ingredients were used up (37, 38). The representative formulation for the preparation of dry mixture is tabulated in Table 1.

For the preparation of the reconstituted syrup formulation, an appropriate amount of water was added to the dry syrup powder in two steps and stirred with spoon until a homogenous product was obtained.

Preparation of the standard solutions

The stock solution of DPH was prepared by dissolving 25 mg of DPH in 100 mL of distilled water. Additionally, the stock solutions of pharmaceutical excipients were prepared by dissolving an appropriate amount of each excipient in 100 mL of distilled water.

In case of the CWT signal processing method, the calibration solutions of DPH in the range of 5.0–40.0 µg/mL were obtained from the stock solution. In case of the PLS and PCR, a training (concentration) set of eight different mixtures containing DPH with pharmaceutical excipients was prepared (Table 2). An independent validation samples containing DPH with pharmaceutical excipients were prepared by using stock solution. Standard addition samples were obtained by adding the DPH stock solutions (at the concentration levels, 5, 10, 20, 30, 35 µg/mL) to the prepared syrup samples.

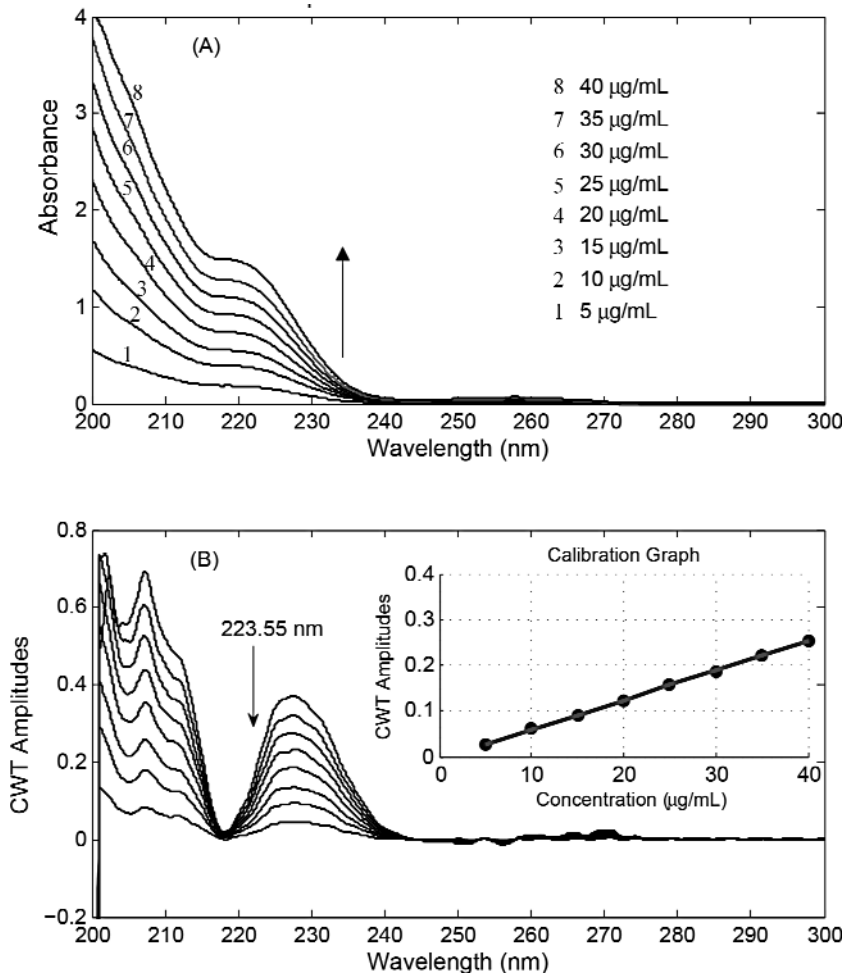

Figure 3. (A) Absorption spectra of DPH in the concentration range of 5.0–40.0 µg/mL in distilled water, (B) CWT spectra and calibration curve of DPH in the concentration range of 5.0–40.0 µg/mL in distilled water

Table 3. Calibration parameters and limits of detection, LOD and LOQ in μg/mL for Bior1.1-CWT calibration.

Parameters	
Concentration range (μg/mL)	5.0–40.0
λ (nm)	223.55
m	6.4×10^{-3}
n	6.4×10^{-3}
r	0.9998
SE (m)	0.0000
SE (n)	1.1×10^{-3}
SE (r)	1.4×10^{-3}
LOD	1.44
LOQ	4.80

*m = slope of the regression function, n = intercept of the regression function, r is correlation coefficient of the regression function, SE (m) = standard error of the slope, SE (n) = standard error of the intercept, SE (r) = standard error of the correlation coefficient, LOD = limits of detection, LOQ = limits of quantification.

Analysis procedure of dry powder and syrup formulation

The samples taken from three different points of dry syrup powder equivalent to 25 mg of DPH were weighed and dissolved in distilled water, filtered into a 100 mL calibrated flask to remove the insoluble matter and diluted to volume with water. The obtained solutions were diluted to the working concentration range with distilled water. The same sample preparation procedure was applied to the reconstituted syrup formulation. Absorption spectra of the resulting solutions of both dry powder and syrup formulation were recorded as well as calibration solutions.

RESULTS, DISCUSSION AND CONCLUSION

As it can be seen from Figure 2a, the absorption spectra of DPH and pharmaceutical excipients strongly overlap in the spectral region of 200–300 nm. Due to the mutual spectral interferences of DPH and pharmaceutical excipients in the same spectral region, the direct spectrophotometric determination of DPH in its syrup samples is not possible by traditional spectral analytical methodologies (13, 18). For the rapid and reliable resolution of this problem, we focused mainly on the application of the CWT signal processing approach to quantitative analysis of DPH in dry powder and syrup forms. Additionally, PLS and PCR calibrations were used

Table 4. Results obtained by the application of Bior1.1-CWT, PLS and PSR methods to the standard addition samples.

	DPH (n = 3)													
	BIOR1.1-CWT					PLS method					PCR method			
Added	Predicted	SD	RSD	BIAS	Recovery (%)	Predicted	SD	RSD	BIAS	Recovery (%)	Predicted	SD	RSD	BIAS	Recovery (%)
5	4.87	0.08	1.66	–2.60	99.8	4.99	0.04	0.76	–0.17	99.8	5.07	0.12	2.38	1.37	101.4
10	10.08	0.20	1.99	0.80	99.7	9.84	0.06	0.64	–1.60	98.4	9.89	0.19	1.88	–1.13	98.9
20	19.91	0.28	1.41	–0.45	99.9	18.97	0.41	2.17	–5.14	94.9	19.01	0.46	2.43	–4.97	95.0
30	30.38	0.19	0.64	1.26	100.7	28.99	0.85	2.93	–3.37	96.6	28.79	0.54	1.86	–4.03	96.0
35	34.93	0.02	0.04	–0.20	100.0	33.02	0.54	1.62	–5.65	94.3	33.39	0.82	2.45	–4.61	95.4

*SD = standard deviation, RSD = relative standard deviation.

for comparison of the assay results provided by the improved CWT approach with those obtained by applying PLS and PCR (32). The proposed methods gave us sensitive, selective, accurate and precise results for the fast analysis of DPH in samples.

In order to get the optimal wavelet analysis, various wavelet families at different scale factor (a) were applied the absorption spectra of DPH and pharmaceutical excipients (Fig. 2) and BIOR1.1-CWT (a = 40) among wavelet families was found to be an optimal signal analysis approach for the DPH. As illustrated in Figure 2b, the concentration of DPH is proportional to the BIOR1.1-CWT signal at the wavelength 223.55 nm, corresponding to the zero-crossing point for all pharmaceutical excipients (D-sorbitol, sodium citrate, sodium benzoate and sodium EDTA). The absorption spectra of the DPH calibration solutions were recorded between 200 and 300 nm as shown in Figure 3a and the BIOR1.1-CWT spectra were obtained using CWT procedure (Fig. 3b). An analogous procedure was applied to all samples. Calibration curve for the related compound was obtained by measuring the BIOR1.1-CWT amplitudes at 223.55 nm. The calibration curve and corresponding statistical results in the application of the linear regression analysis to the concentrations and BIOR1.1-CWT amplitudes were summarized in Figure 3b and Table 3. The content of DPH in samples was determined by the computed calibration curve.

In case of the PLS and PCR applications, a training (concentration) set of the mixture solutions in the concentration range of 5.0–40.0 μg/mL of DPH and pharmaceutical excipients (D-sorbitol, sodium citrate, sodium benzoate and sodium EDTA) in possible combinations were prepared to construct the PLS and PCR calibrations. Table 2 shows the training set of DPH in the presence of pharmaceutical excipients. The absorbance data matrix corresponding to the training set was obtained by measuring the absorbance values at the wavelengths set with the intervals of $\Delta\lambda$ = 0.05 nm in the spectral range 200.0–300.0 nm. Two chemometric calibrations based on the relationship between the training

Table 5. Stastistical parameters for PLS and PCR calibration.

	DPH	
Parameters	PLS	PCR
SEC	0.278	0.269
PRESS	0.540	0.577
SEP	0.894	0.832

*SEC = standard error of calibration,
PRESS = prediction residual error sum-of-squares,
SEP = standard error of prediction.

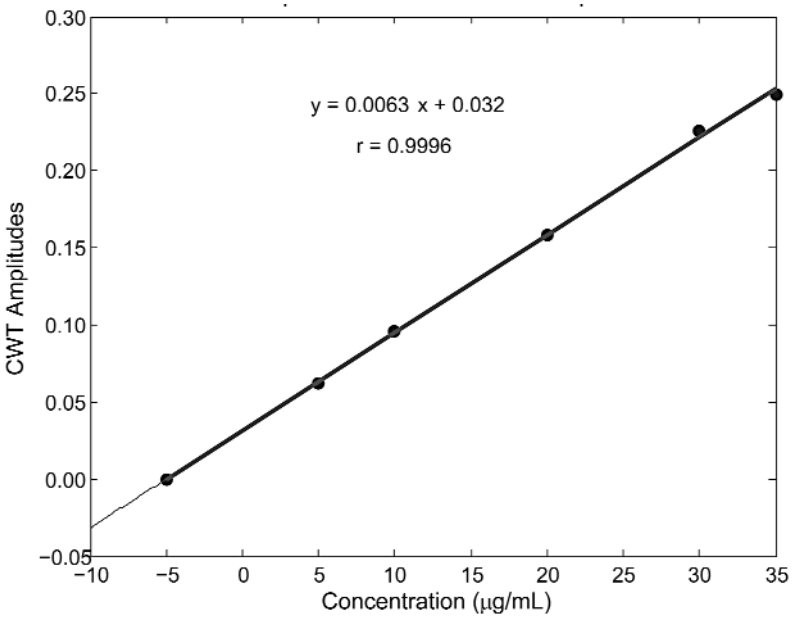

Figure 4. A plot of the standard addition samples, obtained by using the relationship between BIOR1.1-CWT amplitudes and concentration

Table 6. DPH content of dry powder and syrup formulation by Bior1.1-CWT, PLS and PCR approach.

	mg/100 mL											
	Dry powder sample 1			Dry powder sample 2			Dry powder sample 3			Syrup		
	BIOR1.1-CWT	PLS	PCR	BIOR1.1-CWT	PLS	PCR	BIOR1.1-CWT	PLS	PCR	BIOR1.1-CWT	PLS	PCR
Mean	25.692	25.261	25.144	27.385	26.330	26.199	26.639	25.389	25.284	25.539	25.549	25.482
SD	0.335	0.377	0.367	0.137	0.533	0.532	0.248	0.724	0.730	0.162	0.388	0.383
RSD	1.305	1.490	1.459	0.501	2.024	2.031	0.930	2.854	2.887	0.635	1.519	1.503

*SD is standard deviation for n = 10 observations, RSD is relative standard deviation.

set and absorbance data matrix were calculated by applying the PLS and PCR algorithms. PLS and PCR calibrations obtained were used for the prediction of the amount of DPH in its samples containing pharmaceutical excipients.

In case of the BIOR1.1-CWT process, a good linearity for regression equation was observed. Correlation coefficient was found to be 0.9998 as shown in Table 3. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated to be 1.44 and 4.80 µg/mL, respectively (Table 3). The applicability and validity of the improved BIOR1.1-CWT were tested by analyzing the mixtures of DPH and pharmaceutical excipients. Recovery and standard deviation for the proposed CWT signal processing method were illustrated in Table 4. It is clear that these results indicate good accuracy and precision for the application of the improved CWT method to the analysis of the mixtures containing active compound and pharmaceutical excipients. For the observation of the pharmaceutical excipients' effects on the analysis of DPH, standard addition technique was applied by adding 5 different concentration levels (5, 10, 20, 30 and 35 µg/mL) to the syrup samples containing 5 µg/mL DPH. The concentration values of the standard addition samples were plotted against the values of the BIOR1.1-CWT amplitudes and a straight line was observed as shown in Figure 4. The results indicate that the linear regression slope of standard addition samples is close to the slope of the calibration equation of DPH (Table 3 and Fig. 4). This uncovers that no interference of pharmaceutical excipients was observed during the application of the proposed BIOR1.1-CWT approach to the standard addition samples, dry powders and syrup samples.

In order to test the applicability and validity of the PLS and PCR methods, the DPH samples containing pharmaceutical excipients were analyzed by PLS and PCR in the calibration and prediction steps. Predictive ability of the proposed chemometric calibration method was checked by the standard error of calibration (SEC), the predicted residual error sum-of-squares (PRESS) and the standard error of prediction (SEP). Table 5 shows the SEC, PRESS and SEP values calculated by using the actual and predicted concentrations. It was observed that the applicability and predictive validity of PLS and PCR gave satisfactory results. As a result, percent mean recoveries with the relative standard deviations were found as 94.3–99.8 ± 0.64–2.93% and 95.0–101.4 ± 1.86–2.45% for DPH using PLS and PCR methods, respectively (Table 4). These experimental results indicate that the proposed chemometric numerical and signal processing meth-

ods are suitable for the simultaneous determination of DPH in dry powder and syrup formulations.

The CWT, PLS and PCR methods were applied to analysis of the dry powder and syrup samples containing DPH and pharmaceutical excipients and the obtained results are presented in Table 6. A good agreement was observed for the assay results with the prepared amount of the DPH in the dry powder and syrup composition. Furthermore, it was observed that the determination results obtained from three different dry powder samples are very close to each other. These results showed that DPH and pharmaceutical excipients were mixed homogeneously. As reported in previous studies (37, 38), mixing of active and inactive compounds according to the principle of the geometric dilution resulted in homogeneous distribution of active compound in dry powder samples.

A reconstitutable syrup formulation of DPH containing D-sorbitol, sodium citrate, sodium benzoate, and sodium EDTA as pharmaceutical excipients was successfully prepared in this study. The proposed CWT, PLS and PCR chemometric methods are rapid, precise, and accurate for the simultaneous resolution of dry powder and syrup formulations containing DPH and pharmaceutical excipients having strongly overlapping spectra. The obtained results indicate that the CWT, PLS and PCR calibration methods are very suitable for the analysis of DPH in the presence of pharmaceutical excipients without using chemical pretreatments.

REFERENCES

1. Rhodes C.T.: in Modern Pharmaceutics, Banker G.S., Rhodes C.T. Eds., p. 339, Dekker, New York 1990.
2. Kolling W.M., Ghosh T.K.: in Theory and Practice of Contemporary Pharmaceutics, Jasti B.R., Ghosh T.K. Eds., p. 367, CRC Press, Boca Raton 2005.
3. Remington: The Science and Practice of Pharmacy, 24th edn., Gennaro A.R. Ed., Lippincott Williams and Wilkins, Philadelphia 2000.
4. Goodman-Hillman A., Rall T., Nier A., Taylor P.: The Pharmacological Basis of Therapeutics, McGraw-Hill, New York 1996.
5. Tu R.H., Grewall P., Leung J.W., Suryaprasad A.G., Sheykhzadeh P.I., Doan C., Garcia J.C. et al.: Gastrointest. Endosc. 63, 87 (2006).
6. Hung C.H., Chu C.C., Chen Y.C., Chen Y.W., Li Z.Y., Wang J.J.: Eur. J. Pharmacol. 673, 20 (2011).
7. Yuan H.P., Locke D.C.: Drug Dev. Ind. Pharm. 17, 2319 (1991).
8. Marchesini A.F., Williner M.R., Mantovani V.E., Robles J.C., Goicoechea H.C.: J. Pharm. Biomed. Anal. 31, 39 (2003).
9. Dong Y., Chen X., Chen Y., Hu Z.: J. Pharm. Biomed. Anal. 39, 285 (2005).
10. Li X., Xu X., Albano D.R., You T.: Analyst 136, 5294 (2011).
11. El Ries M.A., Khalil S.: J. Pharm. Biomed. Anal. 25, 3 (2001).
12. Reguera I.P., Rubio M.G., Diaz A.M.: Anal. Sci. 20, 799 (2004).
13. Tipparat P., Lapanantnoppakhun S., Jakmunee J., Grudpan K.: J. Pharm. Biomed. Anal. 30, 105 (2002).
14. Zhao C., Chai X., Tao S., Li M., Jiao K.: Anal. Sci. 24, 535 (2008).
15. Basavaiah K., Charan V.S.: Farmaco 57, 9 (2002).
16. Desai J., Alexander K., Riga A.: Int. J. Pharm. 308, 115 (2006).
17. Ulu S.T., Elmali F.T.: Spectrochim. Acta A. Mol. Biomol. Spectrosc. 77, 324 (2010).
18. Raj S.V., Kapadia S.U., Argekar A.P.: Talanta 46, 221 (1998).
19. Kumar S., Rurak D.W., Riggs K.W.: J. Mass Spectrom. 33, 1171 (1998).
20. Martínez-Algaba C., Bermúdez-Saldaña J.M., Villanueva-Camañas R.M., Sagrado S., Medina-Hernández M.J.: J. Pharm. Biomed. Anal. 40, 312 (2006).
21. Toothaker R.D., Barker S.H., Gillen M.V., Helsinger S.A., Kindberg C.G., Hunt T.L., Powell J.H.: Biopharm. Drug Dispos. 21, 229 (2000).
22. Deconinck E., Sacré P.Y., Baudewyns S., Courselle P., De Beer J.: J. Pharm. Biomed. Anal. 56, 200 (2011).
23. Daubechies I.: in Ten Lectures on Wavelets, Society for Industrial and Applied Mathematics, Philadelphia 1992.
24. Walczak B.: in Wavelets in Chemistry, Elsevier Press, Amsterdam 2000.
25. Ehrentreich F.: Anal. Bioanal. Chem. 372, 115 (2002).
26. Dinç E., Baleanu D.: in Mathematical Methods in Engineering. Taş K., Tenreiro Machado J.A., Balanu D. Eds., p. 265, Springer, Amsterdam 2007.
27. Dinç E., Baleanu D., Aboul-Enein H.Y.: Farmaco 59, 335 (2004).
28. Dinç E., Özdemir A., Baleanu D.: Talanta 65, 36 (2005).

29. Dinç E., Kaya S., Doganay T., Baleanu D.: J. Pharm. Biomed. Anal. 44, 991 (2007).
30. Afkhami A., Abbasi-Tarighat M.: Talanta 78, 424 (2009).
31. Sohrabi M.R., Kamali N., Khakpour M.: Anal. Sci. 27, 1037 (2011).
32. Dinç E., Baleanu D.: J. AOAC Int. 95, 903 (2012).
33. Kramer R.: Chemometric techniques for quantitative analysis, Marcel Dekker, New York 1998.
34. El-Gindy A., Emara S., Mostafa A.: J. Pharm. Biomed. Anal. 41, 421 (2006).
35. Dinç E., Üstündağ Ö., Baleanu D.: Drug Test. Anal. 2, 383 (2010).
36. Dönmez O.A., Aşçi B., Bozdoğan A., Sungur S.: Talanta 83, 1601 (2011).
37. Morales M.E., Ruiz M.A., López G., Gallardo V.: Drug Dev. Ind. Pharm. 36, 885 (2010).
38. Devrim B., Bozkır A., Canefe K.: Acta Pol. Pharm. Drug Res. 68, 593 (2011).

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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPLC METHOD FOR DETERMINATION OF CEFPIROME SULFATE

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Abstract: The stability-indicating LC assay method was developed and validated for quantitative determination of cefpirome sulfate (CPS) in the presence of degradation products formed during the forced degradation studies. An isocratic HPLC method was developed with Lichrospher RP-18 column, 5 µm particle size, 125 mm × 4 mm column and 12 mM ammonium acetate-acetonitrile (90 : 10 v/v) as a mobile phase. The flow rate of the mobile phase was 1.0 mL/min. Detection wavelength was 270 nm and temperature was 30°C. Cefpirome sulfate as other cephalosporins was subjected to stress conditions of degradation in aqueous solutions including hydrolysis, oxidation, photolysis and thermal degradation. The developed method was validated with regard to linearity, accuracy, precision, selectivity and robustness. The method was applied successfully for identification and determination of cefpirome sulfate in pharmaceuticals and during kinetic studies.

Keywords: method validation, stability-indicating method, cefpirome sulfate

Cefpirome sulfate (CPS, Fig. 1) is a new, parenteral, fourth generation cephalosporin. CPS is effective against Gram-positive bacteria including *Staphylococcus aureus* and Gram-negative microorganisms, such as *Pseudomonas aeruginosa* (1–3). The elements responsible for such spectrum of activity are 2-amino-thiazolylmethoxyimino group in a side-chain in position 7 and cyclopentapyridine in position 3. CPS is used in treatment of various infections such as pneumonia, sepsis, urinary tract infections, and intra-abdominal infections in adult patients (4–7). The suggested administration of cefpirome is 1–6 g daily from two to four divided doses (7). CPS like other cephalosporins have surprisingly few serious side effects, which make it attractive for use in the treatment of a wide variety of serious infections (8). The most common adverse symptoms are nonspecific circulatory disorders (chills, tachycardia, hypertension, nausea, dyspnea, cold perspiration, weak concentration and dizziness). All adverse effects are mild or have moderate severity, are of a short period, improve spontaneously, and recovery is complete. Simultaneously, most of the side effects of β-lactams are caused by their degra-

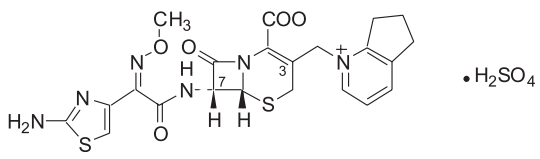

Figure 1. The chemical structure of cefpirome sulfate

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dation products thus it is so important to estimate the stability and mechanism of the degradation of this group of drugs. Stability studies are an integral part of the drug development process and are widely recognized as one of the most important procedures in pharmaceutical products registration (9–12). Previous studies confirm that cephalosporins are susceptible to degradation in aqueous solutions (13–19) and in a solid state (20–28). CPS in solution is stable in pH 4–7, slightly unstable below pH 3 and promptly degraded at pH 9 and higher (17). The degradation pathways in aqueous solutions were described (Fig. 2) (17). Developed chromatographic method for the determination of CPS had many disadvantages like significant organic solvent consumption or incompatibility to HPLC-MS water phase (29–31). The aim of this work was to develop and validate HPLC method with UV detection suitable for identification, determination, and stability study of CPS and its degradation products.

EXPERIMENTAL

Standards and reagents

CPS was obtained from CHEMOS GmbH Werner-von-Siemens Str. 3 D-93128 Regenstauf, Germany. It is a white or pale yellowish white, crystalline powder soluble in water and conforms Japanese Pharmacopeia XV standards.

All other chemicals and solvents were obtained from Merck (Germany) and were of analytical grade. High quality pure water was prepared by using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120).

Kinetic analysis

For the kinetic study, the Dionex Ultimate 3000 analytical system consisted of a quaternary pump, an autosampler, a column oven and diode array detector was used. As the stationary phase a Lichrospher RP-18 column, 5 µm particle size, 125

Table 1. Recovery studies (n = 3).

Spiked concentration (mg/L)	Measured concentration ± S.D. (mg/L) and recovery (%)
100.0 (~ 50%)	104.85 ± 0.37, 104.85
200.0 (~ 100%)	202.73 ± 6.51, 101.36
300.0 (~ 200%)	298.31 ± 1.10, 98.87

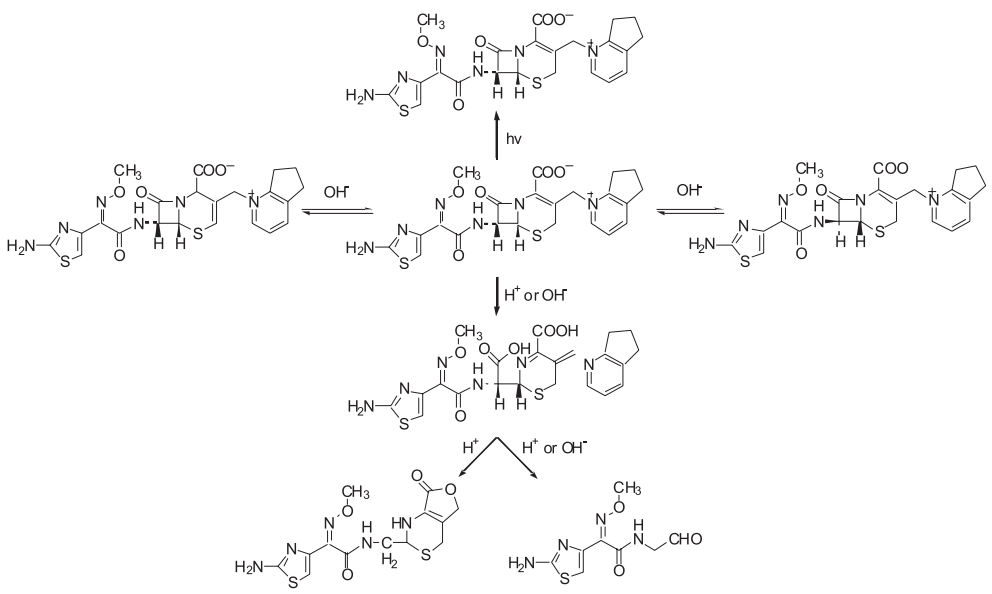

Figure 2. Degradation pathways of CPS in aqueous solutions (17)

× 4 mm (Merck, Darmstadt, Germany) was used. The mobile phase composed of acetonitrile – 12 mM ammonium acetate (10 : 90 v/v). The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 10 µL. The wavelength of the DAD detector was set at 270 nm. Separation was performed at 30°C. Photodegradation stability studies were performed using Suntest CPS+ (Atlas®) with filter Solar ID65.

Procedure for forced degradation study of cefpirome sulfate

Stability tests were performed according to International Conference on Harmonization Guidelines (32).

Degradation in aqueous solutions

The degradation of cefpirome sulfate in aqueous solutions was studied in hydrochloric acid (1 mol/L) at 298 K, in sodium hydroxide (0.1 mol/L) at 298 K and in water at 373 K. Degradation was initiated by dissolving an accurately weighed 5.0 mg of CPS in 25.0 mL of the solution equilibrated to desired temperature in stoppered flasks.

Oxidative degradation

Degradation was initiated by dissolving an accurately weighed 5.0 mg of CPS in 25.0 mL solution of 3% H_2O_2 equilibrated to 298 K.

Thermal degradation

Five milligram samples of CPS were weighed into glass vials. In order to achieve the degradation in solid state, the samples were immersed in heat chambers at 393 K at RH = 0%, at 369 K at RH ~ 50.9% and at 369 K at RH ~ 90.0%. At specified time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and their contents were dissolved in the mixture of acetonitrile and water (1 : 1 v/v). The obtained solutions were quantitatively transferred into measuring flasks and diluted with the same mixture of solvents to 25.0 mL.

UV degradation

Five milligrams of CPS were accurately weighed and dissolved in 25.0 mL of water and then exposed to light according ICHQ1b directions.

RESULTS AND DISCUSSION

It was observed that satisfactory resolution of CPS (retention time 4.59 min) and four degradation products (retention time from 2.10 to 8.05 min) formed under various stress conditions was achieved when analysis of stressed samples were performed on an HPLC system using the C-18 column and a mobile phase composed of 10 volumes of acetonitrile and 90 volumes of ammonium acetate, 12 mmol/L (Fig. 3). Shorter column (125 mm) than in other HPLC methods (250 mm) (29–31) resulted in lower amounts of organic phase wasted, while simultaneously peak asymmetry (1.351) and resolution (2.088) were still on satisfactory level.

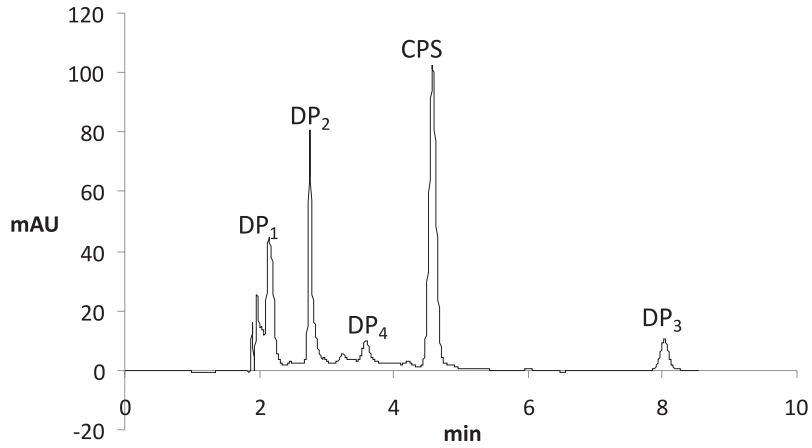

Figure 3. The HPLC chromatogram of CPS (t_R = 4.59 min) in the presence of degradation products DP (t_R from 2.10 to 8.05 min) following incubation at 363 K for 180 min

Method validation

HPLC method was validated according to International Conference on Harmonization Guidelines. The method was validated for specificity, linearity, precision, accuracy and robustness.

Selectivity

The selectivity was examined for non-degraded and degraded samples (the solutions of CPS after stress conditions of hydrolysis (acid, base and neutral), photolysis, oxidation (H_2O_2) and thermal degradation.

The HPLC method for determination of CPS was found selective in the presence of degradation products as shown in Figure 3. Peaks were symmetrical and distinctly separated from each other (Fig. 3).

Linearity

Linearity was evaluated in the concentration range of 20–300 mg/L (10–150% of the nominal concentration of CPS during degradation studies). The samples of each solution were injected three times and each series comprised 7 experimental points.

The calibration plots were linear in the concentration range 20–300 mg/L (n = 7, r = 0.9999). The calibration curve was described by the equation y = ac; y = (40346 ± 666) c. The b value, calculated from equation y = ac + b, was not significant. Statistical analysis using Mandel's fitting test confirmed linearity of the calibration curves.

Accuracy, as recovery test

The accuracy of the method was determined by recovering CPS from the placebo. The recovery test was performed at three levels: 50, 100 and 150% of the nominal concentration of CPS during degradation studies. Three samples were prepared for each recovery level. The solutions were analyzed and the percentage of recoveries was calculated. Good recoveries were obtained for each concentration, confirming that the method was accurate (Table 1).

Precision

Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate the repeatability of the methods, six samples were determined during the same day for three concentrations

Table 2. Intra-day and inter-day precision (n = 6) studies.

Spiked concentration (mg/L)	Measured concentration ± S.D. (mg/L) and RSD (%)
Intra-day precision	
100.00	105.00 ± 0.59, 0.48
200.00	200.83 ± 0.71, 0.31
300.00	298.99 ± 1.22, 0.36
Inter-day precision	
100.00	201.99 ± 3.04, 1.43

Table 3. Results of forced degradation studies.

Stress conditions and time studies	Degradation [%]
Acidic (1 mol/L HCl; 298 K; 72 h)	37.27
Basic (0.1 mol/L NaOH; 298 K; 12 min)	56.30
Neutral (373 K; 70 min)	81.73
Oxidizing (3% H_2O_2; 298 K; 3 h)	19.34
Thermal (solid state; 393 K; RH~0%; 38 days)	69.48
Thermal (solid state; 369 K; RH~50.9%; 73.5 h)	32.91
Thermal (solid state; 369 K; RH~90.0%; 4 h)	63.00
1.2 million lux h (solution)	26.39
6.0 million lux h (solution)	94.38

of CPS. Intermediate precision was studied comparing the assays performed on two different days.

The intra-day and inter-day precision values of measured concentration of CPS, as calculated from linearity plots, are given in Table 2. The RSD values were 0.31 and 1.43%, respectively, demonstrating that the method was precise.

Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ parameters were determined from the regression equation of CPS: LOD = 3.3 S_y/a, LOQ = 10 S_y/a; where S_y is a standard error and a is the slope of the corresponding calibration curve.

Under applied chromatographic conditions, the LOD of CPS was 2.38 mg/L and LOQ of CPS was 7.22 mg/L.

Robustness

The robustness of the procedure was evaluated after changing the following parameters: the composition of the mobile phase; content of acetonitrile 10 ± 2%, the mobile phase flow rate 1.0 ± 0.2 mL/min; wavelength of absorption 270 ± 5 nm and temperature 30 ± 2°C. For each parameter change its influence on the retention time, resolution, area and asymmetry of peak was evaluated. No significant changes in resolution and shapes of peak, areas of peak and retention time were observed when above parameters were modified. Modifications of the composition of the mobile phase: organic-to-inorganic component ratio and pH resulted in the essential changes of retention time and resolution in determination of CPS.

Results of forced degradation experiments

In previous studies, concerning the stability of cephalosporins, it was observed that basic hydrolysis was a fast reaction (13–19). Also in the case of CPS, significant degradation was observed at basic hydrolysis. Photodegradation of CPS was observed after exposition even on 1.2 million lux h (solution). It was observed that around 26% of CPS degraded under these conditions. CPS was susceptible for degradation in solid state. At increased RH the degradation was much faster than in dry air. CPS was more stable than other 4th generation cephalosporin – cefozopran hydrochloride (33). The results of forced degradations in various conditions are summarized in Table 3.

CONCLUSION

The isocratic RP-LC method developed for the analysis of CPS in its pharmaceutical preparations is selective, precise and accurate. The method is useful for routine analysis due to short run time and low amounts of solvent (acetonitrile) used in the mobile phase. Low acetonitrile consumption is consistent with the current worldwide trend of green (sustainable) chemistry. This method can be used for determination of stability of CPS in its pharmaceutical preparations.

REFERENCES

1. Chaudhury A.: Indian J. Med. Microbiol. 21, 52 (2003).
2. Tumah H.N.: Pharmazie 59, 854 (2004).
3. Bergeret M., Boutros N., Raymond J.: Int. J. Antimicrob. Agents 23, 247 (2004).
4. Lindenmann J., Kugler S.A., Matzi V., Porubsky C., Maier A., Dittrich P., Graninger W. et al.: J. Antimicrob. Chemother. 66, 160 (2011).
5. Timmers G.J., van Vuurden D.G., Swart E.L., Simoons-Smit A.M., Huijgens P.C.: Haematologica 90, 1005(2005).
6. Hayashi H., Yamashita T., Nakata T., Huji T., Kojima T., Katayama H., Ishikawa M.: Int. J. Antimicrob. Agents 19, 405 (2002).
7. Sauermann R., Delle-Karth G., Marsik C., Steiner I., Zeitlinger M., Mayer-Helm B.X., Georgopoulos A. et al.: Antimicrob. Agents Chemother. 49, 650 (2005).
8. Zaffiri L., Gardner J., Toledo-Pereyra L.H.: J. Invest. Surg. 25, 67 (2012).
9. Bakshi M., Singh B., Singh A., Singh S.: J. Pharm. Biomed. Anal. 26, 891 (2001).
10. Bakshi M., Singh S.: J. Pharm. Biomed. Anal. 28, 1011 (2002).
11. Sehrawat R., Maithani M., Singh R.: Chromatographia 72, 1 (2010).
12. Singh S., Handa T., Narayanam M., Sahu A., Junwal M., Shah R. P.: J. Pharm. Biomed. Anal. 69, 148 (2012).
13. Patel G., Rajput S.: Acta Chromatogr. 23, 215 (2011).
14. Ikeda Y., Ban J., Ishikawa T., Hashiguchi S., Urayama S., Horibe H.: Chem. Pharm. Bull. 56, 1406 (2008).
15. Jelińska A., Dobrowolski L., Oszczapowicz I.: J. Pharm. Biomed. Anal. 35, 1273 (2004).
16. Zalewski P., Cielecka-Piontek J., Jelińska A.: React. Kinet. Mech. Cat. 108, 285 (2013).
17. Sugioka T., Asano T., Chikaraishi Y., Suzuki E., Sano A., Kuriki T., Shirotsuka M., Saito K.: Chem. Pharm. Bull. 38, 1998 (1990).
18. Fubara J.O., Notari R.E., J. Pharm. Sci. 87, 1572 (1998).

19. Zalewski P., Cielecka-Piontek J., Jelińska A.: Asian J. Chem. 25, 7596 (2013).
20. Medenecka B., Jelińska A., Zając M., Bałdyka M., Juszkiewicz K., Oszczapowicz I.: Acta Pol. Pharm. Drug Res. 66, 563 (2009).
21. Jelińska A., Medenecka B., Zając M., Knajsiak M.: Acta Pol. Pharm. Drug Res. 65, 261 (2008).
22. Zając M., Jelińska A., Zalewski P.: Acta Pol. Pharm. Drug Res. 62, 89 (2005).
23. Jelińska A., Dudzińska I., Zając M., Oszczapowicz I., Krzewski W.: Acta Pol. Pharm. Drug Res. 62, 183 (2005).
24. Zalewski P., Cielecka-Piontek J., Garbacki P., Jelińska A., Karaźniewicz-Łada M.: Chromatographia 76, 387 (2013).
25. Zając M., Jelińska A., Dobrowolski L., Oszczapowicz I.: J. Pharm. Biomed. Anal. 32, 1181 (2003).
26. Jelińska A., Zając M., Gostomska J., Szczepaniak M.: Farmaco 58, 309 (2003).
27. Zalewski P., Cielecka-Piontek J., Jelińska A.: Centr. Eur. J. Chem. 10, 121 (2012).
28. Jelińska A., Zając M., Jakubowska M.: React. Kinet. Catal. Lett. 73, 325 (2001).
29. Rao K.S., Kumar K.N., Joydeep D.: Sci. Pharm. 79, 899 (2011).
30. Sriwiriyajan S., Mahatthanatrakul W., Arzneimittelforschung 60, 336 (2010).
31. Elghobashy M.R., Bebawy L.I., Abbas S.S., Shokry R.F.: Chromatographia 76, 1141 (2013).
32. ICH, Stability Testing of New Drug Substances and Products (Q1AR). International Conference on Harmonization, IFPMA, Geneva 2000.
33. Zalewski P., Skibiński R. Cielecka-Piontek J., Bednarek-Rajewska K.: Chem. Ind. Chem. Eng. Q. (under review).

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RAPID AND SENSITIVE BIOANALYTICAL STABILITY-INDICATING METHOD FOR QUANTIFICATION OF TALINOLOL, A SELECTIVE β1 ADRENOCEPTOR ANTAGONIST IN LIPID BASED FORMULATIONS USING ULTRAFAST UHPLC SYSTEMS

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Abstract: The current study evaluates the ultra high performance liquid chromatography (UHPLC) method for the quantification of talinolol in lipid-based formulations. A simple, rapid, reliable and precise reversed phase UHPLC method has been developed and validated according to the regulatory guidelines, which was composed of isocratic mobile phase; acetonitrile and phosphate buffer saline (pH 4.5) with a flow rate of 0.4 mL/min, and column HSS C_{18} (2.1 × 50 mm, 1.8 µm). The detection was carried out at 245 nm. The developed UHPLC method was found to be rapid (1.8 min run time), selective with high resolution of talinolol peak (0.88 min) from different lipid matrices and highly sensitive (limit of detection and lower limit of quantification were 0.14 ppm and 0.5 ppm, respectively). The linearity, accuracy and precision were determined as acceptable over the concentration range of 0.5–100 ppm for talinolol. The results showed that the proposed UHPLC method can be used for the estimation of talinolol in lipid-based formulation by indicating its purity and stability with no interference of excipients or related substances of active pharmaceutical ingredient.

Keywords: talinolol, UHPLC systems, lipid based formulation, method validation

Talinolol is chemically known as [1-(4-cyclohexylureidophenoxy)-2-hydroxy-3-tert-butylaminopropane], categorized as a selective β1 adrenoceptor antagonist (Fig. 1), which is structurally related to practolol (1). It is widely used in the treatment of various cardiovascular diseases such as arterial hypertension, acute and chronic tachycardiac heart arrhythmia, and hyperkinetic heart syndrome (2). Talinolol was introduced into clinical practice in 1975 under the trade-mark "Cordanum" in Germany. Talinolol is only available as 50/100 mg immediate release tablets and 5 mL *i.v.* solution (contains 10 mg of talinolol). The recommended daily doses of talinolol range from 25 to 300 mg in healthy volunteers. According to the pharmacokinetics, it is a poorly water soluble drug with solubility of 0.14 mg/mL (BCS II compound, weak base with pKa 9.4, melting point 160–162°C) and it has low, variable and incomplete absorption which leads to poor bioavailability of around 40–55% (3). Thus, it is a suitable candidate for lipid based formulation,

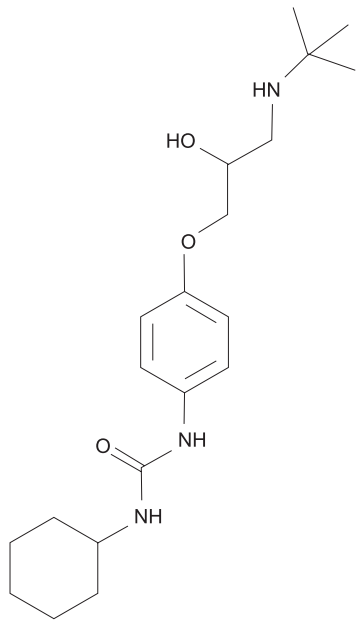

Figure 1. Chemical structure of talinolol (M.w.: 363.5, pKa: 9.4)

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which could improve the aqueous solubility and absorption rate.

In the literature, various analytical methods have been developed using reversed phase (RP) high performance liquid chromatography (HPLC) (4, 5), capillary electrophoresis (6), liquid chromatography (7), for determination of talinolol. Mostly all of these methods were used to quantify talinolol and its metabolites in plasma, urine and feces. Till date, not a single method has been developed in lipid based formulations except biological fluids and caused lengthy and tedious process, lack of required sensitivity or required special reagents and detectors. Thus, an improved analytical method for talinolol should be developed demanding more sensitivity, fast and also required to meet the stability indicating parameters. Within the experimental method development, it was also needed to carry out stability studies under forced acidic, alkaline, thermal, and oxidative degradation processes. The aim of this work was to develop a fast, reliable analysis method for the determination of talinolol using UHPLC.

Within the scope of the current analytical method talinolol was assayed and validated using an advanced UHPLC system, which reduced the time of analysis and the use of solvent. Apart from reducing time and solvent, the current instrument method carries many advantages such as the device, which allows the system to withstand high back pressure without any harmful effect to the analytical column or the whole device. Therefore, the column used in the UHPLC systems can last longer than any other HPLC columns. The proposed method was also successfully applied to the analysis of lipid-based formulations containing talinolol with no interference from dosage form excipients. The method was validated with respect to the standard FDA guidelines for bioanalytical method.

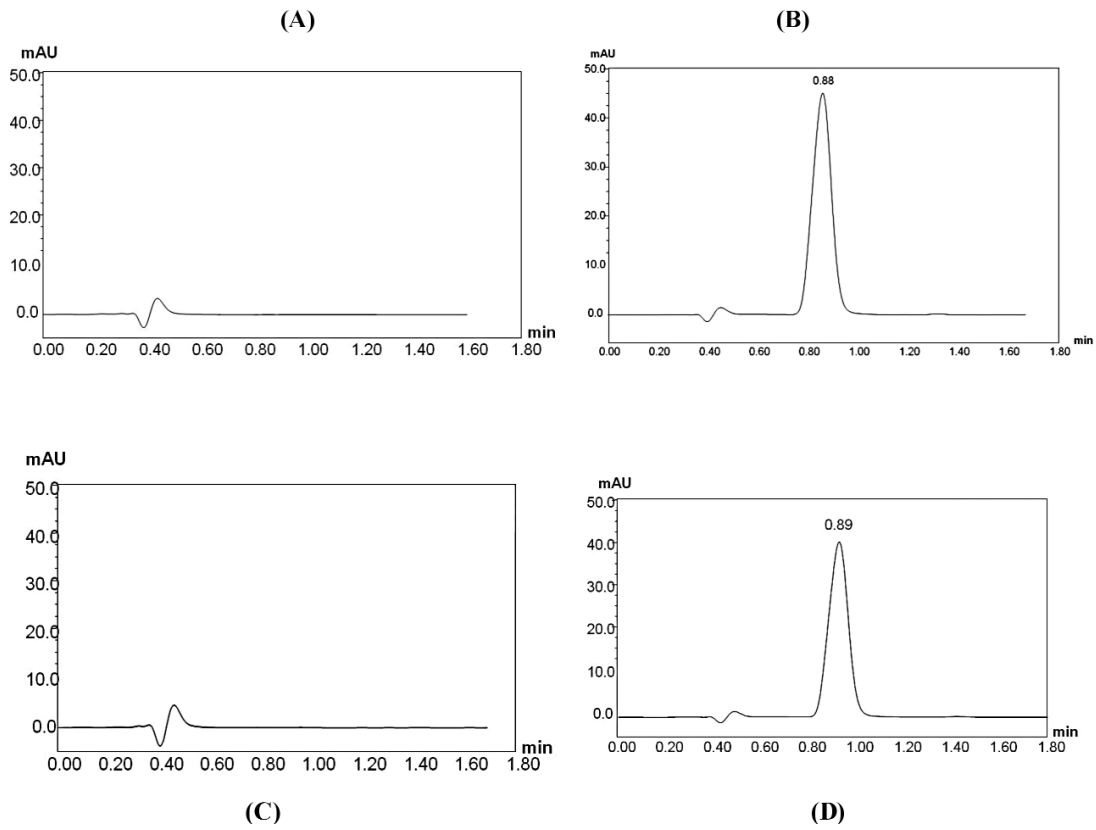

Figure 2. UHPLC chromatograms of blank sample (**A**), standard solution of talinolol at concentration 50 ppm (**B**), drug-free lipid formulation sample (Tween 80, QC_{zero}) (**C**), and drug containing lipid formulation (Tween80, QC) sample (**D**)

EXPERIMENTAL

Materials

All chemicals used in the studies were obtained from commercial suppliers. Talinolol (purity > 99.2%) was obtained as a gift sample from Alfa Aesar, a Johnson Metthey Company (Ward Hill, MA). Acetonitrile, potassium dihydrogen orthophosphate and hydrochloric acid were obtained from BDH Chemicals Ltd., Poole, U.K. Sodium hydroxide and hydrogen peroxide were used as analytical grade reagents. Imwitor 308 (medium chain monoglycerides) and Tween 80 (non ionic surfactant) were used to prepare self emulsifying lipid formulations, supplied by Sasol Germany GmbH, Werk Witten, Germany. The high purity milli-Q water was obtained through a Milli-Q Integral Water Purification System (Millipore, Bedford, MA). All other reagents were of analytical grade and used without further purification.

METHODS

UHPLC chromatographic conditions

Chromatographic separation was developed and optimized with respect to the stationary/ mobile phase compositions, flow-rate, sample volume, column temperature and detection wavelength. The study employed a highly sensitive UHPLC system that consisted of a Dionex ® UHPLC binary solvent manager equipped with a Dionex® automatic sample manager and a photodiode array (PDA) eλ detector obtained from Thermo Scientific, Bedford, MA, USA. The mobile phase was an isocratic mix of HPLC-grade acetonitrile (ACN) and potassium dihydrogen orthophosphate in a ratio of 30 : 70 (v/v) and maintained at pH 4.5. The flow rate was 0.4 mL/min delivered through an Acquity® UPLC HSS C_{18} column (2.1 × 50 mm, 1.8 µm) kept at 30°C. The total run time was 1.8 min. Freshly prepared mobile phase was filtered through an online 0.20 µm filter and degassed continuously by an online degasser within the UHPLC system. The detector wavelength was set at 245 nm and the injection volume was 1.0 µL.

Preparation of buffer solution

Phosphate buffer solution was prepared by dissolving 13.61 g of potassium dihydrogen orthophosphate in 900 mL of milliQ water. The pH of the solution was adjusted to 4.5 with 0.1 M hydrochloric acid and the buffer was further diluted up to 1000 mL with water. The buffer solution was found stable without having any changes with pH and visual clarity for more than a week.

Preparation of stock solution, calibration standards and QC samples

Standard stock solution was prepared by dissolving accurately weighted 50 mg of talinolol powder in 50 mL of milliQ water, resulting in a solution containing 1000 ppm. For the purpose of calibration, the standard solutions of talinolol at seven points were prepared by appropriate serial dilutions in ACN to cover the concentration range of 0.5–100 ppm. These standards solutions were stored at 4°C temperature before use. Calibration curves were obtained by plotting peak area against standard drug concentration and regression equations were computed thereby. Four quality control (QC) samples with the selected concentration levels (2.3, 6.5, 35, 70 ppm) were prepared from stock solution to cover the desired range. QC samples were prepared by spiking the self emulsifying lipid-formulation with known amount of talinolol, and then diluting the mixture with appropriate volume of ACN.

Method validation

The above mentioned developed method in the present study has been validated for linearity, sensitivity, precision, accuracy, selectivity, recovery and stability according to the standard guidelines of bioanalytical method validation (8–10) by US Food and Drug Administration (FDA). The acceptance criteria of precision and accuracy were evaluated as the relative standard deviation of the mean expressed as a percent (coefficient of variation, CV% determined precision of the method).

Linearity and range

Appropriate volume of talinolol stock solution (1000 ppm) was utilized in the preparation of seven non-zero standard drug concentrations covering the calibration range of 0.5–100 ppm. Four different QC samples were prepared by spiking known concentrations of talinolol within the same detection range (0.5– 100 ppm).

Each standard solution (0.5, 1, 5, 10, 20, 50, and 100 ppm) has been injected as six replicates daily on three consecutive days for validation. Calibration solutions were injected in ascending order in each validation run and the other samples were distributed randomly through the run.

Linear regression equation and correlation coefficient (R^2) were employed to statistically calculate the linearity of the results (11).

Specificity

Specificity of the method was required to assess the matrix effect by comparing peak areas

between the drug and different lipid-based formulations.

The specificity of the method was evaluated through the whole assay period using drug free lipid formulation samples (QC_{zero}) and talinolol analyte. The retention times of the drug free lipid components were compared with that of talinolol analyte. In addition, towards the establishment of the method, specificity was also studied by determination of the intact drug in terms of resolution (R) between the drug peak and the nearest degradation product if present (12).

Accuracy and precision

The intra-day accuracy and precision were assessed by analyzing six replicates of each seven talinolol standards within the same day. Similarly, the inter-day accuracy and precision were also determined during the three consecutive days using six replicates analysis of the LLOQ, low, medium and high QC samples. The overall precision of the method was expressed as relative standard deviation (RSD) and accuracy of the method was expressed as % drug recovered.

Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LOD and LLOQ levels were determined by serial dilutions of talinolol stock solutions in order to obtain signal to noise (S/N) ratio of at least ≈ 3 : 1 for LOD and ≈ 10 : 1 for LLOQ (13).

RESULTS AND DISCUSSION

UHPLC peak separation

Separation and detection of talinolol peak without any minimum interference was ideal by the developed UHPLC assay. The chromatographic results of UHPLC technique in the current analysis

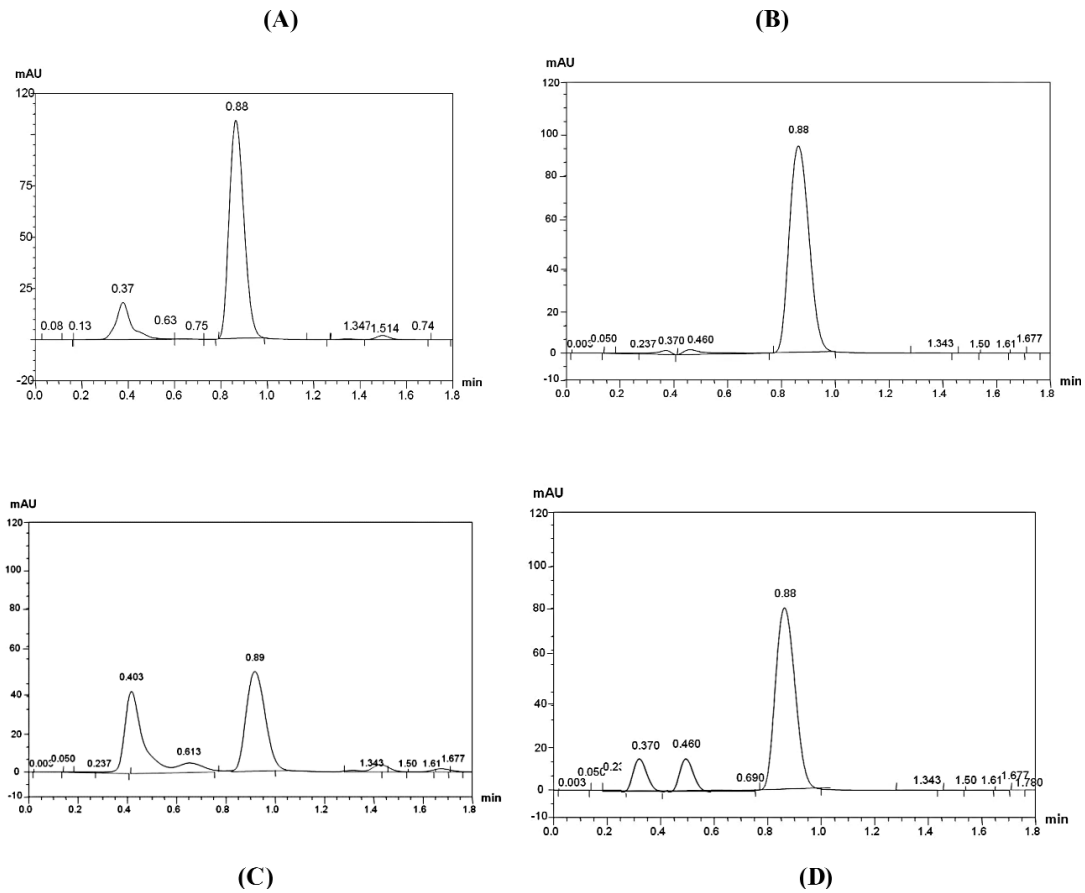

Figure 3. Typical UHPLC chromatograms of: (**A**) acid hydrolysis degraded talinolol, (**B**) base hydrolysis degraded talinolol, (**C**) oxidative degraded talinolol and (**D**) thermally degraded talinolol

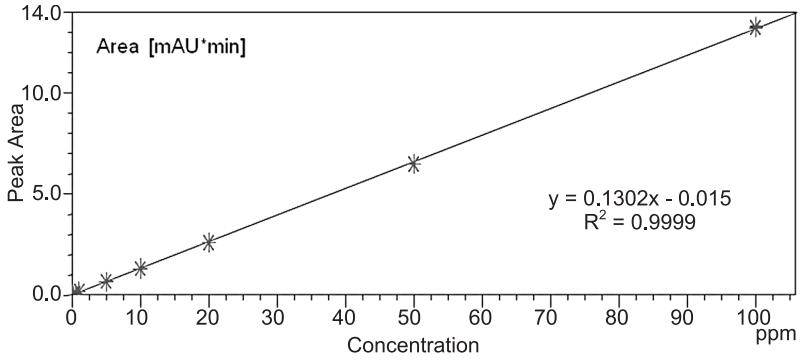

Figure 4. UHPLC calibration curve of talinolol in ACN

Table 1. UHPLC data of intra-day back-calculated talinolol concentrations of the calibration standards in ACN.

Nominal concentration (ppm)	Back calculated concentrations (ppm)									
	1st	2nd	3rd	4th	5th	6th	Mean	S.D.	Precision %	Accuracy %
0.5	0.55	0.53	0.56	0.56	0.57	0.52	0.55	0.022	3.378	109.59
1	1.05	1.02	1.06	1.06	1.08	1.04	1.05	0.022	2.095	105.16
5	5.08	5.06	5.12	5.10	5.10	5.09	5.09	0.020	0.399	101.84
10	9.96	9.92	9.91	9.90	9.94	9.92	9.93	0.019	0.194	99.26
20	19.57	19.71	19.65	19.63	19.53	19.66	19.62	0.065	0.331	98.12
50	49.23	49.33	48.98	49.16	49.00	49.24	49.16	0.138	0.281	98.31
100	100.46	100.41	100.55	100.54	100.57	100.45	100.50	0.067	0.066	100.50

show that talinolol can be determined well enough in the self emulsifying lipid formulations within the highest sensitivity and selectivity of this analytical procedure and in a very short time. In the previous studies, Sinha and Damanjeet (5) developed talinolol method using conventional HPLC and showed the talinolol peak at 12.5 min. Our current analysis has a great advantage over the previous studies, which reduced the analysis time and solvent consumption significantly. Figure 2 shows the representative chromatograms of blank sample (2A), standard solution of talinolol (2B), drug-free lipid formulation (QC_{zero}) sample (2C), and drug-containing lipid formulation QC sample (2D). The talinolol analyte was well separated from the solvent peak (used as mobile phase as well as sample dilutions) at retention time of ~0.88 min, while there was no degradation product detected in the sample formulation (Fig. 2 A–D). The total chromatographic run time was ~ 1.8 min and the talinolol peak was of good shape and completely resolved.

Forced degradation study

The forced degradation study was conducted by treating model drug talinolol with 1 M HCl, 1 M NaOH, 3% H_2O_2 under 60°C temperature for 12 h in water bath. In addition, the thermal degradation experiment was carried out under 120°C temperature in an oven. All the talinolol sample solutions used in forced degradation studies were prepared with final concentrations of 100 µg/mL (100 ppm). The results showed by the degradation study that the amounts of talinolol in the samples were lowered compared to the original concentrations. This degradation study was particularly important for the analysis of lipid based formulation, which was not studied previously (5).

Acid degradation

Ten milligrams of talinolol powder was transferred into a 100 mL round bottom flask and then10 mL of 1 M hydrochloric acid solution was added. The contents of the flasks were mixed well and kept for overnight at 60°C temperature. After cooling the solution at room temperature, 10 mL of 1 M sodium hydroxide solution was added in 100 mL volumetric flask for neutralization and then diluted to 100 mL with ACN. The chromatogram of the acid degradation is shown in Figure 3A.

Basic degradation

Ten milligrams of talinolol powder was transferred into a 100 mL round bottom flask and then10 mL of 1 M sodium hydroxide solution was added. The contents of the flasks were mixed well and kept for overnight at 60°C. After cooling the solution to room temperature, 10 mL of 1 M hydrochloric acid solution was added in 100 mL volumetric flask for neutralization and then diluted to 100 mL with ACN. The result of the basic degradation is shown in Figure 3B.

Oxidation

Ten milligrams of talinolol powder was transferred into a 100 mL round bottom flask with the addition of 10 mL of 3% hydrogen peroxide solution. The contents of the flasks were mixed well and kept for overnight at 60°C. After cooling the solution to room temperature, it was diluted to 100 mL with ACN. The chromatogram of the oxidation data is shown in Figure 3C.

Thermal degradation

Ten milligrams of talinolol powder was transferred into a 100 mL round bottom flask and kept in oven at 120°C for 24 h. After cooling the powder yo room temperature, the ACN was added up to 100 mL as diluent. The chromatogram of the thermal degradation study is shown in Figure 3D.

Table 2. UHPLC data of inter-day accuracy and precision of talinolol QC samples.

Day of analysis	QC1	QC2	QC3	QC4
	2.30 ppm	6.50 ppm	35 ppm	70 ppm
1st	2.23	6.61	34.93	69.68
	2.09	6.67	35.08	70.48
	2.33	6.47	35.05	69.32
	2.36	6.55	34.94	69.98
	2.33	6.60	35.08	69.43
	2.31	6.66	34.93	69.76
2nd	2.38	6.99	35.15	70.04
	2.29	6.70	35.38	70.28
	2.35	6.49	35.06	70.08
	2.26	6.55	35.33	69.89
	2.35	6.71	35.15	69.48
	2.31	6.60	35.19	70.19
3rd	2.39	6.44	34.37	68.42
	2.35	6.39	34.74	69.06
	2.33	6.44	34.93	69.05
	2.41	6.33	34.33	68.73
	2.45	6.38	34.16	68.17
	2.38	6.65	34.36	68.88
Mean	2.327	6.567	34.897	69.497
SD	0.060	0.117	0.376	0.681
Precision %	2.591	1.778	1.077	0.980
Accuracy %	101.159	101.026	99.705	99.281

Table 3. Statistical data of the regression equation for the determination of talinolol obtained from the proposed method.

Parameters	UHPLC Method
Concentration range	0.5–100 ppm
Intercept	0.015
Slope	0.1302
Correlation coefficient (r)	0.9999
Limit of detection (LOD)	0.14 ppm
Limit of quantification (LOQ)	0.5 ppm

Table 4. Systems suitability parameters of talinolol.

System suitability parameter	Talinolol
Retention time	0.88 min
% RSD	0.067%
Peak tailing	1.05
Theoretical plate number	2829

Bioanalytical method validation

The precision and accuracy of the method show an excellent workability of the method. The precision was 20% or better for LLOQ (% RSD) and 15% or better for the remaining concentrations and the acceptable accuracies were 100 ± 20% or better for LLOQ and 100 ± 15% or better for the remaining concentrations.

Linearity and range

A seven point standard calibration curve was constructed to see the linearity within the concentration range. The peak area response of talinolol was linear over the concentration range between 0.5 and 100 ppm (Fig. 4). The result of linear regression gives the following mean equation:

$$y = 0.1302x - 0.015$$

where y and x denote: the peak area and the concentration of the analyte, respectively. This result shows an excellent linearity (approaching a straight line function) over the interval studied. The correlation coefficient (r) was higher than 0.9995 with an average value of 0.9999 (Table 3) (11).

Accuracy and precision

The intra-day and inter-day accuracies were calculated as the % of drug recovered after analyzing six replicates of the QC samples at four nominal concentration levels. The intra-day (Table 1) accuracies were found in between 98 and 109.59% and the inter-day (Table 2) accuracies were in between 99.70 and 101.154%. The results from the drug recovery studies suggest that the accuracy of the assay method was within the acceptable limits according to the FDA guidelines (11).

Precision

The developed method was found to be precise as the intra-day standard deviation (SD) values (Table 1) of six replicate analyses were within the range of 0.02–1.38 ppm. Within the analytical concentration range of 0.5–100 ppm, % CV values were less than 3.38%. In addition, the inter-day (Table 2) accuracies of six replicates during the three consecutive days were between 0.06 and 0.68 ppm, whereas the % CV values were less than 2.59%. These low values of both SD and % CV during the intra-day and inter-day analysis thus met the accepted requirements of precision for the current method (14).

Specificity

The specificity of the developed UHPLC method was investigated in order to measure the analyte response in the presence of its degradation products. Specificity was established by determining the purity of the talinolol peak using a PDA detector. In addition, the resolution factor of the drug peak was determined with respect to the near-

est resolving peak. The developed method was found to be specific for talinolol without having any possible interference from the degradation product(s). The result from a sample of lipid solubility studies (talinolol loaded in lipid formulation) in Figure 2D shows that there was no degradation products present in the sample containing talinolol. It seems that talinolol compound can be recovered completely from the lipid formulation (Fig. 2D). Therefore, the R value in this assay can be calculated based on the availability of the talinolol peak only. In addition, there were no significant interfering peaks present in randomly selected drug free lipid formulation (QC_{zero}) samples at talinolol retention time (Fig. 2C), that suggests that talinolol compound can be analyzed predominantly from lipid based formulations.

Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LLOQ in the assay was 0.5 ppm, which was estimated to be the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision for the talinolol analyte with S/N ratio of 79.7. To the best of our knowledge, this concentration was relatively low as compared to other available bioanalytical methods that developed using the similar instruments. On the other hand, LOD was 0.14 ppm with S/N ratio of 27.8. Both LLOQ and LOD were experimentally verified by six replicate injections of talinolol standard concentrations (Table 3).

Suitability of the systems

Systems suitability parameters were taken into consideration to confirm the highest precision of the systems. The variation (% RSD) in the peak area from six replicates injections was around 0.067%, which proves that the system is precise. The results of other chromatographic parameters such as peak tailing and theoretical plate numbers (showing column efficiency) are shown in Table 4. The overall analysis results show the acceptable performance of the system as the % RSD and the tailing peak are not more than 2.0 % and 1.5, respectively, along with the theoretical plates, which are not less than 2000.

Application

Within the scope of the current research, the developed UHPLC method has been successfully used for the quantification of talinolol compound in the studies of equilibrium solubility, and dissolution profiles of self emulsifying lipid-based formulations (SEDDS/SMEDDS) (15).

CONCLUSION

The developed UHPLC analytical method provides a reliable, reproducible and specific assay for talinolol in pure form and pharmaceutical formulations. The described method is sensitive enough to detect as low as 0.14 ppm and exclusively offer a rapid determination of talinolol (peak at 0.88 min within 1.8 min run time). No significant interferences were caused by the formulation excipients, diluents and/or degradation products.

The validation of the method allows quantification of talinolol in the range between 0.5 to 100 ppm. Compared to previously reported methods, the present assay method assessed extensive validation parameters according to FDA guidelines. The method has shown acceptable precision, accuracy and adequate sensitivity and demands to be in use for further studies.

The established method satisfies the system suitability criteria, peak integrity, and resolution of the drug peak. The overall results clearly indicate that the current method is attractive due to the good selectivity for quantitative determination of talinolol in lipid-based formulation and also suitable for stability measurements.

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REFERENCES

1. Pathak S.M., Musmade P.B., Bhat K.M., Udupa N.: Bioanalysis 2, 95 (2010).
2. Campeanu A.: Eur. J. Heart Fail. 3, 377 (2001).
3. Trausch B., Oertel R., Richter K., Gramatte T.: Biopharm. Drug Dispos. 16, 403 (1995).
4. Oertel R., Richter K., Trausch B., Gramatte T.: Pharmazie 49, 291 (1994).
5. Sinha V.R., Damanjeet G.: J. Chromatogr. Sci. 49, 786 (2011).
6. Awadallah B., Schmidt P. C., Holzgrabe U., Wahl M.A.: Electrophoresis 24, 2627 (2003).
7. Li Y., Shirasaka Y., Langguth P., Tamai I.: J. Chromatogr. Sci. 48, 367 (2010).
8. Lang J. R., Bolton S.: J. Pharm. Biomed. Anal. 9, 357 (1991).
9. FDA. Guidance for Industry: Centre for Drug Evaluation and Research (CDER), Rockville, MD, http://www.fda.gov/cder/guidance/index.htm/ (2001).

10. Dadgar D., Burnett P.E., Choc M.G., Gallicano K., Hooper J.W.: J. Pharm. Biomed. Anal. 13, 89 (1995).
11. Mulholland M., Hibbert D.B.: J. Chromatogr. A 762, 73 (1997).
12. Lister A.S.: Validation of HPLC Methods in Pharmaceutical Analysis, in Separation Science and Technology, vol. 6, Ahuja S., Dong M.W. Eds., pp. 191-217, Elsevier, Academic Press, Amsterdam 2005.
13. Dongre V. G., Karmuse P. P., Rao P. P., Kumar A.: J. Pharm. Biomed. Anal. 46, 236 (2008).
14. Karnes H. T., March C.: Pharm. Res. 10, 1420 (1993).
15. Mohsin K.: AAPS PharmSciTech 13, 637 (2012).

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DRUG BIOCHEMISTRY

ENRICHMENT OF MATERNAL DIET WITH CONJUGATED LINOLEIC ACIDS INFLUENCES DESATURASES ACTIVITY AND FATTY ACIDS PROFILE IN LIVERS AND HEPATIC MICROSOMES OF THE OFFSPRING WITH 7,12-DIMETHYLBENZ[A]ANTHRACENE-INDUCED MAMMARY TUMORS

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Abstract: The aim of this study was to assess the influence of diet supplementation of pregnant and breast-feeding female Sprague-Dawley rats with conjugated linoleic acids (CLA) on the Δ6- and Δ5-desaturase activity in hepatic microsomes as well as on fatty acids profile and lipids peroxidation in liver and hepatic microsomes of the progeny with chemically induced mammary tumors. Rats were divided into two groups with different diet supplementation (vegetable oil (which did not contain CLA) or CLA). Their female offspring was divided within these groups into two subgroups: (1) – fed the same diet as mothers (K1 – oil, O1 – CLA), and (2) – fed the standard fodder (K2, O2). At 50th day of life, the progeny obtained carcinogenic agent (7,12-dimethylbenz[a]anthracene). Higher supply of CLA in diet of mothers resulted in lower susceptibility to chemically induced mammary tumors in their offspring ($p = 0.0322$). It also influenced the fatty acids profile in livers and in hepatic microsomes, especially polyunsaturated n3 and n6 fatty acids. CLA inhibited the activity of the desaturases, which confirmed that CLA can reduce the level of arachidonic acid directly, reducing linoleic acid content in membranes, or indirectly, through the regulation of its metabolism. We were unable to confirm or deny the antioxidative properties of CLA. Our results indicate that the higher supply of CLA in mothers' diet during pregnancy and breastfeeding causes their incorporation into tissues of children, changes the efficiency of fatty acids metabolism and exerts health-promoting effect in their adult life reducing the breast cancer risk.

Keywords: conjugated linoleic acids, Δ5-desaturase, Δ6-desaturase, breast cancer, rats

Abbreviations: AA – arachidonic acid, ALA – α-linoleic acid, CLA – conjugated linoleic acids, D5D – Δ5-desaturase, D6D – Δ6-desaturase, DHA – docosahexaenoic acid, DMBA – 7,12-dimethylbenz[a]anthracene, EPA – eicosapentaenoic acid, FA – fatty acids, FAME – fatty acids methyl esters, LA – linoleic acid, MDA – malonyldialdehyde, OL – oleic acid, RA – rumenic acid, TBARS – thiobarbituric acid reactive substances, VA – vaccenic acid

Dietary fat is the most concentrated source of energy for organism as 1 g of fat provides 9 kcal of energy. It is also the carrier of fat-soluble vitamins and essential fatty acids. Liver plays an important role in the metabolism of lipids. It produces the bile, which facilitates the digestion and intestinal absorption of lipids. The synthesis and oxidation of fatty acids take place in liver. Formation of ketone bodies from fatty acids also occurs in liver. Moreover this organ integrates the synthesis and metabolism of plasma lipoproteins. Desaturases are the enzymes which are involved in transformation of fatty acids. They can insert the double bonds into different positions of fatty acids (Δ4, Δ5, Δ6, Δ9) but not higher than Δ9, and that is why linoleic (LA) and α-linolenic (ALA) essential fatty acids should be supplied with diet (1).

Breast cancer is the most frequent type of cancer among women and the third in global population. Numerous nutritional factors are associated with elevated or reduced risk of this type of cancer (2, 3). Despite the fact that the etiology of most cases of this disease is unknown (4), quantity and quality of fat, especially the fatty acids ratio in diet,

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are associated with many cancers (e.g., breast, colon) (3).

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid which contain two conjugated double bonds in their chain. They occur especially in milk, dairy products and meat from ruminants. These types of food are the richest dietary sources of CLA. *Cis*-9, *trans*-11 octadecadienoic acid (rumenic acid – RA) constitutes over 90% of all CLA isomers in dietary products (5). The second important CLA isomer – *trans*-10, *cis*-12 octadecadienoic acid, is present in many dietary supplements (6). Despite the fact, that rumenic acid can be endogenously synthesized from *trans*-11 octadecenoic acid (vaccenic acid – VA) by the action of Δ9-desaturase, dietary intake is the main source of CLA for people (7). Results of scientific research show the positive impact of CLA in different pathological conditions, especially different types of cancer, e.g., breast cancer (7–9). CLA are active in each step of cancer development, from initiation to metastasis. They can reduce the cancerous process risk and there are many possible mechanism of this action: antioxidative properties, influence on eicosanoids synthesis, apoptosis etc. (11). Polyunsaturated fatty acids have beneficial effect in many pathological conditions, e.g., in many types of cancer. The content of unsaturated fatty acids in cellular structures is the result of intake of essential polyunsaturated fatty acids from diet and their endogenous synthesis and subsequent utilization in body building. Elongation and desaturation depend on both the absolute content of these fatty acids and the activity of various enzymes, e.g., desaturases, which control this metabolic pathway. Activity of Δ6- and Δ5-desaturases is the main factor controlling the conversion of dietary linoleic acid (LA) to arachidonic acid (AA). Therefore, in the examination of fatty acids composition in mammalian tissues it is essential to take into account not only diet but also the activity of crucial enzymes. Many dietary factors can influence this activity (12–14).

Table 1. Fatty acids composition of applied diets (% of FA).

Fatty acid	Fodder	Fodder + oil	Fodder + CLA
C6:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C12:0	< 0.1	< 0.1	0.1 ± 0.1
C14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C15:0	0.1 ± 0.0	0.1 ± 0.0	–
C16:0	13.3 ± 0.1	10.5 ± 0.1	11.2 ± 0.2
C16:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C17:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C18:0	2.7 ± 0.0	2.5 ± 0.0	2.5 ± 0.0
C18:1 n9 *cis*	16.6 ± 0.1	34.5 ± 0.1	15.9 ± 0.2
C18:2 n6 *cis*	40.5 ± 0.1	35.1 ± 0.1	31.5 ± 0.3
C18:3 n3	22.7 ± 0.1	14.1 ± 0.0	16.7 ± 0.2
C20:0	0.2 ± 0.0	0.2 ± 0.1	–
cis-9, *trans*-11 CLA	–	–	8.6 ± 0.3
trans-10, *cis*-12 CLA	–	–	8.5 ± 0.4
C20:1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:2	–	–	0.1 ± 0.0
C22:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
C20:5 n3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C22:2	0.2 ± 0.0	0.1 ± 0.0	–
C23:0	–	–	0.1 ± 0.0
C24:0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
C24:1	< 0.1	0.1 ± 0.0	–

The aim of this study was to assess the influence of diet supplementation of pregnant and breastfeeding female Sprague-Dawley rats with conjugated linoleic acids on thiobarbituric acid reactive substances (TBARS) concentration and fatty acids profile in the liver and hepatic microsomes as well as on the Δ6- and Δ5-desaturase (E.C. 1.14.19.) activity in hepatic microsomes of the progeny with chemically induced mammary tumors.

EXPERIMENTAL

Animals

This research and guiding principles in the care and use of laboratory animals were approved by The Local Ethical Committee on Animal Experiments. The whole experiment was thoroughly described previously (15). Briefly, maiden adult female Sprague-Dawley rats (n = 8), which came from Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Warszawa, Poland), after 1-week adaptation period were randomly divided into two groups of 4 each. Each group had a different dietary supplementation: group K received vegetable oil whereas group O – Bio-CLA (Pharma Nord, Vojens, Denmark) given intragastrically in the amount 0.15 mL per day, which corresponds to 100 mg of CLA. Applied vegetable oil, purchased from the Pharma Nord, did not contain the CLA and was a substrate for the CLA synthesis. Detailed composition of fatty acids of applied oil was: C16:0 (5.7 ± 0.0)%, C18:0 (0.4 ± 0.0)%, C18:1 n9 (64.5 ± 0.1)%, C18:2 n6 (25.5 ± 0.0)%, C20:04 n6 (0.3 ± 0.0)% C24:1 (0.2 ± 0.0)%. Supplementation lasted for the whole period of pregnancy and breastfeeding. The female offspring was separated from their mothers at the 30th day of age and within each group of supplementation was divided into two subgroups of 8–10 individuals each: 1 – with diet enriched with the same dietary supplement that had been previously given to their mothers (group K1 obtained vegetable oil and group O1 – Bio-C.L.A. and 2 – fed exclusively the standard Labofeed H fodder H (fodder producer "Morawski", Żurawia 19, Kcynia, Poland). Table 1 shows the fatty acids composition of applied diets. Dietary supplementation of subgroups was conducted for the following 21 weeks from 30 days of life. The offspring received *via* gavage at 50th day of life a single dose of 80.00 mg/kg body weight of carcinogenic agent – DMBA (7,12-dimethylbenz[a]anthracene, approx. 95%, Sigma-Aldrich, Saint Louis, Missouri, USA) for the induction of mammary tumors. Rats were weighed and palpated weekly to detect the appearance of mammary tumors. The animals were decapitated and exsanguinated, and livers and tumors were collected. Isolated tumors were histopathologically evaluated as adenocarcinomas and papillary adenocarcinomas of mammary gland. Spontaneous tumors were not found in maternal rats during the whole experiment.

Preparation of experimental material

Samples of liver were collected during necropsy and stored at –70°C before the analysis.

Hepatic microsomes were prepared immediately after decapitation according to the slightly modified method of Kłyszejko-Stefanowicz (16). A sample of liver (4 g) was mechanically homogenized in saccharose solution (16 mL; 0.25 mol/L) buffered with Tris-buffer (pH 7.4). The obtained homogenate was centrifuged three times: (1) for 10 min at 1000 × g at 4°C, (2) for 20 min at 16000 × g at 4°C, and (3) for 75 min at 100000 × g at 4°C. The applied modification refers to the fact, that as we were interested only in microsomal fraction, each time the sediment was discarded and the supernatant was again centrifuged with increasing speed. Final pellet was resuspended in 4 mL 0.25 mol/L saccharose solution and obtained suspension of hepatic microsomes was stored at –70°C until further analysis.

Fatty acids analysis

Fatty acid analysis was made with gas chromatography (GC) using gas chromatograph (GC-17A gas chromatograph, Shimadzu, Kyoto, Japan) with capillary column (BPX 70; 60 m × 0.25 mm i.d., film thickness 0.20 μm, SGE, Ringwood, Australia) and flame-ionization detection.

Samples of liver were thawed only once and three parallel samples of 0.2 g were taken for lipids extraction according to Folch et al. with slight modification (17). Applied modification refers to the volume and sequence of organic solvents, which were used in extraction procedure. Fatty acids methyl esters (FAME) were prepared according to procedure of Bondia-Pons et al. (18) with slight modifications, which were thoroughly described elsewhere (19).

The hepatic microsomes were thawed only once and three parallel samples of 200 μL of microsomal suspensions were taken for lipids extraction. Lipids were extracted according to Folch et al. with slight modification (17). Briefly, a sample of microsomal suspension was mixed with 2.5 mL of chloroform : methanol (2 : 1, v/v) and after vigorous shaking the chloroform layer was separated. The residue

was mixed with 1.5 mL of chloroform : methanol (2 : 1, v/v) and the extraction was repeated. Combined chloroform layers were centrifuged for 10 min at 1000 × g and the sediment was discarded. The organic extract was evaporated under stream of nitrogen and the residue was taken for the preparation of FAME according to procedure of Bondia-Pons et al. (18) with slight modifications, which was also used for liver samples. The detailed procedure of FAME analysis was previously described and applied modification refers to the volumes of solvents (19).

TBARS analysis

Samples of liver were thawed only once and sample of 0.5 g was taken for TBARS analysis with spectrophotometric method (20). This sample was mechanically homogenized with teflon homogenizer in 2.5 mL of sodium chloride solution (0.9%). Afterwards, 2.5 mL of phosphate buffer (pH 7.0) and 1.5 mL of trichloroacetic acid (1.7 mol/L), were added to 0.5 mL of previously obtained homogenate. The whole sample was shaken vigorously and centrifuged for 15 min at 1000 × g at 0°C, to separate the protein sediment. The 1.0 mL of 2-thiobarbituric acid solution (69 mmol/L) was added to 3.0 mL of supernatant; the whole sample was shaken vigorously and heated for 15 min on boiling water bath. The absorbance was measured at $\lambda = 530$ nm after cooling to room temperature. The reference sample was prepared analogously with 0.5 mL of sodium chloride solution (0.9%). Three parallel samples were prepared for each liver sample.

Enzymes activity analysis

Enzymes activity was determined in an indirect way because the amount of AA formed *in vitro* from LA correlates with the activity of the investigated enzymes (21). The measurement of these activities was carried out according to previously published method (22), with slight modifications. We decided not to use the radioisotopes, which were applied in original procedure. Each 1.0 mL reaction mixture contained 5 mmol ATP, 0.1 mmol CoA, 1.25 mmol NADH, 0.5 mmol niacinamide, 2.25 mmol glutathione and 5 mmol $MgCl_2$, dissolved in phosphate buffer pH 7.4 and 200 nmol sodium salt linoleic acid. The reaction mixture was preincubated for 5 min at 37°C. The enzymatic reaction was started by the addition of 0.2 mL of hepatic microsomes suspension. The whole mixture was incubated in a shaking water bath for 90 min at 37°C. Further analytical procedure consisted of lipids extraction according to Folch et al. (17) and esterification (22).

The fatty acids concentrations in samples were determined with high performance liquid chromatography (HPLC) with UV/VIS detection (Merck Hitachi, L-7100 pump, UV/VIS L-74200 detector, CTO-10 AS oven, YMC-Pack ODS-AM S-5 μm column, the column temp. 30°C, $\lambda = 198$ nm). The activity index of Δ6-desaturase (D6D) was calculated as the ratio of γ-linolenic acid (GLA) concentration to linoleic acid (LA) concentration and the activity index of Δ5-desaturase (D5D) was expressed as the ratio of arachidonic acid (AA) concentration to dihomo-γ-linolenic acid (DGLA) concentration in liver microsomes. The content of AA in liver microsomes was calculated in protein content, determined using the method of Lowry et al. (23). The differences in AA concentrations between incubated and non-incubated samples indicate the activity of investigated enzymes.

Table 2. Characterization of experimental groups.

Group (number of individuals)	K1 (n = 10)	K2 (n = 9)	O1 (n = 8)	O2 (n = 9)
Mothers' diet	Lab. H + oil	Lab. H + oil	Lab. H + CLA	Lab. H + CLA
Progeny's diet	Lab. H + oil	Lab. H	Lab. H + CLA	Lab. H
Number of individuals with tumors	8	7	2	3
Age of first tumor appearance [week]	19	17	15	21
Total number of tumors	14	11	4	5
Maximal number of tumors per individual	0–3	0–4	0–3	0–2
Maximal weight of tumors per individual [g]	31.77	16.19	11.81	4.27

Table 3. Comparison of TBARS concentration in livers of investigated groups.

	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
Liver [g]	7.21 ± 1.31	5.98 ± 0.59	6.65 ± 0.58	6.15 ± 0.58	0.0079
Liver *vs.* total body weight [%]	3.2 ± 0.7	2.7 ± 0.2	3.0 ± 0.2	2.8 ± 0.3	0.0224
Fat [%]	2.6 ± 0.4	2.8 ± 0.4	2.3 ± 0.4	2.3 ± 1.0	0.0094
TBARS [nM/g of tissue]	60.10 ± 30.86	46.04 ± 44.25	53.95 ± 47.36	50.03 ± 37.00	NS
TBARS [μM/g of fat]	2.39 ± 1.39	1.63 ± 1.55	2.46 ± 2.18	2.84 ± 2.31	NS

All data are shown as the mean values ± standard deviation; p value < 0.05 for variables with significant differences among groups in Kruskal-Wallis test; NS – not significant differences among groups in Kruskal-Wallis test (p value > 0.05).

Statistical analysis

All data are shown as the mean values ± standard deviation. For variables with skew distribution, obtained data were transformed into logarithms and retransformed after calculations. They are presented as the mean and confidence interval. Results obtained for fatty acids and TBARS content were evaluated with Statistica 10.0 (StatSoft, Kraków, Poland). Due to the relatively small number of individuals in each group, the data were tested with Kruskal-Wallis test and p-value ≤ 0.05 was considered significant.

Results of enzymes activity were evaluated with statistical methods such as: PCA (principal component analysis), ANOVA with permutation test and ASCA (analysis of variance-simultaneous component analysis).

RESULTS

All four groups of the progeny received DMBA administered intragastrically in a single dose of 80 mg/kg body weight and the breast tumors, identified as adenocarcinomas and papillary adenocarcinomas of mammary gland, appeared in all investigated groups. The characterization of experimental groups, as well as the effectiveness of cancer induction are shown in Table 2. CLA decreased the incidence of mammary cancer in investigated animals (p = 0.0322). Moreover, the tumors' number and weight were smaller than in two oil groups. In O1 breast tumors appeared much earlier (on average at 15th week of life) than in other groups, but their number per individual was the smallest. We observed strong cancer preventive properties of CLA also in O2 group, where both the number and mass of tumors were similar to those in O1, which suggests that dietary intake of CLA from the very early period of life is very important for reducing the risk of carcinogenesis (15).

Comparison of average mass of livers revealed that applied supplementation significantly influenced their weight (Table 3). We observed the highest mass of livers obtained from animals from K1, whereas the mass of these organs from K2 was the smallest. Two-step supplementation with CLA seems not to affect the weight of examined organs, as their mass was slightly lower than in K1 but slightly higher in O2 and K2.

We compared the fat content in livers from examined groups. There were significant differences in amount of fat among them (Table 3). The highest content of fat occurred in livers from K2, whereas its content in both CLA groups was lower. Mean con-

Table 4. Fatty acids profile in livers of experimental groups.

Fatty acid	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
	[% of FA]				
C12:0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	NS
C14:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	NS
C15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.002
C16:0	18.1 ± 1.5	19.1 ± 1.7	16.8 ± 0.5	19.5 ± 1.1	0.001
C16:1	0.4 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.000
C17:0	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	NS
C17:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:0	23.0 ± 2.1	21.4 ± 2.0	25.2 ± 1.0	21.1 ± 1.3	0.000
C18:1 n9 *cis*	5.8 ± 1.2	5.5 ± 1.5	4.4 ± 0.7	5.6 ± 1.1	0.042
C18:2 n6 *trans*	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.000
C18:2 n6 *cis*	15.6 ± 2.3	16.1 ± 1.5	13.4 ± 1.3	17.0 ± 1.6	0.003
C18:3 n6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	NS
C18:3 n3	0.7 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	0.000
cis-9, *trans*-11 CLA	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.000
trans-10, *cis*-12 CLA	nd	nd	0.1 ± 0.0	nd	NS
C20:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C21:0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.015
C20:2	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.016
C20:4 n6	18.3 ± 2.6	17.7 ± 1.6	19.8 ± 0.6	17.7 ± 0.8	0.006
C20:5 n3	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	NS
C24:0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	NS
C24:1	1.4 ± 0.3	1.5 ± 0.4	1.0 ± 0.2	1.5 ± 0.3	0.039
C22:6 n3	10.0 ± 0.8	9.5 ± 1.4	11.2 ± 1.1	9.2 ± 1.3	NS

All data are shown as the mean values ± standard deviation. Percentage share of fatty acids: C13:0, C14:1, C15:1, C20:0, C20:3 n6, C20:3 n3, C22:0, C22:2, was < 0.1% and they are not included into table. For variables with skew distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as the mean and confidence interval; p value < 0.05 for those fatty acids with significant differences among groups in Kruskal-Wallis test; nd – not detected; NS – not significant differences among groups in Kruskal-Wallis test (p value > 0.05)

tent of fat in O2 was the lowest but we observed significant variations among individuals. No connection was found between liver mass and its fat content in examined groups.

Concentration of TBARS, in respect both to the mass of in liver tissue and to in its fat content, did not differed among examined groups. Moreover, in all groups great differences among individuals were observed, which suggests that the influence of applied dietary supplementation on the oxidative stress is marginal.

FAME profiles of livers were measured using gas chromatography (GC). In our experiment we analyzed 31 fatty acids. C18:0, C20:4 n6 (AA), C16:0, C18:2 n6 *cis* (LA), and C22:6 n3 (DHA) were found to be the main fatty acids in livers of all investigated groups (Table 4). There were significant differences in concentration of some fatty acids among examined groups. Those differences were caused mainly by two-step supplementation of diet with conjugated linoleic acids. In O1 we detected the highest concentration of C18:0, C20:1, AA and DHA acid and the lowest concentration of C16:0, C16:1, C18:1 n9 *cis* (OL), C18:2 n6 *trans*, LA, C18:3 n3 (ALA), C20:2 and C24:1 acids. The two-step supplementation of diet with vegetable oil increased the mean concentration of C21:0 and decreased the concentration of C20:1 in livers of K1 group. Unlimited consumption of standard fodder caused visible similarity in content of most fatty acids in livers of K2 and O2.

FAME profiles of hepatic microsomes were also investigated using GC and 29 fatty acids were identified and determined in them. The most common fatty acids in microsomes obtained from livers from animals of all experimental groups were: C18:0, C20:4 n6 (AA), C16:0, C18:2 n6 *cis* (LA) and C22:6 n3 (DHA). The concentration of many of them differed significantly among experimental groups (Table 2). Concentration of some of the saturated fatty acids (C14:0, C15:0, C16:0 and C24:0) was decreased, whereas concentration of others (C18:0) was increased by the two-step supplementation of diet with CLA. In microsomes of O1 group we also detected the lowest concentration of C16:1, OL, LA, ALA and C24:1 and the highest concentration of C20:1, C20:3 n3 and DHA. The two-step supplementation of diet with vegetable oil had also great influence on fatty acids concentration in hepatic microsomes and it increased concentration of some fatty acids (C14:0, OL, C21:0, C20:2, EPA, C24:0) and decreased the AA content.

In our experiment, we used Bio-C.L.A. as a source of CLA. As previously described, it consists of several fatty acids, mostly *trans*-10, *cis*-12 CLA (33%) and *cis*-9, *trans*-11 CLA (31%) (18). These two main CLA isomers were detected only in all livers of O1 group. In livers acquired from other investigated groups only rumenic acid (RA) was present, but in much lower amount. In O1 group, with two-step CLA supplementation, the percentage share of RA in total fatty acids amount in liver was much higher than in other groups (Table 4) and its mean content was $0.2 \pm 0.1\%$ of total fatty acids. The other CLA isomer: *trans*-10, *cis*-12 CLA constituted only $0.1 \pm 0.0\%$ of total fatty acids in livers. In microsomal fraction of hepatic tissue both CLA isomers were identified only in O1 group (Table 5). Comparison of their distribution in hepatic microsomes revealed the great similarity to their distribution in whole tissue, because *cis*-9, *trans*-11 CLA concentration tended to be much higher than *trans*-10, *cis*-12 CLA ($0.2 \pm 0.0\%$ *versus* $0.1 \pm 0.0\%$).

Our experiment allowed to test the statistical differences in dietary supplementation and the differences in supplements administration to mothers and children (K1, O1) or only to mothers (K2, O2) on parameters describing the desaturation effectiveness. Results of permutation test for individual effects and their interactions are shown in Figure 1.

Results of the analysis of the main factors and interactions are shown in Figure 2.

In applied model systems the differences in arachidonic acid concentrations between incubated and non-incubated samples indicated the enzymes' activity. The highest enzymes activity was found in K1 group, which proves that the addition of vegetable oil increases the levels of AA and increases the activity of D6D. If supplementation is carried out only for mothers (K2), this effect is less pronounced. In case of CLA supplementation, the observed effect was opposite (Table 6).

Two-step supplementation of diet with vegetable oil caused the increase in AA concentration, whereas the effect of two-step supplementation with CLA was opposite. If the supplementation was limited only to mothers, CLA increased AA concentration, whereas oil decreased it.

DISCUSSION AND CONCLUSION

The liver is a key organ in lipids metabolism. It integrates the pathways of fatty acids, triacylglycerols and phospholipids synthesis and degradation. Many processes of lipid metabolism, e.g., fatty acids esterification or desaturation, are related to microsomal fraction of cells where necessary enzymes are located (24) and therefore liver is an appropriate

Table 5. Fatty acids profile in hepatic microsomes of experimental groups.

Fatty acid	Groups of animals				Kruskal-Wallis test p value
	K1	K2	O1	O2	
	[% of FA]				
C12:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	NS
C14:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.049
C15:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.010
C16:0	18.5 ± 1.4	18.2 ± 1.4	16.3 ± 1.1	18.4 ± 1.2	0.013
C16:1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.000
C17:0	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	NS
C17:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:0	26.2 ± 1.4	25.9 ± 1.3	29.0 ± 1.1	24.6 ± 1.2	0.000
C18:1 n9 *cis*	5.4 ± 0.7	4.8 ± 1.0	4.1 ± 0.3	4.7 ± 0.8	0.001
C18:2 n6 *trans*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C18:2 n6 *cis*	12.6 ± 1.4	12.6 ± 1.4	10.8 ± 1.2	13.4 ± 1.3	0.005
C18:3 n6	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	NS
C18:3 n3	0.5 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.1	0.002
C20:0	0.1 ± 0.0	0.0 (0.0-0.1)*	0.0 ± 0.0	0.0 ± 0.0	0.005
cis-9, *trans*-11 CLA	0.0 (0.0-0.0)*	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.005
trans-10, *cis*-12 CLA	nd	nd	0.1 ± 0.0	nd	NS
C20:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.001
C21:0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.043
C20:2	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	NS
C20:4 n6	19.2 ± 2.3	20.2 ± 0.8	21.4 ± 0.9	19.8 ± 0.7	0.013
C20:3 n3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.012
C20:5 n3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	NS
C22:2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C24:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.005
C24:1	1.4 ± 0.4	1.3 ± 0.3	1.0 ± 0.2	1.4 ± 0.3	0.034
C22:6 n3	9.3 ± 1.0	9.6 ± 1.2	10.4 ± 0.9	8.99 ± 1.05	NS

All data are shown as the mean values ± standard deviation. Percentage share of fatty acids: C10:0, C14:1, C20:3 n6 was < 0.1% and they are not included in the Table. For variables with skew distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as the mean and confidence interval; p value < 0.05 for those fatty acids with significant differences among groups in Kruskal-Wallis test; nd - not detected; NS - not significant differences among groups in Kruskal-Wallis test (p value > 0.05).

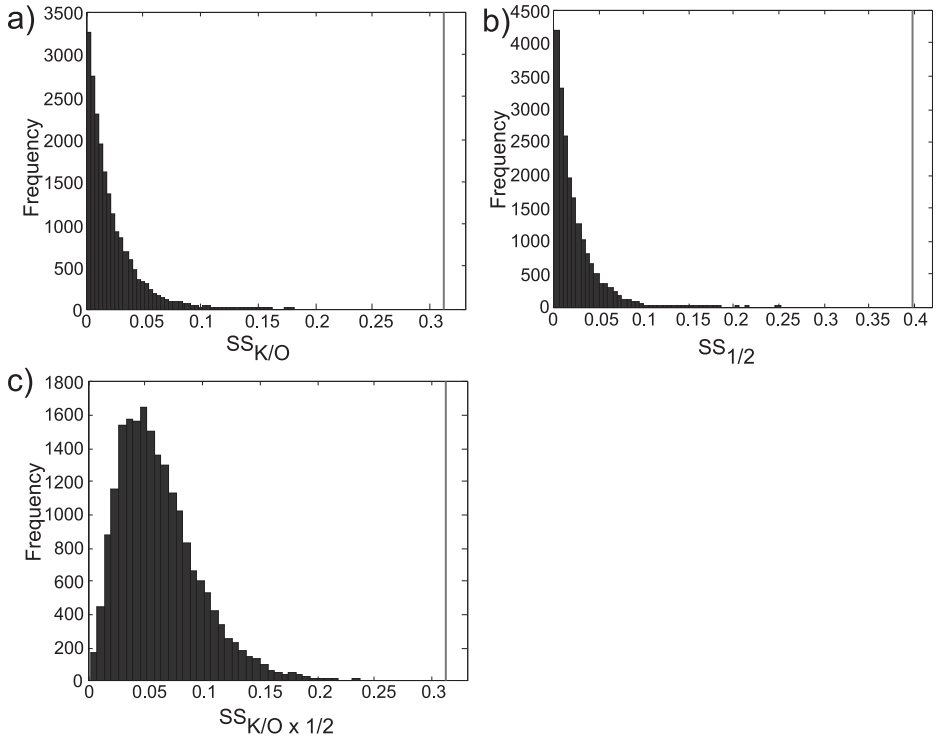

Figure 1. Results of permutation test for the various effects

organ to investigate the profile of CLA and other fatty acids (25).

Conjugated linoleic acids are a group of isomers of linoleic acid with wide range of biological activity. Their beneficial properties in many pathological conditions e.g., obesity, arteriosclerosis, cardiovascular diseases, osteoporosis, diabetes, insulin resistance, inflammation and different types of cancer are the subject of numerous scientific studies. Their ability to lower the breast cancer risk draws especially much attention. Many potential mechanisms of anticancer activity of CLA are investigated, e.g., antiproliferative, proapoptotic, antiangiogenic, antiestrogenic and competition with linoleic acid in metabolic pathway (11). Activity of Δ6- and Δ5-desaturases is the main factor which controls the conversion of LA to AA, however the effectiveness of this reactions depends not only on the activity of crucial enzymes but also on the absolute content of fatty acids, as the substrates. Since many dietary factors can regulate this pathway, we decided to check whether CLA can influence these enzymes, directly and indirectly. It seems to be especially important in cancerous process. As rats show gender differences, with females responding better than males (26), in our study we used female Sprague-Dawley rats as model organisms. We previously showed that supplementation of diet with 1.0% CLA not only decreases the risk of chemically induced breast cancer in female Sprague-Dawley rats but also influences the fatty acids profile in serum and in hepatic microsomes (19). In this study, we decided to use one or two step supplementation of diet to verify if the overall dose of CLA and the time of supplementation influenced the effectiveness of chemopreventive action.

Addition of CLA to the diet of pregnant and breastfeeding rats increases their concentration in maternal milk and as a consequence – their intake by the offspring (27, 28). The same correlation was shown for women who consumed dietary products enriched with CLA. Their milk contained much more CLA (29). We also previously proved that numerous dietary and environmental factors can elevate CLA content in maternal milk of breastfeeding women (30, 31). In our experiment, the higher supply of conjugated linoleic acids in diet of pregnant and breastfeeding females revealed the lower susceptibility to chemically induced mammary tumors in their offspring (Table 2). Effectiveness of chemo-

preventive action was comparable in both CLA groups (O1 and O2), which suggests that optimal dosage of conjugated diens of linoleic acid in early stage of life influences health in adulthood. Our results are in line with those of Ip et al. (32), who supplemented the diet of young rats for 5 weeks before the carcinogenic agent administration and demonstrated the inhibition of mammary carcinogenesis. They claimed that CLA given during the maturation of mammary glands can change their structure, e.g., diminish the number and differentiation of terminal end buds and can reduce the number of places of potential cancer induction. Moreover, numerous studies confirm the anticancerogenic properties of CLA in breast cancer model (9, 33, 34).

We observed significant differences in liver mass among examined groups. Feeding vegetable oil during the whole life resulted in increase in the liver weights, whereas influence of two-step CLA supplementation seemed to be similar but not so evident. Our observations are in line with those obtained by others. Javadi et al. (35) detected an increase in liver weights only after 12 weeks of feeding mice with CLA, but there were no differences after 3 weeks period. Akahosi et al. (36) did not observe any significant differences in inner organs weight, however, mass of livers seemed to be slightly bigger in groups of male Sprague-Dawley rats supplemented with single CLA isomers, or with their mixture. Ip et al. (9) did not note any differences in organs' mass either when the mixture of CLA isomers was applied. Long term feeding of male Fisher rats with 1.0% of CLA mixture did not change the liver weights (26). Turpeinen et al. (37), who applied single CLA isomer – *cis*-9, *trans*-11, claimed that rumenic acid did not influence the inner organs' mass. These findings are in line with those of Ip et al. (38), who used in their experiment mice with cancer, and observed that *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA caused the significant increase of mass of such organs as liver, heart and spleen.

As far as fatty acids profile in liver is concerned, our observations were similar to those of other authors. Javadi et al. (35) found the same fatty acids to be the most prominent in mice livers and confirmed that both CLA supplementation and the feeding period significantly changed the content of some of the fatty acids in examined tissue. Twelve weeks supplementation reduced the concentration of AA, 22:4 n6, C20:3 n6 and DHA, and elevated the content of C18:4 n3, C20:1 n9 and EPA. Moreover, the addition of CLA to diet changed the amount of C16:0, C18:3 n6, C20:2 n6 and C22:5 n3. The authors concluded that these changes could be the result of lower effectiveness of desaturation and elongation processes, caused by CLA. Also Kostogrys et al. (39), who checked whether CLA can reverse the harmful effect of high-fructose diet on fatty acids profile in liver, emphasized the beneficial impact of CLA on the concentration of some of the fatty acids.

As far as fatty acids concentration in microsomal fraction of livers are concerned, we detected the highest amounts of the same fatty acids as other researchers did (40–42). They proved that fatty acids profile in liver microsomes depended on the fatty acids profile in diet and dietary supplementation, especially with long chain polyunsaturated fatty acids, significantly influence not only the fatty acids composition of microsomes but also their function, e.g., activity of numerous enzymes.

We observed some interesting tendencies in fatty acids content, especially in n3 and n6 polyunsaturated fatty acids. The highest content of DHA and its substrate ALA was detected in O1, whereas their lowest content was in O2. For EPA, which is the intermediate in conversion of ALA to DHA (43), a similar result might be expected. However, we did not detect such correlation, but obtained results confirm our previous observation. CLA seem to

Table 6. Influence of the diet on AA concentration increase, D6D and D5D activity in hepatic microsomes.

Group	Increase of AA concentration [mg/100 mg of protein]	D6D	D5D
K1	0.43 ± 0.13	$(4.39 \pm 0.17) \times 10^{-3}$	1.73 ± 0.06
K2	0.13 ± 0.07	$(4.38 \pm 0.11) \times 10^{-3}$	1.71 ± 0.08
O1	0.16 ± 0.06	$(2.41 \pm 0.22) \times 10^{-3}$	1.68 ± 0.12
O2	0.13 ± 0.08	$(2.71 \pm 0.15) \times 10^{-3}$	1.71 ± 0.07

All data are shown as the mean values ± standard deviation.

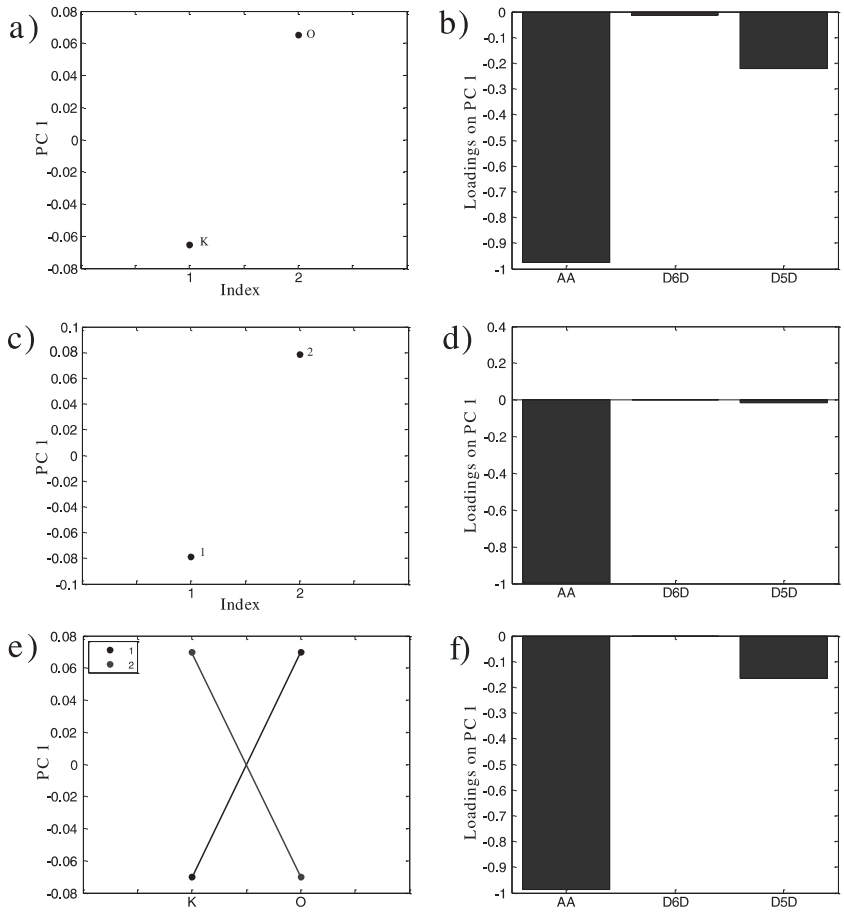

Figure 2. Results of the analysis of the principal components and their interactions

increase DHA and decrease ALA and EPA content in hepatic microsomes (19). Cao et al. (28) also observed elevated levels of DHA in liver phospholipids of suckling rats after supplementation of their mothers' diet with CLA. Also Martins et al. (25) detected similar increase in DHA content both in polar and in neutral lipids of rats' livers after CLA mixture administration, while single isomers did not exert such an effect. Eder et al. (44) observed elevated levels of DHA as well as the sum of n-3 FA in liver phospholipids as a result of CLA supplementation.

LA, which starts the n6 fatty acids family, is converted into AA, which in turn is the precursor for eicosanoids synthesis. The highest concentration of AA was detected in O1, whereas its lowest amount was in K1. These results are in line with those for LA content in microsomes, which was the highest in K1 and the lowest in O1. It confirms that supplementation of diet with CLA influences the fatty acids profile in hepatic microsomes, especially polyunsaturated n6 fatty acids. Belury et al. (45), who detected the decreased content of LA and AA in liver phospholipids in mice receiving CLA, also proved that CLA can compete with n6 fatty acids and determine their metabolism. Results of Javadi et al. (35) also confirm the ability of CLA to decrease the LA and AA content in livers of mice. Some differences in our results concerning fatty acids profiles in hepatic tissue and its microsomal fraction after CLA supplementation in relation to results obtained by others can be caused by the coexisting cancerous process. Our results indicate that not only diet but also coexisting factors such as pathological conditions or diseases e.g., cancers affect the levels of fatty acids in tissues. We previously reported (19) the significant differences in fatty acids profile in serum and hepatic microsomes of rats treated with DMBA and those not treated with DMBA. It has been observed that the profile of fatty acids in serum

of patients suffering from different types of cancer differs from distribution of fatty acids in wealthy people. Plasma phospholipids from patients suffering from bladder cancer contained much lower levels of LA and its metabolites, whereas the levels of ALA did not differ (46). There are many possible explanations for this fact but the most probable one is the changes in lipids metabolism, e.g., enhanced lipolysis or lipids peroxidation (47) or inhibited action of desaturases, mainly Δ6- desaturase, which was characteristic of cancer cells (46).

Although CLA concentration in breast tissues is generally higher than in liver (48), phospholipids of livers are richer in CLA isomers than phospholipids of mammary glands (9). Conjugated linoleic acids are preferentially incorporated into triacylglycerols rather than into phospholipids (25, 49). Liver possesses a reasonable ratio between phospholipids and neutral lipids and is an appropriate organ for studying of CLA incorporation (25). In this study, we detected both CLA isomers only in livers and in their microsomal fraction of the O1 group – constantly supplemented with CLA. In other groups only *cis*-9, *trans*-11 CLA was present in examined material, but its concentration was much lower than in O1. Although the higher supply of CLA in mothers' diet during pregnancy and breastfeeding caused only minor incorporation of CLA into hepatic tissues of children, CLA health-promoting effects were significant. *Trans*-10, *cis*-12 CLA content in hepatic microsomes and liver tissue was much lower than the content of rumenic acid despite their similar distribution in fatty acids pool of O1 group diet (Table 1). Our results are in line with those of Martins et al. (25), who detected that *cis*-9, *trans*-11 CLA was much more incorporated into both polar and neutral hepatic lipid fractions than *trans*-10, *cis*-12 CLA. However, Cao et al. (28), who used equimolar mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in two dosages (1.0 and 2.0%), found higher concentration of *trans*-10, *cis*-12 CLA isomer in liver phospholipids of both groups and in phospholipids from livers of their progeny. According to Tsuzuki (50) such diversity emerges from differences in metabolism and not in the bioavailability. *Trans*-10 *cis*-12 octadecadienoic acid activates β-oxidation and facilitates its own metabolism. Differences in CLA isomers incorporation are observed not only in liver but also in other tissues. Huot et al. (51) indicated in caveolae phospholipids of MCF-7 breast cancer cells lower amounts of *trans*-10, *cis*-12 CLA than *cis*-9, *trans*-11 CLA, despite their similar content in applied mixture. Our previous results also confirmed preferential incorporation of *cis*-9, *trans*-11 CLA into hepatic microsomes (19) and breast cancer tissues (15).

Lipid peroxidation is the result of oxidative stress and can lead to cell and tissue damage (39). Cancerous process is related to the increase in lipids peroxidation. Polyunsaturated fatty acids in phospholipids are especially prone to this reaction (52). Other sources suggest that chemopreventive properties of PUFA are the result of toxic effect of their peroxidation products on cancer cells (53). Results of numerous studies showed the increased levels of TBARS in serum of patients suffering from cancer of different organs: breast, lungs, stomach or small intestine (52, 54). However, some researchers claim that decreased lipids peroxidation stimulates the proliferation of cancer cells and promotes their malignancy (55). Many researches emphasize the antioxidative properties of CLA. Ha et al. (56) compared the antioxidative potential and concluded that CLA is as strong an antioxidant as α-tocopherole and almost as strong as butylhydroxytoluene. We did not detect any significant changes in TBARS concentration in livers among examined groups, which could confirm or deny the antioxidative properties of CLA. Sugano et al. (57) did not detect any significant differences in TBARS content in serum and in hepatic tissue after CLA addition either. Kostogrys et al. (39), who indicated the elevated MDA levels in livers after fructose administration, did not observe any influence of CLA on MDA, in comparison to control group. Ip et al. (9) also did not indicate any differences in TBARS content in livers of animals treated with CLA mixture, although they observed their decreased amount in mammary glands. Moreover, the comparison of the strongest anticancerogenic effect of 1.0% CLA and the strongest antioxidative action of 0.25% CLA separated the toxic effect on cancer cells and the potential antioxidative properties of CLA. Our results seem to confirm these findings.

The highest enzyme activities, measured by the growth of AA, D6D and D5D, was reported in K1 group. Vegetable oil, which was administered to K1, contained significantly higher amount of LA than Bio-CLA (almost three times – 27%) (19) and caused the greatest increase in enzyme activity. This indicates that supplementation of diet with oils rich in n6 fatty acids stimulates the metabolism of polyunsaturated fatty acids and formation of AA by increasing the activity of desaturases. This is turn may promote the elevated synthesis of eicosanoids, produced from AA. One of them is prostaglandin E_2 (PGE_2), which has strong immunosuppressive prop-

erties and inhibits formation of antibodies and immune cells. It also shows the pro-inflammatory and pro-tumorigenic activities (58, 59). The experimental material received from CLA supplemented animals was characterized by the significantly lower concentrations of AA and D6D in comparison to group receiving only vegetable oil. This clearly shows the inhibitory effect of conjugated linoleic acid on the activity of the enzymes, that confirms the assumption that CLA reduces the level of AA in cells directly by the influence on LA content in membranes, or indirectly, through the regulation of metabolism. Conjugated linoleic acid can be incorporated instead of LA to phospholipids and neutral fats, which are the part of cell membranes and in this way reduced the availability of the n6 substrates for the transition. However, CLA is affecting the metabolism of LA, by competing for key enzymes controlling transitions of both compounds simultaneously, which leads to decreased levels of LA metabolites, including AA and prostaglandins emerging from this fatty acid. Similar results were obtained by Bretillon et al. (60), who showed an inhibitory effect of both CLA isomers: *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on Δ6-desaturase activity. Thijssen et al. (61) in the *in vivo* studies also indicated the reduced activity of Δ6-desaturase in humans, due to the early supplementation with a mixture of CLA, while CLA had no effect on Δ5-desaturase activity. Similar results were obtained in studies with human hepatoma cell line HepG2, but only for the *trans*-10, *cis*-12 CLA (65). Also Javadi et al. (35) suggest lower desaturation and elongation activity in the liver of the CLA-fed mice. Moreover inhibited action of desaturases, mainly Δ6-desaturase, is characteristic for cancer cells (46).

It is suggested that the desaturase activity affects the inflammatory process. The increase in the concentration of AA, which correlates with the eicosanoids formulation, plays an important role in the development of inflammation (63). Reduction of the concentration of AA and its metabolites, leading to the weakening of the inflammatory response, is one of the proposed mechanism of anticancerogenic effect of CLA isomers (64–67).

Our results indicate that conjugated linoleic acids can inhibit the development of chemically induced mammary tumors. Their higher concentration in diet influence the fatty acids profile in livers and in their microsomal fraction, as well as the enzymes' activity. The decrease in D6D activity and the increase in AA concentration due to the presence of CLA in the diet of animals can confirm their anticancer properties. The higher supply of conjugated linoleic acid in mothers diet during pregnancy and breastfeeding not only causes their incorporation into tissues of children but also exerts health-promoting effect in their adult life.

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REFERENCES

1. Achremowicz K., Szary-Sworst K.: Zywn. Nauka Technol. Jakość 3 (44), 23 (2005).
2. Food, Nutrition, Physical Activity and the Prevention of Cancer; a Global Perspective. World Cancer Research Fund/American Institute for Cancer Research, Washington DC 2007.
3. Doll R., Petro R.: J. Natl. Cancer Inst. 6, 1191 (1981).
4. Recommendations of diagnostic-therapeutic conduct in malignant tumors. Breast cancer. (Polish). Wydawnictwo Medyczne Via-Medica, Gdańsk 2009. [accessed 10 June 2011]. <http://onkologia.zalecenia.med.pl/tom1/ptok_2011___05_Rak_piersi.pdf>
5. Kramer J.K.G., Parodi P.W., Jensen R.G., Mossoba M. M., Yurawecz M.P., Adlof R.O.: Lipids 33, 835 (1998).
6. Silveira M.B., Carraro R., Monero S., Tebar J.: Public Health Nutr. 10, 1181 (2007).
7. Pariza M. W., Loretz L. J., Storkson J. M., Holland N. C.: Toxicol. Sci. 52 (Suppl. 2), 107 (1999).
8. Bougnoux P., Hajjaji N., Maheo K., Couet C., Chevalier S.: Prog. Lipid Res. 49, 76 (2010).
9. Ip C., Chin S.F., Scimeca J.A., Pariza M.W.: Cancer Res. 51, 6118 (1999).
10. Masso-Welch P.A., Zangani D., Ip C., Vaughan M.M., Shoemaker S., Ramirez R.A., Ip M.M.: Cancer Res. 62, 4383 (2002).
11. Białek A., Tokarz A.: Postepy Hig. Med. Dosw. 67, 6 (2013).
12. Ebbesson S.O.E., Lopez-Alvarenga J.C., Okin P.M., Devereux R.B., Tejero M.E.: Int. J. Circumpolar Health, 71, 17343 (2012). (doi: 10.3402/ijch.v71i0.17343)
13. Behrouzian B., Baist P.H.: Prostaglandins Leukot. Essent. Fatty Acids 68, 107 (2003).

14. Wadhwani N.S., Manglekar R.R., Dangat K.D., Kulkarni A.V., Joshi S.R.: Prostaglandins Leukot. Essent. Fatty Acids 86, 21 (2012).
15. Białek A., Tokarz A., Zagrodzki P.: J. Food Nutr. Res. 3, 39 (2014).
16. Wojtkowiak Z, Lipińska A, Kłyszejko-Stefanowicz L.: Gen. Physiol. Biophys. 9, 29 (1990).
17. Folch J., Lees M., Sloane Stanley G.H.: J. Biol. Chem. 226, 497 (1957).
18. Bondia-Pons I., Molto-Puigmarti C., Castellote A.I., Lopez-Sabater M.C.: J. Chromatogr. A 1157, 422 (2007).
19. Białek A., Tokarz A., Dudek A., Kazimierska W., Bielecki, W.: Lipids Health Dis. 9, 126 (2010).
20. Asakawa T., Matsushito S.: Lipids 15, 137 (1980).
21. Warensjö E., Risérus U., Gustafsson I. B., Mohsen R., Cederholm T.: Nutr. Metab. Cardiovasc. Dis. 18, 683 (2008).
22. Keelan M., Clandinin M.T., Thomson A.B.: Canadian J. Physiol. Pharmacol. 75, 1009 (1997).
23. Lowry D.H., Rosenbrough J.J., Farr A.A., Randal R.J.: J. Biol. Chem. 193, 265 (1951).
24. Nguyen P., Leray V., Diez M., Serisier S., Le Bloch J.: J. Anim. Physiol. Anim. Nutr. 92, 272 (2008).
25. Martins S.V., Lops P.A., Alves S.P., Alfaia C.M., Nascimento M.: Nutr. Res. 31, 246 (2011).
26. Park Y., Albright K.J., Pariza M.: Food Chem. Toxicol. 4, 1273 (2005).
27. Chin S.F., Storkson J.M., Albright K.J., Cook M.E., Pariza, M.W.: J. Nutr. 124, 2344 (1994).
28. Cao Y., Chen J., Yang L., Chen Z.Y.: J. Nutr. Biochem. 20, 685 (2009).
29. Moutsioulis A.A., Rule D.C., Murrieta C.M., Bauman D.E., Lock A.L., Barbano D.M., Carey G.B.: Nutr. Res., 28, 437 (2008).
30. Białek A., Tokarz A., Romanowicz M.: Probl. Hig. Epidemiol. 92, 819 (2011).
31. Tokarz A., Białek A., Romanowicz M.: Probl. Hig. Epidemiol. 92, 823 (2011).
32. Ip C., Singh M., Thompson H.J., Scimeca J.A.: Cancer Res. 54, 212 (1994).
33. Ip C., Dong Y., Ip M.M., Banni S., Carta G., Angioni E., Murru E. et al.: Nutr. Cancer 43, 52 (2002).
34. Thompson H., Zhu Z., Banni S., Darcy K., Lotus T., Ip C.: Cancer Res. 57, 5067 (1997).
35. Javadi M., Beynen A.C., Hovenier R., Lankhorst E., Lemmens A.G.: J. Nutr. Biochem. 15, 680 (2004).
36. Akahoshi A., Koba K., Ohkura-Kaku S., Kaneda N., Goto C.: Nutr. Res. 23, 1691 (2003).
37. Turpeinen A.M., von Willebrand E., Salminen I., Linden J., Basu S.: Lipids 41, 669 (2006).
38. Ip M.M., McGee S.O., Masso-Welch P.A., Ip C., Meng X.: Carcinogenesis 28, 1269 (2007).
39. Kostogrys R.B., Pisulewski P.: Environ. Toxicol. Pharmacol. 10, 245 (2010).
40. Fremont L., Gozzelino M. T.: Lipids 31, 871 (1996).
41. Jimenez J., Boza J., Suarez M.D., Gil A.: J. Nutr. Biochem. 8, 217 (1997)
42. Suarez, A. Faus, M.J. Gil A.: J. Nutr. Biochem. 7, 252 (1996).
43. Jelińska M.: Biul. Wydz. Farm. WUM 1, (2005).
44. Eder K., Slomma N., Becker K., Brandsch C.: J. Anim. Physiol. Anim. Nutr. 89, 45 (2005).
45. Belury M.A., Kempa-Steczko A.: Lipids 32, 199 (1997).
46. McClinton S., Moffat L.E.F., Horrobin D.F., Manku M.S.: Br. J. Cancer 63, 314 (1991).
47. Pratt V.C., Watanabe S., Bruera E., Mabkey J., Clandinin M.T.: Br. J. Cancer 87, 1370 (2002).
48. Banni S., Angioni E., Casu V., Melis M.P., Carta G., Corongiu F.P., Thompson H., Ip C.: Carcinogenesis 20, 1019 (1999).
49. Ip C., Jiang C., Thompson H.J., Scimeca J.A.: Carcinogenesis 18, 755 (1997).
50. Tsuzuki T., Ikeda I.: Biosci. Biotech. Biochem. 71, 2034 (2007).
51. Huot P.S.P., Sarkar B., Ma D.W.L.: Nutr. Res. 30, 179 (2010).
52. Gaweł S., Wardas M., Niedworok E., Wardas P.: Wiad. Lek. 57, 453 (2004).
53. Bougnoux P.: Curr. Opin. Clin. Nutr. 2, 121 (1999).
54. Del Rio D., Stewart A. J., Pellegrini N.: Nutr. Metab. Cardiovasc. Dis. 15, 316 (2005).
55. Czeczot H., Ścibor-Bentkowska D., Skrzycki M., Majewski M., Podsiad M.: Pol. Merkuriusz Lek. 29 (173), 309 (2010).
56. Ha Y. L., Storkson J., Pariza M. W.: Cancer Res. 50, 1097 (1990).
57. Sugano M., Tsujita A., Yamasaki M., Yamada K., Ikeda I.: J. Nutr. Biochem. 8, 38 (1997).
58. Bartach H., Nair J., Owen R. W.: Carcinogenesis 20, 2209 (1999).
59. Woutersen R.A., Appel M.J., Ven Garderen-Hoetmer A., Wijnands M.V.W.: Mutat. Res. 443, 111 (1999).
60. Bretillon L., Chardigny J.M., Gregoire S., Berdeaux O., Sebedio J.L.: Lipids 34, 965 (1999).

61. Thijssen M.A., Malpuech-Brugere C., Gregoire S., Chardigny J.M., Sebedio J.L.: Lipids 40, 137 (2005).
62. Eder K., Slomma N., Becker K.: J. Nutr. 132, 1115 (2002).
63. He C., Qu X., Wan J., Rong R., Huang L.: PloS One, 7, e47567 (2012). (doi: 10.1371/journal.pone. 0047567).
64. Bhattacharya A., Banu J., Rahman M., Causey J., Fernandes G.: J. Nutr. Biochem. 17, 789 (2006).
65. Białek A., Tokarz A., Kazimierska W., Bielecki W.: Bromat. Chem. Toksykol. 43, 314 (2010).
66. Park H.S., Ryu J.H., Ha Y.L., Park J.H.: Br. J. Nutr. 86, 549 (2001).
67. Huang G., Zhong X., Cao Y., Chen Y.: Asia Pac. J. Clin. Nutr. 16 (Suppl. 1), 432 (2007).

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DRUG SYNTHESIS

SYNTHESIS AND ANTI-MYCOBACTERIAL ACTIVITY OF NEW 4-THIAZOLIDINONE AND 1,3,4-OXADIAZOLE DERIVATIVES OF ISONIAZID

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Abstract: A new series of 4-thiazolidinone **(3a–e)** and 1,3,4-oxadiazole **(4a–e)** derivatives of isoniazid were synthesized and evaluated for their *in vitro* anti-mycobacterial activity. The structures of the compounds were confirmed on the basis of spectral data and elemental analysis. Some compounds showed interesting activity against four *Mycobacterium* strains: *M. intercellulari* (ATCC 35743), *M. xenopi* (ATCC 14470), *M. cheleneo* (ATCC 35751) and *M. smegmatis* (ATCC 35797). Compounds **3e**, N-(4-oxo-2-undecylthiazolidin-3-yl) isonicotinamide and **4e** N-acetyl-4-(5-undecyl-1,3,4-oxadiazol-2-yl) pyridine with minimum inhibitory concentration (MIC), 6.0 μg/mL were found to be more potent than isoniazid under the *in vitro* investigational conditions. Compound **3e** and **4e** bear a high lipophilic chain bonded to the 5-position of the thiazolidinone and 1,3,4-oxadiazole moiety, respectively. This fact indicates that there exists a contribution of lipophilicity, which would facilitate the transport of these molecules through membranes.

Keywords: 4-thiazolidinone, 1,3,4-oxadiazole, isoniazid, anti-mycobacterium agents

Tuberculosis is a chronic disease caused by several species of *Mycobacteriae*. The major issue is the increase of multidrug resistant tuberculosis. The appearance of multidrug resistant strains of *Mycobacterium tuberculosis*, which exhibit *in vitro* resistance to at least two major anti-tubercular drugs (usually isoniazid and rifampicin) and cause intractable tuberculosis, has greatly contributed to the increased incidence of tuberculosis. Another serious problem is the extensively drug-resistant tuberculosis, which are strains resistant to first and second line anti-tubercular drugs. The emergence of drug-resistant tuberculosis is an important fact that made the resurgence of tuberculosis especially alarming. Due to the high impact of multidrug resistant and extensively drug-resistant tuberculosis treatment, there is an urgent need for new drugs to treat this disease efficiently. Hence, there is emerging demand for the development of new anti-tubercular agents effective against pathogens resistant to current treatment regimens, which are limited to five drugs including rifampicin, isoniazid, ethambutol, streptomycin and pyrazinamide. In spite of major advances that have been made in the discovery process, no new drugs have been introduced in clinic since the discovery of rifampicin (1). Isoniazid (INH) is still maintaining its importance as a first line drug for treatment of tuberculosis. There are many reports on synthesis and anti-tuberculosis screening of a large number of compounds containing the isoniazid moiety (2–8). Several recent experiments indicate that incorporation of hydrophobic moieties into the framework of INH can enhance the penetration of drug into the tissues of mammalian host and into waxy cell wall of bacterium. This strategy for drug design has been proposed as a vehicle for controlled study of the growth cycle of the pathogen, as these compounds have demonstrated good activities (9–12). The need for newer compounds of this kind remains urgent due to increasing resistance of *Mycobacterial* strains to certain type of currently used anti-mycobacterials. Reports suggest that INH, a pro-drug, is converted into its active form by mycobacterial catalase peroxidase and acts on mycobacterial cell wall by inhibiting the fatty acid synthetase-II system to produce long chain fatty acid precursors for mycolic acid syn-

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thesis (13). Modification of INH at N^2 atom blocks the resulting molecule against the action of *N*-arylaminoacetyl transferases (NATs) and structurally blocking INH towards the action of NAT at N^2 may thus combat the rise of resistance. 4-Thiazolidinone derivatives have many interesting activity profiles; have been reported to possess antifungal (14), anti-TB (15), anti HIV (16), antihistaminic (17) and anticonvulsant properties (18). 4-Thiazolidinones have been reported to exhibit their anti-mycobacterial effect *via* a different route, by inhibiting bacterial enzyme Mur B, which is a precursor acting during biosynthesis of peptidoglycan (19). Newly designed molecules might be regarded as double action molecules and components involved may act synergistically. It has also been reported that conversion of INH to oxadiazoles produces the corresponding 5-substituted-3*H*-1,3,4-oxadiazol-2-thione and 3*H*-1,3,4-oxadiazol-2-one derivatives, which are characterized by high activity against *M. tuberculosis* strain H37Rv (20). Literature survey revealed that 4-(5-pentadecyl-1,3,4-oxadiazole-2-yl)pyridine was ten times more active than INH and 4-(5-heptadecyl-1,3,4-oxadiazol-2-yl)pyridine also showed the same activity, because these compounds bear a high lipophilic chain bonded to the 5-position of oxadiazole moiety (21). Also oxadiazoles conform to an important class of heterocyclic compounds with a wide range of biological activities such as: anticonvulsant (22), tyrosinase inhibitors (23), antimicrobial (24), cathepsin K inhibitors (25) and anti-neoplastic properties (26). There is an urgent need for anti-TB drugs with improved properties such as enhanced activity against multidrug resistant strains, reduced toxicity, shortened duration of therapy, rapid mycobactericidal mechanism of action and the ability to penetrate host cells and exert anti-mycobacterial effects in the intracellular environment.

Chemical modification of hydrazine unit of INH with a functional group that blocks acetylation, while maintain strong anti TB action has the potential to improve clinical outcomes and reduce the emergence in patients of acquired INH resistance.

To investigate the effect of length of lipophilic chain at 5-position and change of 1,3,4-oxadiazole scaffold with thiazolidinone moiety on the anti-mycobacterial activity, in the present study we incorporated the 4-thiazolidinone and 1,3,4-oxadazole scaffolds with different lengths of side chain at 5-position in the INH moiety and evaluated their *in vitro* anti-mycobacterial activity.

EXPERIMENTAL

All the solvents were obtained from Merck. The elemental analyses (C, H, N and S) of all compounds were performed on the CHNS Elementar (Analysen Systeme GmbH, Germany) and Vario EL III (Elementar Americas Corporation) and were within a limit of ± 0.4% and ± 0.3%, respectively, of the theoretical values. The homogeneity of the compounds was checked by TLC performed on silica gel G coated plates (Merck). Iodine chamber was used for visualization of TLC spots. The FT-IR spectra were recorded in Shimadzu spectrophotometer by dissolving samples in carbon tetrachloride (CCl_4). Melting points were determined on a Gallenkamp melting point apparatus, and thermometer was uncorrected. NMR spectra were scanned in DMSO-d_6 on a Bruker NMR spectrophotometer operating at 500 MHz for ^1H and 125.76 MHz for ^{13}C at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. Chemical shifts are expressed in δ values (ppm) relative to TMS as an internal standard and D_2O was added to confirm the exchangeable protons. Mass spectra were measured on Agilent Triple Quadrupole 6410 QQQ LC/MS apparatus with ESI (electrospray ionization) source.

N'-[(1*E*)-propylidene]pyridine-4-carbohydrazide (2a)

The isonicotinoyl hydrazide Schiff base (**2a**) was prepared by reaction between propanal (1.0 equiv.) with INH (1.0 equiv.) in ethanol/H_2O (10 mL), initially dissolving the INH in H_2O and adding the respective solution to a solution of the propanal in ethanol. After stirring for 1–3 h at room temperature, the resulting mixture was concentrated under reduced pressure. The residue was purified by washing with cold ethyl alcohol and diethyl ether and afforded pure derivatives (**2a**). The compound was assigned (*E*) configuration (27). The other Schiff bases (**2b–e**) were synthesized similarly.

Yield: 75%; m.p.: 170–172°C. FT-IR (ν, cm^{-1}): 3430 (NH, str.), 2970 (C-H, str.), 1660 (C=O, str.), 1606 (C=C, str.), 1555 (C=N, str.). ^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.29 (2H, m, CH_2), 7.70 (1H, s, CH), 7.75 (2H, d, J = 4 Hz, CH pyridyl), 8.75 (2H, d, J = 4 Hz, CH pyridyl), 11.6 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1), 149.8 (C-3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 163 (C-7), 158.3 (C-11), 22.2 (C-12), 10.4 (C-13). MS (ESI) m/z = 177.0 $[M]^+$. Analysis: calcd. for $C_9H_{11}N_3O$ (177.2): C 61.00, H 6.26, N 23.71%; found: C 61.20, H 6.24, N 23.61%.

N'-[(1*E*)-octylidene]pyridine-4-carbohydrazide (2b)

Yield: 80%; m.p.: 90–92°C. FT-IR (ν, cm^{-1}): 3430 (NH, str.), 2970 (C-H, str.), 1660 (C=O, str.), 1606 (C=C, str.), 1555 (C=N, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.4 (12H, m, CH_2), 7.70 (1H, s, CH), 7.8 (2H, d, J = 4 Hz, CH pyridyl), 8.70 (2H, d, J = 4 Hz, CH pyridyl), 11.6 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1), 149.8 (C-3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 163 (C-7), 158.3 (C-11), 26.1 (C-12), 26.1 (C-13), 29.5 (C-14), 29.1 (C-15), 31.9 (C-16), 22.8 (C-17), 14.1 (C-18). MS (ESI) m/z = 246.9 $[M]^+$. Analysis: calcd. for $C_{14}H_{21}N_3O$ (247.3): C 67.98, H 8.56, N 16.99%; found: C 67.75, H 8.54, N 16.93%.

N'-[(1*E*)-decylidene]pyridine-4-carbohydrazide (2c)

Yield: 65%; m.p.: 78–80°C. FT-IR (ν, cm^{-1}): 3430 (NH, str.), 2972 (C-H, str.), 1661 (C=O, str.), 1556 (C=C, str.), 1496 (C=N, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.3 (16H, m, CH_2), 7.70 (1H, s, CH), 7.85 (2H, d, J = 4 Hz, CH pyridyl), 8.7 (2H, d, J = 4 Hz, CH pyridyl), 11.6 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1), 149.8 (C-3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 163 (C-7), 158.3 (C-11), 26.1 (C-12), 26.1 (C-13), 29.5 (C-14), 29.4 (C-15), 29.7 (C-16), 29.4 (C-17), 31.9 (C-18), 22.8 (C-19), 14.1 (C-20). MS (ESI) m/z = 275.3 $[M]^+$. Analysis: calcd. for $C_{16}H_{25}N_3O$ (275.3): C 69.78, H 9.15, N 15.26%; found: C 69.88, H 9.37, N 15.20%.

N'-[(1*E*)-undecylidene]pyridine-4-carbohydrazide (2d)

Yield: 70%; m.p.: 80–82°C. FT-IR (ν, cm^{-1}): 3261 (NH, str.), 2923 (C-H, str.), 1655 (C=O, str.), 1535 (C=C, str.), 1464 (C=N, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.29 (18H, m, CH_2) 7.50 (1H, s, CH), 7.8 (2H, d, J = 4 Hz, CH pyridyl), 8.6 (2H, d, J = 4 Hz, CH pyridyl), 11.6 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1), 149.8 (C-3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 163 (C-7), 158.3 (C-11), 26.1 (C-12), 26.1 (C-13), 29.5 (C-14), 29.4 (C-15), 29.7 (C-16), 29.7 (C-17), 29.7 (C-18), 31.9 (C-19), 22.8 (C-20), 14.1 (C-21). MS (ESI) m/z = 290.0 $[M + 1]^+$. Analysis: calcd. for $C_{17}H_{27}N_3O$ (289): C 70.5, H 9.4, N 14.5%; found: C 70.75, H 9.38, N 14.40%.

N'-[(1*E*)-dodecylidene]pyridine-4-carbohydrazide (2e)

Yield: 60%; m.p.: 85–87°C. FT-IR (ν, cm^{-1}): 3257 (NH, str.), 2923 (C-H, str.), 1655 (C=O, str.), 1546 (C=N, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.4 (20H, m, CH_2), 7.75 (1H, s, CH), 7.8 (2H, d, J = 4 Hz, CH pyridyl), 8.7 (2H, d, J = 4 Hz, CH pyridyl), 11.6 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1), 149.8 (C-3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 163 (C-7), 158.3 (C-11), 26.1 (C-12), 26.1 (C-13), 29.5 (C-14), 29.4 (C-15), 29.7 (C-16), 29.7 (C-17), 29.7 (C-18), 29.4 (C-19), 31.9 (C-20), 22.8 (C-21), 14.1 (C-22). MS (ESI) m/z = 304.0 $[M + 1]^+$. Analysis: calcd. for $C_{18}H_{29}N_3O$ (303.4), C 71.25, H 9.63, N 13.85%; found: C 71.03, H 9.60, N 13.90.

N-(2-ethyl-4-oxothiazolidin-3-yl)isonicotinamide (3a)

A mixture of Schiff base of INH **2a** (0.1 mol), mercaptoacetic acid (0.15 mol) and silica chloride (0.025 mol) was heated at 50°C under solvent-free condition for 1 h. The progress of the reaction was monitored by TLC using hexane-ethyl acetate (7 : 3, v/v). After the completion of reaction, the reaction mixture was extracted with ethyl acetate and organic layer was washed with 5% sodium bicarbonate solution and brine. Organic layer was separated and dried over anhydrous sodium sulfate. From the organic extract the solvent was removed under reduced pressure and the residual crude solid was crystallized from ethanol. The other thiazolidinone derivatives (**3b–e**) were synthesized similarly.

Yield: 60%; semisolid. FT-IR (ν, cm^{-1}): 3300 (NH, str.), 3000 (C-H, str.), 1700 (C=O, str., thiazolidinone), 1680 (C=O, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 1.0 (3H, t, CH_3), 1.8 (2H, m, CH_2), 3.7 (2H, s, -CH_2, thiazolidinone), 5.0 (1H, t, CH, thiazolidinone), 7.7 (2H, d, J = 4 Hz, CH pyridyl), 8.7 (2H, d, J = 4 Hz, CH pyridyl), 11.1 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ ppm): 168.8 (C-1), 164.0 (C-3), 138.0 (C-4), 150.0 (C-5), 122.3 (C-6), 171.7 (C-7), 170.3 (C-10), 63.0 (C-13), 39.0 (C-11), 28.4 (C-16), 12.6 (C17). MS (ESI) m/z = 252.2 $[M + 1]^+$. Analysis: calcd. for $C_{18}H_{27}N_3O_2S$ (251.3): C 61.86, H 7.79, N 12.0, S 9.17%; found: C 61.76, H 7.8, N 12.02, S 9.18%.

N-(2-heptyl-4-oxothiazolidin-3-yl)isonicotinamide (3b)

Yield: 70%; semisolid. FT-IR (ν, cm^{-1}): 3300 (NH, str.), 3000 (C-H, str.), 1710 (C=O, str., thiazolidinone), 1680 (C=O, str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.7 (3H, t, CH_3), 1.8 (12H, m,

CH_2), 3.7 (2H, s, -CH_2, thiazolidinone), 4.8 (1H, t, CH, thiazolidinone), 7.8 (2H, d, *J* = 4 Hz, CH pyridyl), 8.7 (2H, d, *J* = 4 Hz, CH pyridyl), 11.1 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 168.7 (C-1), 164.2 (C-3), 139.4 (C-4), 150.0 (C-5), 121.8 (C-6), 171.2 (C-7), 170.2 (C-10), 62.3 (C-13), 34.2 (C-11), 28.5 (C-16), 23.9 (C-17), 22.0 (C-18, 19, 20, 21), 13.7 (C-22). MS (ESI) m/z = 321.1 $[M]^+$. Analysis: calcd. for $C_{18}H_{27}N_3O_2S$ (321.4): C 61.86, H 7.79, N 12.0, S 9.17%; found: C 61.76, H 7.8, N 12.02, S 9.18%.

N-(2-nonyl-4-oxothiazolidin-3-yl)isonicotinamide (3c)

Yield: 70%; semisolid. FT-IR (*v*, cm^{-1}): 3300 (NH, str.), 3000 (C-H, str.), 1700 (C=O, str., thiazolidinone), 1670 (C=O, str.). ^1H NMR (500 MHz, DMSO-d_6, *δ,* ppm): 0.8 (3H, t, CH_3), 1.2 (16 H, m, CH_2), 3.7 (2H, s, -CH_2, thiazolidinone), 4.87 (1H, t, CH, thiazolidinone), 7.3 (2H, d, *J* = 4 Hz, CH pyridyl), 8.8 (2H, d, *J* = 4 Hz, CH pyridyl), 11.7 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 164.0 (C-1), 161.1 (C-3), 155.7 (C-5), 153.7 (C-4), 150.4 (C-6), 174.4 (C-7), 168.9 (C-10), 62.9 (C-13), 48.5 (C-11), 34.5 (C-16), 31.2 (C-17), 28.9 (C-18), 27.9 (C-18), 23.9 (C19), 22.0 (C-20), 18.5 (C-21), 13.8 (C-22), 12.6 (C-23). MS (ESI) *m/z* = 349.1 $[M]^+$. Analysis: calcd. for $C_{18}H_{27}N_3O_2S$ (349.4): C 61.86, H 7.79, N 12.0, S 9.17%; found: C 61.76, H 7.8, N 12.02, S 9.18%.

N-(2-decyl-4-oxothiazolidin-3-yl)isonicotinamide (3d)

Yield: 75%; semisolid. FT-IR (*v*, cm^{-1}): 3320 (NH, str.), 3010 (C-H, str.), 1700 (C=O, str., thiazolidinone), 1680 (C=O, str.).^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.2 (18 H, m, CH_2), 3.6 (2H, s, -CH_2, thiazolidinone), 4.87 (1H, t, CH, thiazolidinone), 7.8 (2H, d, *J* = 4 Hz, CH pyridyl), 8.8 (2H, d, *J* = 4 Hz, CH pyridyl), 11.7 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 149.8 (C-1,3), 122.8 (C-4), 140.9 (C-5), 122.8 (C-6), 164.9 (C-7), 168.5 (C-10), 36.1 (C-11), 54.3 (C-13), 35.5 (C-16), 23.2 (C-17), 29.0 (C-18), 29.7 (C-19, 20), 29.4 (C-21), 31.9 (C-22), 22.8 (C-23),14.1 (C-24). MS (ESI) m/z = 363.5 $[M]^+$. Analysis: calcd. for $C_{19}H_{29}N_3O_2S$ (363.5): C 62.78, H 8.04, N 11.5, S 8.82%; found: C 62.80, H 8.05, N 11.52, S 8.80%.

N-(4-oxo-2-undecylthiazolidin-3-yl)isonicotinamide (3e)

Yield: 80%; semisolid. FT-IR (*v*, cm^{-1}): 3330 (NH, str.), 3010 (C-H, str.), 1710 (C=O, str., thiazolidinone), 1680 (C=O, str.). ^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.8 (3H, t, CH_3), 1.2 (20 H, m, CH_2), 3.6 (2H, s, -CH_2, thiazolidinone), 4.86 (1H, t, CH, thiazolidinone), 7.8 (2H, d, *J* = 4 Hz, CH pyridyl), 8.8 (2H, d, *J* = 4 Hz, CH pyridyl), 11.1 (1H, s, -NH, D_2O exch.). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ. ppm): 149.5 (C-1,3), 122.0 (C-4), 140.5 (C-5), 122.0 (C-6), 164.4 (C-7), 168.0 (C-10), 36.0 (C-11), 54.1 (C-13), 35.5 (C-16), 23.1 (C-17), 29.0 (C-18), 29.5 (C-19, 20, 21), 29.1 (C-22), 31.5 (C-23), 22.8 (C-24), 14.1 (C-25). MS (ESI) m/z = 377.5 $[M]^+$. Analysis: calcd. for $C_{20}H_{31}N_3O_2S$ (377.5): C 63.6, H 8.28, N 11.13, S 8.49%; found: C 62.58, H 8.06, N 11.52, S 8.80%.

N-acetyl-4-(5-ethyl-1,3,4-oxadiazol-2-yl)pyridine (4a)

A mixture of Schiff base of INH **2a** (0.1 mol) and anhydrous acetic anhydride (10 mL) was refluxed for 4 h. After completion of the reaction, the excessive acetic anhydride was distilled off at reduced pressure; the residue was poured into ice cooled water and stirred for 30 min. The solid product was filtered and recrystallized from ethanol to give final compound (**4a**). The other 1,3,4-oxadiazole derivatives (**4b**–**e**) were synthesized similarly.

Yield: 65%; semisolid. FT-IR (*v*, cm^{-1}): 1564 (C=N), 1670 (C=O), 1620, 1369, 1173, 1097, 1014 (oxadiazole nucleus). ^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.7 (3H, t, CH_3), 1.9 (3H, s, $COCH_3$), 2.2 (2H, m, CH_2), 6.3 (1H, s, -CH, oxadiazole), 7.8 (2H, d, *J* = 4 Hz, CH pyridyl), 8.7 (2H, d, *J* = 4 Hz, CH pyridyl). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 168.3 (C-12), 155.0 (C-7), 149.5 (C-1, 3), 138.4 (C-5), 124.1 (C-4, 6), 73.4 (C-9), 29.5 (C-15), 23.4 (C-14), 5.0 (C-16). MS (ESI) m/z = 219.1 $[M]^+$. Analysis: calcd. for $C_{11}H_{13}N_3O_2$ (219.2): C 60.26, H 5.98, N 19.17%; found: C 60.02, H 5.60, N 19.11%.

N-acetyl-4-(5-heptyl-1,3,4-oxadiazol-2-yl)pyridine (4b)

Yield: 67%; semisolid. FT-IR (*v*, cm^{-1}): 1560 (C=N), 1680 (C=O), 1630, 1470, 1350, 1011 (oxadiazole nucleus). ^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.85 (3H, t, CH_3), 1.2 (10H, m, CH_2), 1.9 (3H, s, $COCH_3$), 2.5 (2H, m, CH_2), 6.3 (1H, s, -CH, oxadiazole), 7.8 (2H, d, *J* = 4 Hz, CH pyridyl), 8.7 (2H, d, *J* = 4 Hz, CH pyridyl). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 166.9 (C-12), 150.6 (C-7), 138.0 (C-1, 3), 131.5 (C-5), 120.0 (C-4, 6), 93.2 (C-9), 32.5 (C-15), 31.2 (C-14), 28.9 (C-16), 22.0 (C-17), 21.1 (C-18), 20.1 (C-19), 19.0 (C-20), 13.9 (C-21). MS (ESI) m/z = 288.2 $[M]^+$. Analysis: calcd.

for $C_{16}H_{23}N_3O_2$ (288.3): C 66.41, H 8.01, N 14.52%; found: C 66.66, H 8.04, N 14.55%.

N-acetyl-4-(5-nonyl-1,3,4-oxadiazol-2-yl)pyridine (4c)

Yield: 80%; semisolid. FT-IR (ν, cm^{-1}): 1564 (C=N), 1670 (C=O), 1640, 1473, 1350, 1011 (oxadiazole nucleus). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.82 (3H, t, CH_3), 1.2 (14H, m, CH_2), 1.9 (3H, s, $COCH_3$), 2.5 (2H, m, CH_2), 6.9 (1H, s, -CH, oxadiazole), 7.8 (2H, d, J = 4 Hz, CH pyridyl), 8.8 (2H, d, J = 4 Hz, CH pyridyl). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 169.9 (C-12), 168.4 (C-7), 167.9 (C-1), 163.3 (C-3), 153.3 (C-5), 150.0 (C-6), 139.4 (C-4), 138.6 (C-9), 31.2 (C-15), 29.4 (C-14), 28.7 (C-16), 28.4 (C-17), 22.0 (C-18, 22), 20.3 (C-20), 18.0 (C-21), 13.8 (C-22), 10.7 (C-23). MS (ESI) m/z = 317.1 $[M]^+$. Analysis: calcd. for $C_{18}H_{27}N_3O_2$ (317.4): C 68.11, H 8.57, N 13.24%; found: C 68.37, H 8.55, N 13.28%.

N-acetyl-4-(5-decyl-1,3,4-oxadiazol-2-yl)pyridine (4d)

Yield: 75%; m.p.:178–180°C. FT-IR (ν, cm^{-1}): 1564 (C=N), 1680 (C=O), 1643, 1470, 1350, 1010 (oxadiazole nucleus). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 1.0 (3H, t, CH_3), 1.2 (16H, m, CH_2), 1.9 (3H, s, $COCH_3$), 2.5 (2H, m, CH_2), 6.3 (1H, s, -CH, oxadiazole), 7.8 (2H, d, J = 4 Hz, CH pyridyl), 8.8 (2H, d, J = 4 Hz, CH pyridyl). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 172.0 (C-12), 166.1 (C-7), 150.8 (C-1, 3), 138.1 (C-5), 122.7 (C-4, 6), 119.9 (C-9), 56.0 (C-15), 20.9 (C-14), 18.4 (C-16, 17, 18, 19), 18.0 (C-20, 21, 22, 23), 10.6 (C-24). MS (ESI) m/z = 331.0 $[M]^+$. Analysis: calcd. for $C_{19}H_{29}N_3O_2$ (331.4): C 68.85, H 8.82, N 12.68%; found: C 68.65, H 8.80, N 12.63%.

N-acetyl-4-(5-undecyl-1,3,4-oxadiazol-2-yl)pyridine (4e)

Yield: 70%; m.p.: 68–70°C. FT-IR (ν, cm^{-1}): 1560 (C=N), 1670 (C=O), 1630, 1373, 1150, 1011 (oxadiazole nucleus). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 0.7 (3H, t, CH_3), 1.2 (18H, m, CH_2), 1.9 (3H, s, $COCH_3$), 2.2 (2H, m, CH_2), 6.3 (1H, s, -CH, oxadiazole), 7.7 (2H, d, J = 4 Hz, CH pyridyl), 8.7 (2H, d, J = 4 Hz, CH pyridyl). ^{13}C NMR (125.76 MHz, DMSO-d_6, δ, ppm): 173.9 (C-12), 170.7 (C-7), 169.3 (C1), 166.9 (C-3), 163.9 (C-5), 157.7 (C-4), 153.2 (C-6), 150.5 (C-9), 33.6 (C-15), 32.4 (C-14), 28.4 (C-16), 22.7 (C-17), 21.9 (C-18), 20.0 (C-19, 20, 21 ,22, 23, 24), 13.7 (C-25). MS (ESI) m/z = 345.2 $[M]^+$. Analysis: calcd. for $C_{20}H_{31}N_3O_2$ (345.4): C 69.53, H 9.04, N 12.16%; found: C 69.80, H 9.07, N 12.11.

Anti-mycobacterial activity

Anti-mycobacterial activity was performed at the Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The tested *Mycobacterium* strains are *M. intercellulari* (ATCC 35734), *M. xenopi* (ATCC 14470), *M. cheleneoi* (ATCC 35751) and *M. smegmatis* (ATCC 35797) using Rist and Grosset proportion method, agar dilution method (28). The synthesized compounds (**3a–e**), (**4a–e**) and INH were dissolved in DMSO at a concentration of 1 mg/mL. The appropriate aliquot of each solution was diluted with 10% molten agar to give concentrations of 100 µg/mL. The agar and the compound solution were mixed thoroughly and the mixture was poured into Petri dishes on a level surface to result in an agar depth of 3 to 4 mm and allowed to harden. The inocula were prepared by growing overnight culture in Mueller-Hinton broth. The cultures were diluted 1 : 100. The tested organisms were streaked in a radial pattern and plates were incubated at 35°C for 48 h to check the growth of the tested strains at this single concentration. Active compounds were further diluted and tested by the same way to determine the minimum inhibitory concentration (MIC) of these compounds. Experiment using the tested strains in a medium free of the investigated compounds was also carried out.

RESULTS AND DISCUSSION

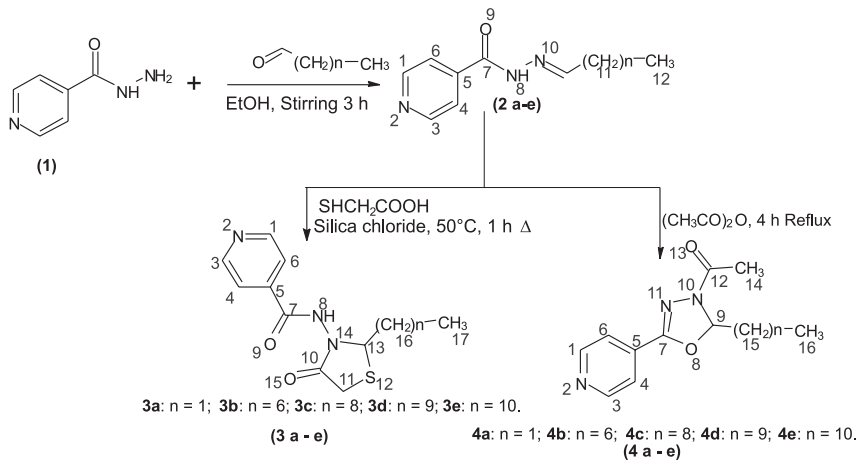
4-Thiazolidinone (**3a–e**) and 1,3,4-oxadiazole (**4a–e**) derivatives of INH were synthesized by reaction of Schiff bases (**2a–e**) with thioglycolic acid in the presence of chlorinated silica and anhydrous acetic anhydride, respectively, to obtain anti-mycobacterial agents in good yield. The condensation was carried by using equimolar amount of the Schiff bases, mercaptoacetic acid, and 25 mmol % of silica chloride. The reaction was monitored by thin layer chromatography (TLC) and was found to reach completion in 4 h giving 60–80% yields of the 4-thiazolidinones (29).

1,3,4 -Oxadiazoles (**4a–e**) were obtained by refluxing Schiff bases (**2a–e**) in excess of anhydrous acetic anhydride for 4 h. The excess of acetic anhydride was distilled off to obtain pure compounds with 65–80% yields. The purity of the synthesized compounds was checked by thin layer chromatography (TLC) and elemental analysis. The structure of the compounds were identified and confirmed by spectral data. The IR spectra of 4-thiazolidinone derivatives (**3a–e**) exhibited in each case, a band in the region of 3300–3200 cm^{-1} due to NH stretching, 3100–3000 cm^{-1} due to CH stretching, 1700–1710

Table 1. Lipophilicity (Clog *P)* and *in vitro* antimycobacterial activities of compounds (**3a–e**) and (**4a–e**).

Compound No.	C log P[a]	MIC (μg/mL)			
		M. intercellulari	*M. xenopi*	*M. cheleneoi*	*M. smegmatis*
3a	0.63	>100	>100	>100	>100
3b	2.03	50	50	50	50
3c	3.09	25	25	25	25
3d	3.62	25	25	25	25
3e	4.16	6.0	6.0	6.0	6.0
4a	0.22	>100	>100	>100	>100
4b	2.88	>100	>100	>100	>100
4c	3.94	25	25	25	25
4d	4.47	25	25	25	25
4e	5.00	6.0	6.0	6.0	6.0
Isoniazid	0.67	12.5	12.5	12.5	12.5

[a]C log P was calculated using software Chem Office 6.0.



Scheme 1. Synthesis of 4-thiazolidinone (**3a–e**) and 1,3,4-oxadiazole (**4a–e**) derivatives of isoniazid.

cm^{-1} due to carbonyl of thiazolidinone and 1680 cm^{-1} due to carbonyl absorption, whereas the IR spectra of 1,3,4-oxadiazole derivatives (**4a–e**) exhibited in each case, a band in the region 1564–1560 cm^{-1} due to C=N stretching, 1680–1670 cm^{-1} due to carbonyl absorption. In the ^1H NMR spectra, the signals of the synthesized compounds were verified on the basis of their chemical shifts, multiplicities and coupling constants. The spectra of the 4-thiazolidinone derivatives (**3a–e**) showed the characteristic D_2O exchangeable NH protons at δ 11.1–11.7 ppm, CH_2 (thiazolidinone) protons at δ 3.6–3.7 ppm, CH (thiazolidinone) proton at δ 4.8–5.0 ppm in addition to aromatic protons at δ 7.3 ppm with *J* value of 4 Hz, and δ 8.8 ppm with *J* value of 4 Hz. The spectra of 1,3,4-oxadiazole derivatives (**4a–e**) showed the characteristic $COCH_3$ protons at δ 1.9 ppm, CH (1,3,4-oxadiazole) proton at 6.3–6.9 ppm in addition to pyridyl protons at δ 7.8 ppm with *J* value of 4 Hz, and δ 8.8 ppm with *J* value of 4 Hz. The mass spec-

tra of the compounds showed the molecular ion peaks $[M]^+$ and $[M + 1]^+$. The elemental analysis of CHN and S were within ± 0.4% and 0.3%, respectively, of the theoretical values.

Anti-mycobacterial activity

The synthesized compounds (**3a–e**) and (**4a–e**) were evaluated for their anti-mycobacterial activity *in vitro* against four *Mycobacterium* strains: *Mycobacterium intercellulari* (ATCC 35734), *Mycobacterium xenopi* (ATCC 14470), *Mycobacterium cheleneo* (ATCC 35751) and *Mycobacterium smegmatis* (ATCC 35797) by agar dilution method according to the protocol described in the experimental section similar to that recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for the determination of MIC (30). INH was used as a reference drug and control experiments were done using a growth media free from drugs or the tested compounds. Results of the *in vitro* anti-mycobacterial activity of the tested compounds along with the standard drug for comparison are given in Table 1. Compound **3e**, N-(4-oxo-2-undecylthiazolidin-3-yl)isonicotinamide and **4e**, N-acetyl-4-(5-undecyl-1,3,4-oxadiazol-2-yl)pyridine presented significant growth inhibition against all strains of *Mycobacterium* with minimum inhibitory concentration (MIC) 6.0 µg/mL, with the highest C log P value of 4.1 and 5.0, respectively. Compounds **3c**, **3d**, **4c** and **4d** showed growth inhibition against all strains of *Mycobacterium* with minimum inhibitory concentration (MIC) 25 µg/mL. Compound **3b** also showed growth inhibition against all strains of *Mycobacterium* with MIC 50 µg/mL, with C log P value of 2.03. Compound **3a**, **4a** and **4b** revealed no activity on the tested strains up to the concentration of 100 µg/mL. The active compounds **3e** and **4e** were found to be more potent than first line anti-tubercular drug INH under investigation conditions. Lipophilicity of the drug molecules may make them more capable of penetrating various biomemebranes, consequently improving their permeation properties towards microbial cell membranes (31). Correlation between lipophilicity and anti TB has been reported (32). Lipophilicity of the synthesized compounds expressed in the term of their C log P values, is shown in Table 1. All the compounds presented lipophilicity higher than that of INH except **3a** and **4a**. Another raised hypothesis explores the possibility that compounds **3b**, **3c**, **3d**, **4c** and **4d** could be acting as INH prodrugs. According to Scior and Garces-Eisele, the pharmacological role of INH derivatives (isonicotinoyl hydrazones, hydrazides, and amides) must be considered as bio-reversible prodrugs of INH or isonicotinic acid. Worse activities showed by these kinds of structures can be explained by the compounds with a structural gain of stability against prodrug hydrolysis (33). None of them showed more potency than INH against the *Mycobacterium*. Although oxadiazole nucleus is very stable to acid hydrolysis, it has been reported that it may be chemically hydrolyzed with a strong base and heating leading thus to the generation of acyl-INH, which are very likely to be completely hydrolyzed to INH (34). It was reported that 5-low alkyl homologues (methyl, ethyl and propyl-1,3,4-oxadiazol-2-yl)pyridines showed a low tuberculostatic *in vitro* effect (35). Apparently, it is necessary to increase the steric hindrance at position 5 of oxadiazole moiety in order to improve the biological activity of these derivatives. It also implies that lipophilicity plays an important role in the bioactivity of these compounds.

CONCLUSION

4-Thiazolidinone (**3a–e**) and 1,3,4-oxadiazole (**4a–e**) derivatives of INH were synthesized and evaluated for their *in vitro* anti-mycobacterial activity against four *Mycobacterium* strains: *M. intercellulari* (ATCC 35743), *M. xenopi* (ATCC 14470), *M. cheleneo* (ATCC 35751) and *M. smegmatis* (ATCC 35797). The present results highlight the importance of lipophilicity of these compounds to present good anti-mycobacterial activity. The high activity of compounds **3e** and **4e** makes them suitable hits for additional *in vitro* and *in vivo* evaluations, in order to develop new anti-mycobacterial drugs or prodrugs with potential use in the tuberculosis treatment. Further studies in this area are in progress in our laboratory.

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REFERENCES

1. Burman W.J., Jones B.E.: Am. J. Respir. Crit. Care Med. 164, 7 (2001).
2. Cocco M.T., Congiu C., Onnis V., Pusceddo M.C., Schivo M.L., De Logu A.: Eur. J. Med. Chem. 34, 1071 (1999).
3. Sriram D., Yogeeswari P., Madhu K.: Bioorg. Med. Chem. Lett. 15, 4502 (2005).
4. Shindikar A.V., Viswanathan C.L.: Bioorg. Med. Chem. Lett. 15, 1803 (2005).

5. Janin Y.L.: Bioorg. Med. Chem. 15, 2479 (2007).
6. Maccari R., Ottana R., Bottari B., Rotondo E., Vigorita M.G.: Bioorg. Med. Chem. Lett. 14, 5731 (2004).
7. Maccari R., Ottana R., Vigorita M.G.: Bioorg. Med. Chem. Lett. 15, 2509 (2005).
8. Sinha N., Jain S., Tilekar A., Upadhayaya R.S., Kishore N., Jana G.H., Arora S.K.: Bioorg. Med. Chem. Lett. 15, 1573 (2005).
9. Mohamed S., Ibrahim P., Sadikun A.: Tuberculosis 84, 56 (2004).
10. Rastogi N., Goh K.S.: Antimicrob. Agents Chemother. 34, 2061 (1990).
11. Hearn M.J., Cynamon M.H.: Drug Des. Discov. 18, 103 (2003).
12. Lourenço M.C., Ferreira L., de Souza M.V., Peralta M.A., Vasconcelos T.R., Henriques M.G.: Eur. J. Med. Chem. 43, 1344 (2008).
13. Fu L.M., Shinnick T.M.: Tuberculosis 87, 63 (2007).
14. Liu H.L., Lieberzeit Z., Anthonsen T.: Molecules 5, 1055 (2000).
15. Babaoglu K., Page M.A., Jones V.C., McNeil M.R., Dong C., Naismith J.H., Lee R.E.: Bioorg. Med. Chem. Lett. 13, 3227 (2003).
16. Barreca M.L., Chimirri A., De Luca L., Monforte A.M., Monforte P., Rao A., Zappalŕ M. et al.: Bioorg. Med. Chem. Lett. 11, 1793 (2001).
17. Diurno M.V., Mazzoni O., Piscopo E., Calignano A., Giordano F., Bolognese A.: J. Med. Chem. 35, 2910 (1992).
18. Archana, Srivastava V.K., Kumar A.: Eur. J. Med. Chem. 37, 873 (2002).
19. Andres C.J., Bronson J.J., D'Andrea S.V., Deshpande M.S., Falk P.J., Grant-Young K.A., Harte W.E. et al.: Bioorg. Med. Chem. Lett. 10, 715 (2000).
20. Mamolo M.G., Zampieri D., Vio L., Fermeglia M., Ferrone M., Pricl S., Scialino G., Banfi E.: Bioorg. Med. Chem. 13, 3797 (2005).
21. Navarrete-Vazquez G., Molina-Salinas G.M., Duarte-Fajardo Z.V., Vargas-Villarreal J., Estrada-Soto S., Lez-Salazar F.G., Hernandez-Nunez E., Said-Fernandez S.: Bioorg. Med. Chem. 15, 5502 (2007).
22. Almasirad A., Tabatabi S.A., Faizi M.: Bioorg. Med. Chem. Lett. 14, 6057 (2004).
23. Tan T., Chen Y., Kong K, Bai J., Li Y., Lim S., Ang T., Lam Y.: Antiviral Res. 71, 7 (2006).
24. Gaonkar S., Rai K., Prabhuswamy B.: Eur. J. Med. Chem. 41, 841 (2006).
25. Palmer J.T., Hirschbein B.L., Cheung H., McCarter J., Janc J.W., Walter Z.Y., Wesolowski G.: Bioorg. Med. Chem. Lett. 16, 2909 (2006).
26. Aboraia A.S., Abdel-Rahman H.M., Mahfouz N.M., El-Gendy M.A.: Bioorg. Med. Chem. 14, 1236 (2006).
27. Bhat M.A., Abdel-Aziz H.A., Ghabbour H.A., Hemamalini M., Fun H.K.: Acta Crystallogr. Sect. E 68 (14), o1144 (2012).
28. Canetti G., Rist N., Grosset J.: Rev. Tuberc. Pneumol. (Paris) 27, 217 (1963).
29. Mali J.R., Pratap U.R., Netankar P.D., Mane R.A.: Tetrahedron Lett. 50, 5025 (2009).
30. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests, 7th edn. Approved standard. M2-A7. NCCLS, Wayne, PA 2000.
31. Sivakumar P.M., Seenivasan S.P., Kumar V., Doble M.: Bioorg. Med. Chem. Lett. 17, 1695 (2007).
32. Imramovský A., Polanc S., Vinšová J., Kočevar M., Jampílek J., Rečková Z., Kaustová J.: Bioorg. Med. Chem. 15, 2551 (2007).
33. Scior T., Garces-Eisele S.J.: Curr. Med. Chem. 13, 2205 (2006).
34. Rekkas S.A., Rodios N.A., Alexandrou N.E.: Synthesis 5, 411 (1986).
35. Novotny A., Brezik Z., Pridal J., Kalfus K.: Cesk. Farm. 7, 517 (1958). see Chem. Abstr. 1958, 53, 10191a.

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SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF NEW NORBORNYL SYSTEM BASED OXADIAZOLE THIOGLYCOSIDES AND ACYCLIC NUCLEOSIDE ANALOGS

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Abstract: New sugar hydrazones linked to norbornyl ring system, their oxadiazole acyclic nucleoside analogs and the corresponding thioglycosides were synthesized. The synthesized compounds were tested for their antimicrobial activity and displayed different degrees of activities or inhibitory actions. Their oxadiazole acyclic nucleoside analogs and thioglycosides showed higher activities.

Keywords: norbornyl, oxadiazole, glycosides, antibacterial, antifungal

The fast and widespread evolution of antimicrobial resistance poses a grave threat to therapy of many microbial infections and necessitates imperative and scrupulous efforts to develop next generation of antibacterial and antifungal agents. Norbornyl ring system is the basic core in many naturally occurring compounds such as monoterpenoids. Camphor, one of the most important compounds within this system, has been widely used as a fragrance in cosmetics, as an active ingredient in some drugs (1). In addition, the monoterpenoid is present in a number of over-the-counter medications, mainly for external application, and is readily available in drugstores (2). Compounds exhibiting norbornyl ring system possess a broad range of biological properties such as insect repellent (3), a bacteriostatic and fungistatic agent (1) and an antitussive (4). Furthermore, compounds incorporating norbornyl ring system showed interesting biological activities with application in the pharmaceutical field such as antispasmodic (5) and as inhibitors of norepinephrine secretion (6). Among the five-membered nitrogen heterocycles, the 1,3,4-oxadiazoles have been associated with a broad spectrum of biological activities (7–10). Their derivatives have been reported to possess antimicrobial (11–14), insecticidal (15), herbicidal, fungicidal (16), anti-inflammatory (17, 18) as well as antiviral (19) and antitumor activities. A number of substituted 1,3,4-oxadiazoles linked to polycyclic alkyl unit **I** (19) and **II** (20) and others attached to sugar moieties **III** (21) and **IV** (22) showed high antimicrobial activities. On the other hand, the glycosylthio heterocycles (23–25) and the acyclic nucleoside analogs with modification of both the glycon part and the heterocyclic base have stimulated extensive research as biological inhibitors (26–28). Nucleosides and their analogs possess a wide range of medicinal properties, including antibiotic, antiviral, and antitumor activity (29–32). We have been interested in the synthesis of new nucleoside analogs by attachment of sugar moieties to newly synthesized heterocycles (22, 33–35) in an ongoing search for new compounds with potential biological activity. Consequently, we have considered the synthesis of norbornyl system based new oxadiazole thioglycosides and their acyclic analogs in addition to the evaluation of their antibacterial and antifungal activity.

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EXPERIMENTAL

Chemistry

Melting points were determined with a Kofler block apparatus and are uncorrected. The IR spectra were recorded on a Perkin-Elmer model 1720 FTIR Spectrometer for KBr discs. NMR spectra were recorded on a Varian Gemini 200 NMR Spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C or on a Bruker Ac-250 FT Spectrometer at 250 MHz for ^{1}H and at 62.9 MHz for ^{13}C with TMS as a standard. The progress of the reactions was monitored by

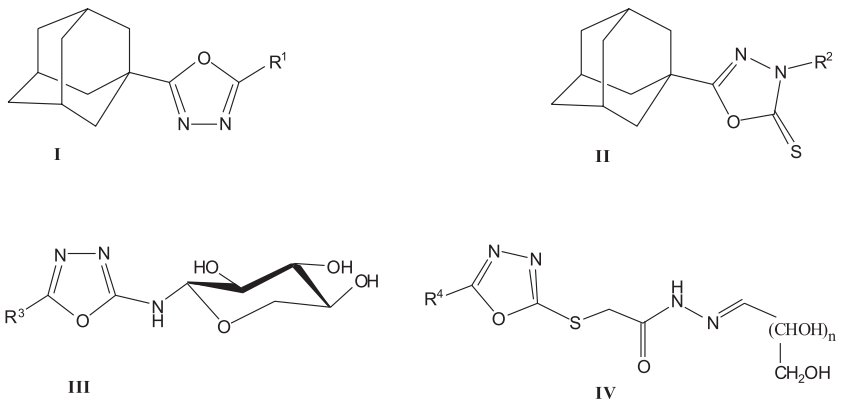
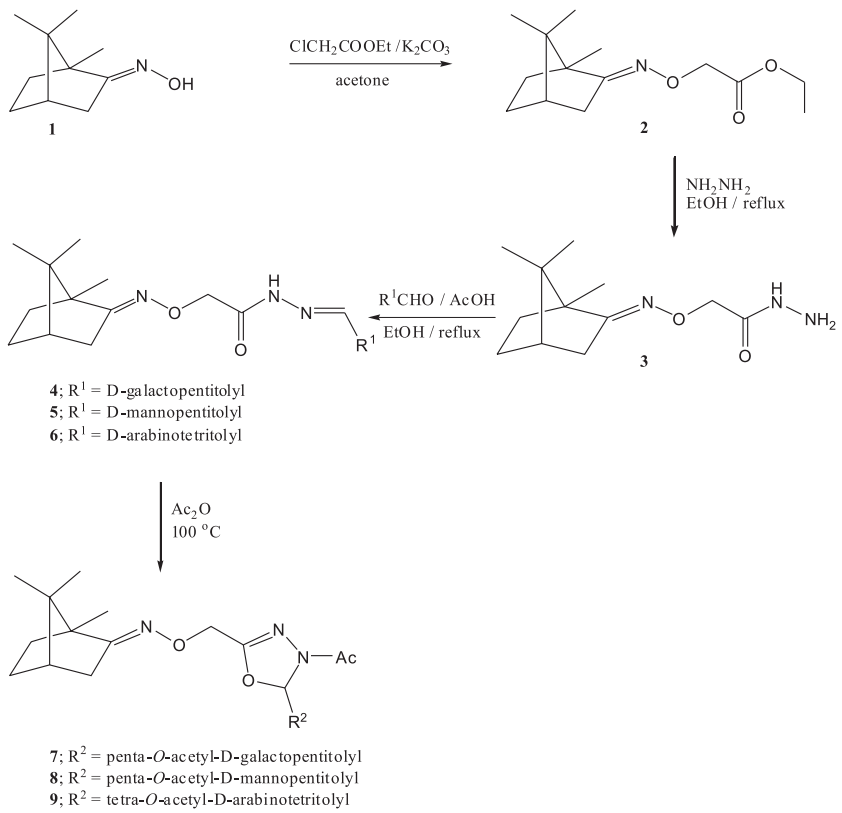


Figure 1. Antimicrobial 1,3,4-oxadiazole derivatives



Scheme 1. Synthesis of oxadiazoline acyclic sugar derivatives

TLC using aluminum silica gel plates 60 F 245. EI-mass spectra were measured on HP D5988 A 1000 MHz spectrometer (Hewlett-Packard, Palo Alto, CA, USA). Elemental analyses were performed at the Micro Analytical Data Center at Faculty of Science, Cairo University, Egypt. Antimicrobial activity was determined at the Biology Department, College of Science, Aljouf University.

Ethyl 2-(1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene)aminooxy)acetate (2)

Ethyl chloroacetate (1.22 g, 10 mmoles) was added to a well stirred solution of compound **1** (1.67 g, 10 mmoles) and dry potassium carbonate (1.38 g, 10 mmoles) in acetone (15 mL). The reaction mixture was stirred at room temperature for 5 h and then poured on ice-cold water. The precipitated solid was filtered, washed with water and recrystallized from ethanol to give compound **2** as white crystals, 2.03 g.

2-[(1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ylidene)aminooxy]acetohydrazide (3)

Hydrazine hydrate (0.5 g, 10 mmoles) was added to a solution of compound **2** (2.54 g, 10 mmoles) in ethanol (25 mL) and the reaction mixture was heated under reflux for 3 h. After cooling, the precipitated solid was filtered, washed with ethanol and recrystallized from ethanol to afford compound **3** as white crystals, 2.03 g.

Sugar-[(1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene)aminooxy]acetohydrazone (4–6)

General procedure: The acetylhydrazone **3** (1.20 g, 5 mmoles) dissolved in ethanol (15 mL) was

Table 1. Physical and analytical data for the synthesized compounds.

Comp. No.	M.p. (°C)	Yield (%)	Mol. formula (m.w.)	Analysis (%) calc. / found.		
				C	**H**	**N**
2	131–132	80	$C_{14}H_{23}NO_3$ (253.34)	66.37 66.02	9.15 9.11	5.53 5.27
3	189–190	85	$C_{12}H_{21}N_3O_2$ (239.31)	60.23 60.39	8.84 8.52	17.56 17.24
4	198–200	78	$C_{18}H_{31}N_3O_7$ (401.45)	53.85 53.49	7.78 8.11	10.47 10.24
5	196–198	80	$C_{18}H_{31}N_3O_7$ (401.45)	53.85 53.51	7.78 7.48	10.47 10.19
6	197–198	80	$C_{17}H_{29}N_3O_6$ (371.43)	54.97 54.62	7.87 7.42	11.31 11.12
7	130–132	74	$C_{30}H_{43}N_3O_{13}$ (653.67)	55.12 54.92	6.63 6.35	6.43 6.20
8	134-135	75	$C_{30}H_{43}N_3O_{13}$ (653.67)	55.12 54.95	6.63 6.38	6.43 6.29
9	137–138	70	$C_{27}H_{39}N_3O_{11}$ (581.61)	55.76 55.58	6.76 6.46	7.22 7.05
10	152–153	72	$C_{13}H_{19}N_3O_2S$ (281.37)	55.49 55.12	6.81 6.50	14.93 14.61
12a	139–141	73	$C_{27}H_{37}N_3O_{11}S$ (611.66)	53.02 52.85	6.10 5.92	6.87 6.59
12b	138–140	71	$C_{27}H_{37}N_3O_{11}S$ (611.66)	53.02 52.89	6.10 5.89	6.87 7.05
12c	141–142	70	$C_{24}H_{33}N_3O_9S$ (539.60)	53.42 53.15	6.16 6.38	7.79 7.45
13a	197–198	75	$C_{19}H_{29}N_3O_7S$ (443.51)	51.45 51.21	6.59 6.28	9.47 9.21
13b	194–195	75	$C_{19}H_{29}N_3O_7S$ (443.51)	51.45 51.19	6.59 6.35	9.47 9.24
13c	192–193	73	$C_{18}H_{27}N_3O_6S$ (413.49)	52.29 52.02	6.58 6.30	10.16 9.96

added to a solution of the respective monosaccharide (5 mmoles) in water (1 mL) and glacial acetic acid (1 mL). The mixture was heated under reflux for 4 h and the resulting solution was concentrated and left to cool. The formed precipitate was filtered off, washed with water and ethanol, then dried and recrystallized from ethanol to afford **4–6**.

O-Acetylsugar-5-[[(1,7,7-trimethylbicyclo[2.2.1]-heptan-2-ylidene)aminooxy]-methyl]-1,3,4-oxadiazol-3(2H)-yl}ethanone (7–9)

General procedure: A solution of sugar hydrazone **4–6** (5 mmol), in acetic anhydride (4 mL), was heated at 100°C for 1.0–1.5 h. The resulting solution was poured onto crushed ice, and the product that separated out was filtered off, washed with sodium hydrogen carbonate solution (50 mL), followed by water (50 mL) and then dried. The products were recrystallized from ethanol-water (2 : 1 v/v) to give oxadiazolines **7–9**.

5-{[-(1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ylidene)aminooxy]methyl}-1,3,4-oxadiazole-2(3H)-thione (10)

Carbon disulfide (3 mL) was added dropwise to a solution of hydrazide **3** (10 mmol, 2.40 g) in pyridine (30 mL). The solution was heated at 100°C for 12 h. The solvent was evaporated to half of its amount under reduced pressure and the residue was poured into ice-cold water containing acetic acid (2 mL). The obtained precipitate was filtered off, washed with water and recrystallized from ethanol to afford the oxadiazole **10** as yellow powder.

1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one-O-[5-glycopyranosylthio)-1,3,4-oxadiazol-2-yl]methyl oxime (12a–c)

General procedure: The bromosugar **11a–c** (6 mmol) was added to a well stirred solution of compound **10** (1.21 g, 5 mmol) in *N*,*N*-dimethylformamide (7 mL) containing Et_3N (0.85 mL, 6 mmol). The reaction mixture was stirred at room temperature until reaction was judged complete by TLC using chloroform/methanol 99.7 : 0.3 v/v). The mixture was concentrated under reduced pressure, diluted with CH_2Cl_2 (40 mL), and washed with water (3 × 30 mL). The organic layer was dried (Na_2SO_4), filtered, evaporated under reduced pressure, and the residue was triturated with petroleum ether (b.p. 40–60°C) (45 mL). The solid product was filtered, dried and recrystallized from ethanol.

1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one-O-[5-(D-glycopyranosylthio)-1,3,4-oxadiazol-2-yl]methyl oxime (13a–c)

General procedure: Dry gaseous ammonia was passed through a solution of the acetylated thioglycosides **12a–c** (5 mmol) in dry methanol (20 mL) at 0°C for 1 h, and then the mixture was stirred at room temperature for 5 h. The solvent was evaporated under reduced pressure at 40°C to give a solid residue, which was recrystallized from ethanol to give the corresponding free glycosides **13a–c**.

Antimicrobial screening

The synthesized compounds were tested for their antimicrobial activity against three microorganisms and the minimal inhibitory concentrations (MICs) of the tested compounds were determined by the dilution method.

Sample preparation

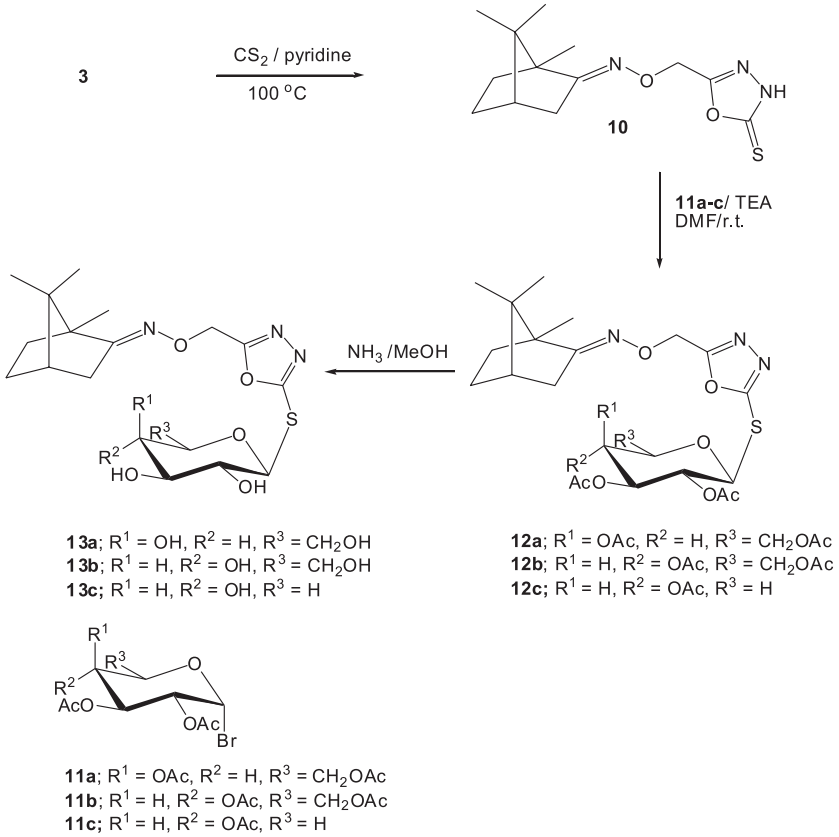
Each of the test compounds and standards were dissolved in 12.5% DMSO, at concentrations of 500 µg/mL. Further dilutions of the compounds and standards in the test medium were prepared at the required quantities.

Culture of microorganisms

Bacteria strains, namely: *Bacillus subtilis* (ATCC 6633) (Gram positive), *Pseudomonas aeruginosa* (ATCC 27853) (Gram negative) and *Streptomyces* species (Actinomycetes) were used in this investigation. The bacterial strains were maintained on MHA (Mueller-Hinton agar) medium (Oxoid, Chemical Co., UK) for 24 h at 37°C. The medium was molten on a water bath, inoculated with 0.5 mL of the culture of the specific microorganism and poured into sterile Petri dishes to form a layer of about 3–4 mm thickness. The layer was allowed to cool and harden. With the aid of corkborer, cups of about 10 mm diameter were produced (36).

Agar diffusion technique

The antibacterial activities of the synthesized compounds were tested against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces* species using MH medium (17.5 g casein hydrolysate, 1.5 g soluble starch, 1000 mL beef extract). A stock solution of each synthesized compound (500 µg/mL) in DMSO was prepared and graded quantities of the test compounds were incorporated in specified quantity of sterilized liquid MH medium. Different concentrations of the test compounds in DMSO were placed separately in cups in the agar medium. All plates were incubated at 37°C overnight. The inhibition zones were measured after 24 h. The minimum inhibitory concentration (MIC) was defined



Scheme 2. Synthesis of 1,3,4-oxadiazole thioglycosides

as the intercept of the grave of logarithm concentrations *versus* diameter of the inhibition zones (37).

RESULTS AND DISCUSSION

Chemistry

Reaction of the bicycloheptanone oxime derivative **1** (38) with ethyl chloroacetate in the presence of potassium carbonate gave the ethyl *O*-substituted acetyl ester **2**. Hydrazinolysis of the latter ester with hydrazine hydrate afforded the corresponding acid hydrazide derivative **3** in 85% yield. The ^{1}H NMR spectrum of **2** showed signals corresponding to the ethyl group where the C*H*$_2$ appeared as quartet and disappeared in the ^{1}H NMR spectrum of the derived hydrazide, which revealed, instead, signals corresponding to NH and NH_2 groups. When the hydrazide **3** was allowed to react with D-galactose, D-mannose or D-arabinose in the presence of catalytic amount of acetic acid, the corresponding sugar hydrazones **4–6** were obtained, respectively. Their ^{1}H NMR spectra showed the signals of the sugar chain protons at δ 3.35–5.69 ppm for the alditolyl sugar protons and the hydroxyl protons in addition to the C-1 methine proton as doublet in the range of δ 7.46–7.51 ppm. It is well known that the reaction of sugar aroylhydrazones with acetic anhydride gives the respective per-*O*-acetyl derivatives. However, it has been reported (40–42) that when the reaction was carried out at high temperature in boiling acetic anhydride, cyclization usually takes place in addition to per-*O*-acetylation to afford acyclic *C*-nucleoside analogs. We reported previously (40, 41) the synthesis of 1,2,4-triazolo[1,3,4]oxadiazole and *N*-acetyl-1,3,4-oxadiazoline acyclic nucleoside analogs by the reaction of hydrazinyl sugars with boiling acetic anhydride. When the hydrazones **4–6** were heated in acetic anhydride at 100°C they gave the 1,3,4-oxadiazoline acyclic nucleoside analogs **7–9**, respectively. The structures of the oxadiazoline acetylated sugar derivatives were confirmed by their spectral and analytical data. Their IR spectra showed absorption bands in the carbonyl frequency region not only for the carbonyl ester but also corresponding to the carbonyl amide groups indicating the presence of *N*-acetyl group in addition to the *O*-

Table 2. Spectral data for the synthesized compounds.

Comp. No.	Spectrum	IR [KBr; ν cm^{-1}] , ^{1}H NMR [(DMSO) δ, ppm], ^{13}C NMR [(DMSO) δ, ppm], MS [(m/z), %]
2	IR	1736 (C=O), 1602 (C=N).
	^{1}H NMR	1.02 (s, 6H, 2 CH_3), 1.25 (s, 3H, CH_3), 1.52–1.77 (m, 7H, CH_3 and 2 CH_2), 2.50–2.55 (m, 3H, CH and CH_2), 3.90 (q, *J* = 5.2 Hz, 2H, CH_2), 4.89 (s, 2H, CH_2).
	^{13}C NMR	11.44, 15.20, 20.35 (4 CH_3), 28.30, 32.05, 33.39, 42.90, 46.28, 53.15 (norbornyl carbons), 62.15, 71.10 (2CH_2), 165.58 (C=N), 169.18 (C=O).
	MS	253 [$(M)^+$, 21].
3	IR	3431–3375 (NH_2 and NH), 1604 (C=N).
	^{1}H NMR	1.04 (s, 6H, 2 CH_3), 1.26 (s, 3H, CH_3), 1.55–1.80 (m, 4H, 2 CH_2), 2.50–2.56 (m, 3H, CH and CH_2), 4.95 (s, 2H, CH_2), 5.03 (bs, 2H, NH_2), 10.12 (bs, IH, NH).
	^{13}C NMR	11.48, 15.24 (3 CH_3), 28.41, 32.24, 33.90, 43.95, 47.02, 54.28 (norbornyl carbons), 72.27 (CH_2), 166.91 (C=N), 169.24 (C=O).
	MS	240 [$(M + H)^+$, 14].
4	IR	3540–3424 (OH), 3311 (NH), 1611 (C=N).
	^{1}H NMR	1.04 (s, 6H, 2 CH_3), 1.26 (s, 3H, CH_3), 1.55–1.80 (m, 4H, 2 CH_2), 2.50–2.59 (m, 3H, CH and CH_2), 3.36–3.39 (m, 2H, H-6,6'), 3.71–3.77 (m, 1H, H-5), 4.25–4.31 (m, 2H, H-4,3), 4.38–4.44 (m, 1H, H-2), 4.49–4.55 (m, 1H, OH), 4.61 (d, *J* = 6.4 Hz, 1H, OH), 4.95–4.08 (m, 3H, CH_2 and OH), 5.19–5.25 (m, 1H, OH), 5.63 (t, *J* = 4.6 Hz, 1H, OH), 7.46 (d, *J* = 7.5 Hz, 1H, H-1), 10.14 (s, IH, NH).
	^{13}C NMR	11.47, 15.22 (3 CH_3), 28.50, 32.29, 33.95, 44.11, 47.42, 54.30 (norbornyl carbons), 62.10 (C-6), 63.05 (C-5), 69.21 (C-4), 74.27 (C-3), 74.94 (CH_2), 75.88 (C-2), 152.16 (C-1), 166.08 (C=N), 169.15 (C=O).
5	IR	3563–3418 (OH), 3305 (NH), 1614 (C=N).
	^{1}H NMR	1.02 (s, 6H, 2 CH_3), 1.25 (s, 3H, CH_3), 1.54–1.78 (m, 4H, 2 CH_2), 2.52–2.58 (m, 3H, CH and CH_2), 3.38–3.41 (m, 2H, H-6,6'), 3.73–3.80 (m, 1H, H-5), 4.25–4.34 (m, 2H, H-4,3), 4.37–4.44 (m, 1H, H-2), 4.49–4.55 (m, 1H, OH), 4.62–4.70 (m, 1H, OH), 4.97–5.08 (m, 3H, CH_2 and OH), 5.20–5.26 (m, 1H, OH), 5.64–5.69 (m, 1H, OH), 7.50 (d, *J* = 7.5 Hz, 1H, H-1), 10.12 (s, IH, NH).
6	IR	3510–3412 (OH), 3310 (NH), 1614 (C=N).
	^{1}H NMR	1.03 (s, 6H, 2 CH_3), 1.24 (s, 3H, CH_3), 1.55–1.77 (m, 4H, 2 CH_2), 2.50–2.58 (m, 3H, CH and CH_2), 3.35–3.42 (m, 2H, H-5,5'), 3.70 (m, 1H, H-4), 4.17–4.26 (m, 2H, H-3), 4.39–4.45 (m, 1H, H-2), 4.48–4.55 (m, 1H, OH), 4.55 (d, *J* = 6.4 Hz, 1H, OH), 4.97–5.08 (m, 2H, CH_2), 5.18–5.24 (m, 1H, OH), 5.65–5.68 (m, 1H, OH), 7.51 (d, *J* = 7.5 Hz, 1H, H-1), 10.14 (s, IH, NH).
	^{13}C NMR	11.21, 15.22 (3 CH_3), 28.50, 32.31, 33.96, 44.12, 47.43, 54.31 (norbornyl carbons), 62.12 (C-5), 69.11 (C-4), 74.27 (C-3), 74.95 (CH_2), 75.73 (C-2), 152.18 (C-1), 166.05 (C=N), 169.20 (C=O).
7	IR	1738 (C=O), 1679 (C=O), 1612 (C=N).
	^{1}H NMR	1.05 (s, 6H, 2 CH_3), 1.27 (s, 3H, CH_3), 1.55–1.80 (m, 4H, 2 CH_2), 1.86, 1.98, 2.03, 2.11, 2.18, 2.28 (6s, 18H, 6CH_3), 2.55–2.59 (m, 3H, CH and CH_2), 4.17 (dd, *J* = 11.4 Hz, *J* = 2.8 Hz, 1H, H-5), 4.22 (dd, *J* = 11.4 Hz, *J* = 3.2 Hz, 1H, H-5'), 4.90–4.95 (m, 1H, H-4), 5.02 (s, 2H, CH_2), 5.27 (dd, *J* = 6.5 Hz, *J* = 7.4 Hz, 1H, H-3), 5.53 (dd, *J* = 7.4 Hz, *J* = 7.2 Hz, 1H, H-2), 5.72 (t, *J* = 7.2 Hz, 1H, H-1), 5.77 (d, *J* = 7.6 Hz, 1H, oxadiazoline-H).
	^{13}C NMR	11.18, 15.20, 20.32, 20.52, 20.64, 20.78, 21.05, 26.12 (9 CH_3), 28.82, 32.30, 33.91, 44.05, 47.40, 54.27 (norbornyl carbons), 72.95 (CH_2), 62.92 (C-5), 64.91 (C-4), 65.38 (C-3), 68.41 (C-2), 71.18 (C-1), 91.24 (*C*-N-Ac), 158.22 (oxadiazoline C-5), 163.02 (C=N), 169.15, 169.82, 170.24, 170.75, 171.10, 172.18 (6CO).
	MS	654 [$(M + H)^+$, 11].
8	IR	1736 (C=O), 1672 (C=O), 1614 (C=N).
	^{1}H NMR	1.04 (s, 6H, 2 CH_3), 1.25 (s, 3H, CH_3), 1.55–1.79 (m, 4H, 2 CH_2), 1.85, 1.97, 2.03, 2.10, 2.18, 2.27 (6s, 18H, 6CH_3), 2.56–2.60 (m, 3H, CH and CH_2), 4.18 (dd, *J* = 11.4 Hz, *J* = 2.8 Hz, 1H, H-5), 4.22 (dd, *J* = 11.4 Hz, *J* = 3.2 Hz, 1H, H-5'), 4.89–4.94 (m, 1H, H-4), 4.99 (s, 2H, CH_2), 5.27 (dd, *J* = 6.5 Hz, *J* = 7.4 Hz, 1H, H-3), 5.57 (dd, *J* = 7.4 Hz, *J* = 7.2 Hz, 1H, H-2), 5.75 (t, *J* = 7.2 Hz, 1H, H-1), 5.79 (d, *J* = 7.8 Hz, 1H, oxadiazoline-H).
	MS	654 [$(M + H)^+$, 9].

Table 2. Cont.

Comp. No.	Spectrum	IR [KBr; ν cm^{-1}], ^{1}H NMR [(DMSO) δ, ppm], ^{13}C NMR [(DMSO) δ, ppm], MS [(m/z), %]
9	IR	1739 (C=O), 1675 (C=O), 1615 (C=N).
	^{1}H NMR	1.03 (s, 6H, 2 CH_3), 1.28 (s, 3H, CH_3), 1.57–1.81 (m, 4H, 2 CH_2), 1.85, 1.97, 2.04, 2.14, 2.29 (5s, 15H, 5CH_3), 2.52-2.57 (m, 3H, CH and CH_2), 4.18 (dd, J = 11.4 Hz, J = 2.8 Hz, 1H, H-4), 4.25 (dd, J = 11.4 Hz, J = 3.2 Hz, 1H, H-4'), 5.11 (s, 2H, CH_2), 5.28 (dd, J = 6.5 Hz, J = 7.4 Hz, 1H, H-3), 5.54 (dd, J = 7.4 Hz, J = 7.2 Hz, 1H, H-2), 5.72 (t, J = 7.2 Hz, 1H, H-1), 5.77 (d, J = 7.6 Hz, 1H, oxadiazoline-H).
	^{13}C NMR	11.16, 15.21, 20.31, 20.50, 20.60, 21.05, 26.14 (8 CH_3), 28.83, 32.30, 33.90, 44.10, 47.40, 54.28 (norbornyl carbons), 74.90 (CH_2), 62.95 (C-4), 65.40 (C-3), 68.40 (C-2), 71.18 (C-1), 91.30 (*C*-N-Ac), 158.25 (oxadiazoline C-5), 162.95 (C=N), 169.10, 170.25, 170.75, 171.15, 172.20 (5CO).
10	IR	2986 (CH), 1615 (C=N).
	^{1}H NMR	1.03 (s, 6H, 2 CH_3), 1.28 (s, 3H, CH_3), 1.59–1.82 (m, 4H, 2 CH_2), 2.52–2.57 (m, 3H, CH and CH_2), 4.92 (s, 2H, CH_2), 12.31 (bs, 1H, NH).
	^{13}C NMR	11.17, 15.29 (3 CH_3), 28.74, 32.38, 33.76, 44.12, 47.46, 54.32 (norbornyl carbons), 72.65 (CH_2), 159.84 (oxadiazoline C-5), 163.95 (C=N), 179.95 (C=S).
	MS	281 [(M)$^+$, 18].
12a	IR	1612 (C=N), 1744 (C=O).
	^{1}H NMR	1.04, 1.25 (2s, 9H, 3 CH_3), 1.54–1.82 (m, 4H, 2 CH_2), 1.89, 1.92, 1.98, 2.03 (4s, 12H, 4CH_3CO), 2.49–2.55 (m, 3H, CH and CH_2), 4.05–4.10 (m, 1H, H-5), 4.14 (dd, $J_{6,6'}$ = 11.4 Hz, $J_{5,6}$ = 2.8 Hz, 1H, H-6), 4.22 (dd, $J_{6,6'}$ = 11.4 Hz, $J_{5,6'}$ = 3.2 Hz, 1H, H-6'), 4.94 (t, $J_{3,4}$ = 9.6 Hz, 1H, H-4), 5.14 (s, 2H, CH_2), 5.20 (dd, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 9.6 Hz, 1H, H-3), 5.28 (t, $J_{2,3}$ = 9.8 Hz, 1H, H-2), 5.79 (d, $J_{1,2}$ = 10.4 Hz, 1H, H-1).
	^{13}C NMR	11.17, 15.29, 19.27, 19.23, 20.44, 20.65 (4CH_3CO and 3 CH_3), 28.74, 32.38, 33.76, 44.12, 47.46, 54.32 (norbornyl carbons), 62.22 (C-6), 66.14 (C-4), 68.85 (C-3), 70.19 (C-2), 71.85 (CH_2), 72.28 (C-5), 91.02 (C-1), 159.05 (oxadiazole C-2), 160.34 (oxadiazole C-5), 163.75 (C=N), 169.41, 170.52, 171.11, 171.80 (4C=O).
	MS	612 [(M + H)$^+$, 8].
12b	IR	1614 (C=N), 1748 (C=O).
	^{1}H NMR	1.05, 1.25 (2s, 9H, 3 CH_3), 1.55–1.80 (m, 4H, 2 CH_2), 1.88, 1.92, 1.97, 2.03 (4s, 12H, 4CH_3CO), 2.48–2.54 (m, 3H, CH and CH_2), 4.09 (m, 1H, H-5), 4.15 (dd, $J_{6,6'}$ = 11.2 Hz, $J_{5,6}$ = 2.8 Hz, 1H, H-6), 4.20 (dd, $J_{6,6'}$ = 11.2 Hz, $J_{5,6'}$ = 3.4 Hz, 1H, H-6'), 5.05 (t, $J_{3,4}$ = 9.4 Hz, 1H, H-4), 5.15 (s, 2H, CH_2), 5.21 (dd, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.4 Hz, 1H, H-3), 5.29 (t, $J_{2,3}$ = 9.6 Hz, 1H, H-2), 5.78 (d, $J_{1,2}$ = 10.4 Hz, 1H, H-1).
	MS	612 [(M + H)$^+$, 19].
12c	IR	1611 (C=N), 1741 (C=O).
	^{1}H NMR	1.03, 1.25 (2s, 9H, 3 CH_3), 1.57–1.81 (m, 4H, 2 CH_2), 1.89, 1.95, 2.03 (3s, 9H, 3CH_3CO), 2.50–2.57 (m, 3H, CH and CH_2), 4.16 (dd, $J_{5,5'}$ = 10.6 Hz, $J_{4,5}$ = 2.8 Hz, 1H, H-5), 4.22 (dd, $J_{5,5'}$ = 10.6 Hz, $J_{4,5'}$ = 3.2 Hz, 1H, H-5'), 5.02 (t, $J_{3,4}$ = 9.2 Hz, 1H, H-4), 5.12 (s, 2H, CH_2), 5.24 (dd, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.2 Hz, 1H, H-3), 5.29 (t, $J_{2,3}$ = 9.6 Hz, 1H, H-2), 5.79 (d, $J_{1,2}$ = 9.8 Hz, 1H, H-1).
	^{13}C NMR	11.17, 15.29, 19.42, 20.44, 20.65 (3CH_3CO and 3 CH_3), 27.81, 32.30, 33.19, 44.35, 47.71, 55.02 (norbornyl carbons), 63.05 (C-5), 65.35 (C-4), 70.61 (C-3), 71.90 (CH_2), 72.97 (C-2), 91.27 (C-1), 158.68 (oxadiazole C-2), 160.21 (oxadiazole C-5), 161.94 (C=N), 169.82, 170.61, 171.91 (3C=O).
	MS	540 [(M + H)$^+$, 18].
13a	IR	1615 (C=N), 3488–3415 (OH).
	^{1}H NMR	1.06 (s, 6H, 2CH_3), 1.26 (s, 3H, CH_3), 1.57–1.85 (m, 4H, 2 CH_2), 2.52–2.64 (m, 3H, CH and CH_2), 3.47–3.56 (m, 2H, H-6,6'), 3.61–3.66 (m, 1H, H-5), 4.14–4.24 (m, 2H, H-3,4), 4.37–4.40 (m, 1H, H-2), 4.76–4.79 (m, 1H, OH), 4.85–4.90 (m, 1H, OH), 5.24–5.31 (m, 1H, OH), 5.38–5.45 (m, 1H, OH), 5.14 (s, 2H, CH_2), 5.80 (d, J = 10.4, 1H, H-1).
	^{13}C NMR	15.11, 23.11 (3CH_3), 27.88, 32.35, 33.21, 44.35, 47.73, 55.42 (norbornyl carbons), 63.60 (C-6), 66.44 (C-4), 68.69 (C-3), 71.29 (C-2), 71.98 (CH_2), 72.88 (C-5), 92.14 (C-1), 158.95 (oxadiazole C-2), 160.05 (oxadiazole C-5), 161.88 (C=N).
13b	IR	1612 (C=N), 3486–3410 (OH).
	^{1}H NMR	1.03 (s, 6H, 2CH_3), 1.25, (s, 3H, CH_3), 1.58–1.84 (m, 4H, 2 CH_2), 2.52–2.68 (m, 3H, CH and CH_2), 3.45–3.54 (m, 2H, H-6,6'), 3.53–3.59 (m, 1H, H-5), 4.14–4.24 (m, 2H, H-3,4), 4.36–4.39 (m, 1H, H-2), 4.75 (m, 1H, OH), 4.85–4.90 (m, 1H, OH), 5.22–5.26 (m, 1H, OH), 5.38–5.43 (m, 1H, OH), 5.11 (s, 2H, CH_2), 5.81 (d, J = 10.2, 1H, H-1).
13c	IR	1615 (C=N), 3481–3449 (OH).
	^{1}H NMR	1.04 (s, 6H, 2CH_3), 1.27 (s, 3H, CH_3), 1.59–1.86 (m, 4H, 2 CH_2), 2.52–2.67 (m, 3H, CH and CH_2), 3.39–3.48 (m, 2H, H-5,5'), 4.22–4.32 (m, 2H, H-3,4), 4.38–4.44 (m, 1H, H-2), 4.77–4.82 (m, 1H, OH), 5.24–5.30 (m, 1H, OH), 5.38–5.42 (m, 1H, OH), 5.08 (s, 2H, CH_2), 5.82 (d, J = 9.8, 1H, H-1).

acetyl groups. The ^{1}H NMR spectra showed signals corresponding to the *O*-acetyl-methyl protons in addition to the *N*-acetyl-methyl protons each as singlet and signals corresponding to the rest of the alditolyl chain protons. The cyclization of the sugar hydrazones was also confirmed by the ^{13}C NMR spectra of the resulting oxadiazoline derivatives. The value of the chemical shift of the *C*-N-Ac (C-1 in the original sugar chain moiety and C-2 in the oxadiazoline ring) appeared at δ 91.24 and 91.30 ppm, which indicated its *N,N*-acetal nature rather than being a C=N. In addition, the resonances of the acetyl-methyl carbons appeared at δ 20.31–26.14 ppm (Scheme 1).

When the acid hydrazide **3** was reacted with CS_2 in pyridine at 100°C it afforded the oxadiazole derivative **10** in 72% yield for which the IR and NMR spectra agreed with the assigned structure. When compound **10** was reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-galacto-, 2,3,4,6-tetra-*O*-acetyl-α-D-gluco- or 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide **11a–c**, the corresponding substituted thioglycoside derivatives **12a–c** were afforded in 70–73% yields. The IR spectra of the resulting glycosides showed absorption bands in the range 1741–1748 cm^{-1} for the acetyl carbonyl groups. The ^{1}H NMR spectra showed signals corresponding to protons of the sugar moiety and carbonyl methyl protons in addition to the bicyclic ring protons. The anomeric proton chemical shift and its coupling constant values in the ^{1}H NMR spectra indicated the β-orientation of the thioglycosidic bond. The absence of a signal corresponding to the C=S in the ^{13}C NMR spectra confirmed that the attachment of the sugar moiety has been taken place at the sulfur atom rather than the nitrogen atom. Also, the anomeric proton of β-*N*-glycosides having an adjacent C=S was reported (43–45) to appear at higher chemical shift (δ 6.9–7.2 ppm) due to the anisotropic deshielding effect of the C=S (44, 46). Deacetylation of the thioglycoside derivatives **12a–c** afforded the free thioglycosides **13a–c** in 73–75% yields. The IR spectra of the deacetylated products showed absorption bands at 3410–3488 cm^{-1} for the hydroxyl groups and also revealed the absence of the acetyl carbonyl bands. Their ^{1}H NMR spectra showed signals corresponding to the hydroxyl protons at δ 4.75–5.45 ppm (Scheme 2).

Table 1. Minimum inhibitory concentration (MIC in μg/mL) of the title compounds. The negative control DMSO showed no activity.

Compound	*Bacillus subtilis* (Gram positive)	*Pseudomonas aeruginosa* (Gram negative)	*Streptomyces species* (Actinomycetes)
2	250	500	–[a]
3	250	250	125
4	100	125	100
5	100	100	75
6	75	75	100
7	125	250	500
8	125	125	125
9	100	125	100
10	125	250	250
12a	500	125	250
12b	250	250	125
12c	250	125	125
13a	125	125	100
13b	100	100	75
13c	75	75	100
Penicillin	31	46	33

[a] Totally inactive (MIC > 500 μg/mL).

Antimicrobial activity

The antimicrobial activity of the synthesized compounds was evaluated against three microorganisms; *Bacillus subtilis* (ATCC 6633) (Gram positive), *Pseudomonas aeruginosa* (ATCC 27853) (Gram negative), and *Streptomyces species* (Actinomycetes). The values of minimal inhibitory concentrations (MICs) of the tested compounds in addition to penicillin (46) as a reference drug are presented in Table 3. Compounds **6**, **13b** and **13c** were the most active against *Bacillus subtilis* whereas **6** and **13c** revealed the highest activity against *Pseudomonas aeruginosa*. Compounds **5** and **13b** showed high activity against the *Streptomyces species* with MIC values of 75 μg/mL.

The antimicrobial activity and structure activity relationship correlation indicated that the attachment of acyclic sugar moieties to the substituted acetyl hydrazinyl group resulted in higher inhibition activities. This is clear as the activity was lost in their preparation precursors. Furthermore, the 1,3,4-oxadiazole ring system linked to cyclic sugar moieties through a thioglycosidic linkage revealed higher activities. Moreover, the attachment of free hydroxyl glycosyl moieties resulted in distinct improvement in activities against *Bacillus subtilis* and *Pseudomonas aeruginosa*. Additionally, the sugar hydrazones with free hydroxyl acyclic sugar moieties showed higher activity values than the corresponding acetylated analogs.

The obtained results also indicated that the acyclic nucleoside analog with the acetylated arabinotetritolyl moiety attached to the oxadiazoline base exhibited relatively higher activity than the corresponding galactopentitolyl or mannopentitolyl residues against *Bacillus subtilis* and *Streptomyces species,* indicating the influence of the number and orientation of the free hydroxyl groups in sugar ring. On the other hand, the effect of attachment of thioglycosyl moiety to 1,3,4-oxadiazole ring was observed especially in the results of the prepared thioglycoside **13c** for *Bacillus subtilis* and *Pseudomonas aeruginosa*.

CONCLUSIONS

New sugar hydrazones linked to norbornyl ring system and their derived oxadiazole acyclic nucleoside analogs were prepared. The produced thioglycosides by glycosylation of the substituted 1,3,4-oxadiazoles showed higher activities. The attachment of acyclic sugar moieties to the substituted acetyl hydrazinyl derivatives as well as attachment of free hydroxyl glycosyl moieties to oxadiazole ring system resulted in relatively improved antimicrobial activities.

REFERENCES

1. Gomes M. R., Felzenszwalb I., Paumgartten, F. J.: Mutat. Res. 416, 129 (1998).
2. Rabl W., Katzgraber F., Steinlechner M.: Forensic Sci. Int. 89, 137 (1997).
3. Ansari M.A., Razdan R.K.: Indian J. Malariol. 32, 104 (1995).
4. Laude E.A., Morice A.H., Grattan T.J.: Pulm. Pharmacol. 7, 179 (1994).
5. Schenonne P., Tasca A., Bignardi G., Mosti L.: Eur. J. Med. Chem.-Chim. Ther. 10, 412 (1975).
6. Park T.J., Seo H.K., Kang B.J., Kima K.T.: Biochem. Pharmacol. 61,787 (2001).
7. El-Sayed W.A., Abbas H.S., Mohamed A.M., Abdel-Rahman A.A.H.: J. Heterocycl. Chem. 48, 1006 (2011).
8. Zareen A., Maimoona R., Choudhary M.I., Supino R., Khalid M. et al.: Biophys. Res. Comm. 319, 1053 (2004).
9. El-Azzouny A.A., Maklad Y.A., Bartsch H., Zaghary W.A., Ibrahim W.M. et al.: Sci. Pharm. 71, 331 (2003).
10. Loetchutinat C., Chau F., Mankhetkorn S.: Chem. Pharm. Bull. 51, 728 (2003).
11. Ates O., Kocabalkanli A., Sanis G.O., Ekinci A.C., Vidin A.: Drug Res. 47, 1134 (1997).
12. Amr A.E., Mohamed A.M., Ibrahim A.A.: Z. Naturforsch. 58b, 861 (2003).
13. Wasfy A.A.F., Mohamed A.M., Khattab R.R.M., El-Sayed W.A., Abdel-Rahman A.A.H.: World J. Chem. 6, 32 (2011).
14. Farghaly A.A.H.: J. Chin. Chem. Soc. 51, 147 (2004).
15. Li X.Z.Z., Wang Y., Chen W., Huang Q., Liu C. et al.: J. Fluor. Chem. 123, 163 (2003).
16. Zou X., Zhang Z., Jin G.J.: J. Chem. Res. 228 (2002).
17. El-Salam O., Fahmy A.F.M., Mohamed A.M., Elnaggar D.H., Hammam A.G.: World J. Chem. 5, 7 (2010).
18. Palaska E., Sahin G., Kelicen P., Durlu N.T., Altinok G.: Farmaco 57, 101 (2002).
19. El-Emam A.A., Al-Deep A.O., Al-Omar M., Lehmann J.: Bioorg. Med. Chem. 12, 5107 (2004).
20. Kadi, A.A., El-Brollosy, N.R. Al-Deeb, O.A., Habib, E.E., Ibrahim, T.M. et al.: Eur. J. Med. Chem. 42, 235 (2007).
21. He Y.-W., Cao L.-H., Zhang J.-B., Wangb D.-Z., Aisa H.A.: Carbohydr. Res. 346, 551 (2011).

22. El-Sayed W.A., Abbas H.S., Ewais A.M.: J. Heterocycl. Chem. 48, 1006 (2011).
23. Kuhn C.S., Lehmann J., Steck J.: Tetrahedron 46, 3129 (1990).
24. Blanc-Muesser M., Vigne L., Driguez H., Lehmann J., Steck J. et al.: Carbohydr. Res. 224, 59 (1992).
25. El Ashry E.S.H., Awad L.F., Atta I.A.: Tetrahedron 62, 2943 (2006).
26. Awad O.M.E., Attia W.E., El Ashry E.S.H.: Carbohydr. Res. 339, 469 (2004).
27. El Ashry E.S.H., El Kilany Y.: Adv. Heterocycl. Chem. 69, 129 (1998).
28. Defaye J., Gelas J.: in Studies in Natural Products Chemistry, Vol 8E, Atta-ur-Rahman Ed., p. 315, Elsevier, Amsterdam 1991.
29. El Ashry E.S.H., El Kilany Y.: Adv. Heterocycl. Chem. 67, 391 (1996).
30. Makara G.M., Keseru G.M.: J. Med. Chem. 40, 4154 (1997).
31. Franchetti P., Cappellacci L., Abu Sheikha G., Jayaram H.N., Gurudutt V.V. et al.: J. Med. Chem. 40, 173 (1997).
32. Hammerschmidt F., Peric B., Ohler E.: Monatsh. Chem. 128, 183 (1997).
33. El-Sayed W.A., El-Kosy S.M., Ali O.M., Emeslem H.M., Abdel Rahman A.H.: Acta Pol. Pharm. Drug Res. 69, 669 (2012).
34. El-Sayed W.A., Ali O.M., Zyada R.A.F., Mohamed A.A., Abdel-Rahman A.A.H.: Acta Pol. Pharm. Drug Res. 69, 439 (2012).
35. El-Sayed W.A., Fathi N.M., Gad W.A., El-Ashry E.S.H.: J. Carbohydr. Chem. 27, 357 (2008).
36. Jorgensen J.H., Jurnide J.D., Washington J.A.: in Manual of Clinical Microbiology, 7^{th} edn., Muarry P.R., Baron E.J., Yolken R.C. Eds., p. 1526, American Society for Microbiology, Washington, DC 1999.
37. Janssen A.M., Scheffer J.J., Svendsen A.B.: Planta Med. 53, 395 (1987).
38. Hukel W., Rieckmann P.: Libigs Ann. Chem. 625, 1 (1959).
39. Rashad A.E., El-Sayed W.A., Mohamed A.M., Ali M.A.: Arch. Pharm. 8, 440 (2010).
40. Abdel-Aal M.T., El-Sayed W.A., El-Kosy S.M., El-Ashry E.S.H.: Arch. Pharm. 341, 307 (2008).
41. Abdel Aal M.T., El-Sayed W.A., El-Ashry E.S.H.: Arch. Pharm. 339, 656 (2006).
42. Ali O.M., Amer H.H., Abdel Rahman A.A.H.: Synthesis 2823 (2007).
43. Ibrahim Y.A., Abbas A.A., Elwahy A.H.M.: Carbohydr. Lett. 3, 331 (1999).
44. Ibrahim, Y.A.: Carbohydr. Lett. 1, 425 (1996).
45. Mansour A.K., Ibrahim Y.A., Khalil N.S.A.M.: Nucleosides Nucleotides Nucleic Acids 18, 2256 (1999).
46. Ramiz, M.M.M., El-Sayed, W.A., El-Tantawy, A.I., Abdel-Rahman, A.A.H.: Arch. Pharm. Res. 33, 647 (2010).

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NATURAL DRUGS

HERBAL PREPARATION EXTRACT FOR SKIN AFTER RADIOTHERAPY TREATMENT. PART ONE – PRECLINICAL TESTS

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Abstract: Naran R is a herbal composition made of *Plantago lanceolate folium*, *Malvae arboreae flos*, *Calendulae flos*, *Chamomillae inflorescentia*, *Lamii albi flos* to prepare compresses or to wash skin with inflammations. The extract of this preparation is mixed to be applied as an ointment on patients' skin after radiotherapy. Experiments performed *in vitro* are part of pre-clinical tests with Naran R ointment. This study examined the impact of the plant composition for ethanol-water extract on human skin fibroblasts (HSF) culture. Samples of extract, prepared from patented amounts of herbs, were in the range of 25–225 µg/mL. Six methods were applied: standard spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, neutral red (NR) uptake assay, DPPH free radical scavenging test, labeling of cytoskeleton F-actin, staining of argyrophilic nucleolar organizer regions (AgNORs) and trypan blue coloration. The extract concentration 75 µg/mL was established as safe for application on human skin. In labeling of F-actin with rhodamine-phalloidin dye at this concentration the cytoskeleton was stable. The extract did not influence the membrane stability and had positive influence on the proliferation activity. It was confirmed in AgNOR test during incubation with extract, which led to formation of larger amount of smaller nucleolins. In DPPH scavenging activity test, the extract revealed over 8% higher free-radical scavenging activity in comparison to control. After trypan blue staining, the extract in concentration 125 µg/mL significantly lowered the cell viability. When the cytotoxic and anti-proliferative activity of the extracts were analyzed, MTT and Neutral Red (NR) methods were used. The cells' viability was maintained on a constant level (80–110%) after 24, 48 and 72 h of incubation. During all time of NR test (72 h) and even when 225 µg/mL of extract was applied, the viability of cells was in range 80–110% of control. Positive influence of the extract on investigated cells structure and proliferation, lack of toxicity and increasing anti-oxidant activity enable to consider this preparation as a natural remedy with potential application in skin therapy after radiation.

Keywords: ethanol-water Naran R extract, biological investigations, skin treatment, HSF cells, radiodermitis

Cells culture is an important element of pre-clinical investigation of new medical formulations. It reflects the influence of biologically active compounds on the whole organism. Knowing how the investigated factor affects the human skin fibroblast cells one may predict its action on patient's skin (1).

Naran R is one of herbal preparation from Naran series. Previous publications were related to investigations of biologically active components of medicinal plants from Naran N formulation (2–4).

The presented publication contains a series of pre-clinical investigations with ethanol-water extract of Naran R. It is a preparation composed of *Plantago lanceolata* L. (Plantaginaceae), *Althaea rosea var. nigra* Cav. (Malvaceae), *Calendula officinalis* L. (Astraceae), *Chamomilla recutita* L. Rauschert (Astraceae) and *Lamium album* L. (Lamiaceae). Medical raw materials of these plants with anti-inflammatory, scavenging free radicals and skin irritations healing activities are still used as the supporting factors in medicine. This natural herbal composition is intended to be applied, in form of ointment with ethanol-water extract, on the skin of patients after radiotherapy.

Radiation, which is used in cancer therapy, damages healthy tissues before it reaches the tumor.

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The ray beam destructs proliferating tissues, for example, skin with the layer of fibroblasts. Disorders of structure and functions of healthy cells, described as radiodermitidis, are skin damages after radiation (5).

Postradiation skin inflammation is difficult to cure. It has a local character but often is transformed to delayed radiation effect, which may be one of cancer causes (6, 7). The main factors that damage the skin are free radicals originating from irradiated water molecules and from granulocytes in the inflammation area (8).

Treatment of radiodermitidis is complicated and clinically relatively weak results are obtained. In our study, we propose that the plant extract of the composition can be used in form of an ointment for external use (9). We suggest that it can enrich the panel of materials used in patients treatment. In biological investigations the safe concentration of Naran R ethanol-water extract was determined and it can be used for water/oil (W/O) emulsion ointment preparation.

MATERIALS AND METHODS

Ethanol-water extract was made of Naran R. It is a medical composition made of dried and crumbled plant materials: *Plantago lanceolate folium*, *Malvae arboreae flos*, *Calendulae flos*, *Chamomillae inflorescentia*, *Lamii albi flos*. These raw materials were derived from natural state and plant crops near Lublin (Dys), collected in July 2011. The amounts of all components are patented (10). Distilled water and ethanol 95% were added (1 :1 v/v). Extraction at temperature of 50°C, under reflux was conducted for 5 h. Then, the extract was filtered and evaporated to dryness in a rotary evaporator HB 4 Basic RV 05-ST (IKA, Łódz, Poland). For investigations with cultures of HSF cells the dry residue was dissolved in dimethyl sulfoxide (DMSO) to obtain appropriate concentrations.

In biological experiments, to prepare the HSF cell culture freshly excised small fragments of human skin were used, which were washed two times using RPMI (1640) medium (Gibco™, Paisley, UK) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) (Gibco) and then placed into wells of 24-well plate. The tissue fragment was cut out from the forearm of one of the study's co-author (Roman Paduch). He agreed to use the tissue for presented experiments and whole procedure of explants preparation and cell culture establishment was performed by himself. The explants were then overlaid with a warm 1 : 1 (v/v) mixture of 1% agarose and RPMI 1640 medium. The culture was performed by adding the culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco) on top of agarose gel and incubated at 37°C in a humidified 5% CO_2/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured. For experiments, HSF cells obtained from two donors were used.

In investigations of extract toxicity, HSF cell cultures in a density of 1×10^5 cell/mL were used. Incubation was conducted for 24 h. When investigations of extract activity were performed, HSF cell cultures with a density of 2×10^4 cell/mL were used. Incubation was conducted for 72 h.

The HSF cells were cultured as monolayers in 25 cm^2 culture flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% FBS (v/v) and antibiotics at 37°C in a humidified atmosphere with 5% CO_2. For experiments, the total number of cells was estimated by counting in hemocytometer. A 100 μL sample of cell suspension (2×10^4 cells/mL for cells proliferation activity tests or 1×10^5 cells/mL for toxicity tests) was added to appropriate wells of 96-well flat-bottomed microtitre plates (MTT and NR methods). After 24 h of incubation, the medium was discarded and new medium containing 2% FBS and appropriate plant extract concentrations in 25–225 μg/mL range was added. As a control, HSF cells suspended in 100 μL of culture medium with 2% FBS without plant extracts was used. The total cell number was equivalent to that in the sample wells. Additional controls without cells but containing appropriate plant extract concentrations in 2% FBS culture medium were prepared to exclude non-specific dye reduction (MTT method) (11). As blank control culture medium with 2% FBS was used. Sensitivity of cells to Naran R extract activity was determined by a standard spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann (11). MTT test is based on conversion of yellow tetrazolium salt by viable cells to purple crystals of formazan. The reaction is catalyzed by mitochondrial succinyl dehydrogenase. The cells grown in 96-well multiplates in 100 mL of culture medium supplemented with 2% FBS were incubated for 3 h with MTT solution (5 mg/mL, 25 mL/well) (Sigma, St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 10% sodium dodecyl sulfate (SDS) (Sigma) in 0.01 M HCl. The product was quantified spectrophotometri-

cally by absorbance measurement at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

NR cytotoxicity assay is based on the uptake and lysosomal accumulation of the supravital dye, neutral red. Dead or damaged cells do not take up the dye (12). In neutral red (NR) uptake assay the cells were grown in 96-well multiplates in 100 µL of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of Naran R extract (25–225 µg/mL). Subsequently, the medium was discarded and 0.4% NR (Sigma) solution in 2% FBS medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, the cells fixed with 1% $CaCl_2$ in 4% paraformaldehyde and thereafter the incorporated dye was solubilized using 1% acetic acid in 50% ethanol solution (100 µL). The plates were gently shaked for 20 min at room temperature and the extracted dye absorbance was spectrophotometrically measured at 540 nm.

Free radical scavenging activity of ethanol-water extract of Naran R was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH· (Sigma) to the yellow colored diphenyl-picrylhydrazine. Briefly, 100 µL of DPPH· solution (0.2 mg/mL in ethanol) was added to 100 µL of different plant extract solution concentrations (25–225 µg/mL) and standards. Trolox (Sigma) at increasing concentrations (1–50 µg/mL) was used as a reference for the free radical scavenging activity. After 2, 5, 10, 15 and 20 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower was the absorbance, the higher was free radical scavenging activity of the plant extracts. The activity of each extract concentration was determined by comparing its absorbance with that of a blank solution (reagents without plant extracts) and standard. The capability to scavenge DPPH· radical was calculated by the following formula:

$$\text{DPPH}^{\cdot}\ \text{scavenging effect (\%)} = [(X_{control} - X_{extract}/X_{control}) \times 100]$$

where $X_{control}$ is the absorbance of the control and $X_{extract}$ is the absorbance in the presence of plant extract (13).

Argyrophilic nucleolar organizer regions (AgNORs) staining was performed after incubation in 4-well Lab-Tek II Chamber slides; cells were rinsed with PBS and fixed with absolute ethanol/acetic acid solution (1 : 1 v/v) for 10 min. The silver colloid solution was prepared by 2% gelatin in 1% formic acid mixed in 1 : 2 volumes with 30% aqueous silver nitrate. Cells were immersed in this solution for 5 min at 37°C, rinsed with deionized water and mounted with glycerol. AgNOR proteins were determined using a computer-assisted image analysis system (14).

Phalloidin staining is the useful tool for investigating the distribution of F-actin in cells (15). In labeling of cytoskeleton F-actin, the cells were incubated in 4-well Lab-Tek chamber slides in 1 mL of culture medium supplemented with 2% FBS and plant extracts. After incubation, the cells were rinsed with RPMI 1640 medium and exposed to paraformaldehyde (10%, v/v) solution for 20 min, rinsed three times with PBS, exposed to Triton X-100 (0.2%, v/v) solution for 5 min and rinsed again three times with PBS. A half milliliter of PBS containing tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin, 1 µg/mL) (Sigma) was added to each well and incubation in the dark at 37°C/5% CO_2 for 30 min was accomplished. Cells observation was conducted under a fluorescence microscope (Olympus, BX51). Quantitative analysis of fluorescent images was performed by AnalySIS imaging software system.

In trypan blue staining, cells were grown in 96-well multiplates in 100 µL of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of Naran R extract (25–225 µg/mL). Subsequently, the medium was discarded and 0.4% trypan blue solution in 0.9% sodium chloride was added to each well. The cell walls of dead cells were colored in blue (16).

RESULTS

After trypan blue staining (Table 1) the viability of cells was not lowered when the cells were incubated with the extract at concentrations up to 75 mg/mL. Analysis with MTT method (Fig. 1) revealed that in cells treated with the extract gradual, slight increase of mitochondrial dehydrogenase activity was observed. Cells disintegration appeared for extract concentrations above 125 µg/mL. NR uptake test showed that HSF cells membranes were stable after fibroblasts incubation with Naran R extract even at the high 225 µg/mL concentration (Fig. 2).

The analysis with trypan blue coloration is presented in Table 2. Three days' incubation of cells with the extract in high concentrations exceeded 125 µg/mL significantly lowered the cell viability. When MTT method was used, the cells' viability main-

tained on a constant level (80–110%) after 24, 48 and 72 h of incubation (Fig. 3). During all time of NR test (72 h) and even when the highest concentration of extract was applied, the viability of cells was in range 80–110% of control (Fig. 4).

The next stage of investigations consisted in determination of cell ability to division under influence of ethanol-water extract of Naran R. Argyrophilic nucleolar organizer regions (AgNORs) staining was analyzed. The cell cultures were observed under visual microscope. In extract concentrations up to 75 μg/mL r-RNA was detected, which suggested cells proliferation (Fig. 5).

Cell cytoskeleton organization was determined by labeling of F-actin with rhodamine-phalloidin dye. Incubation with plant extract showed positive

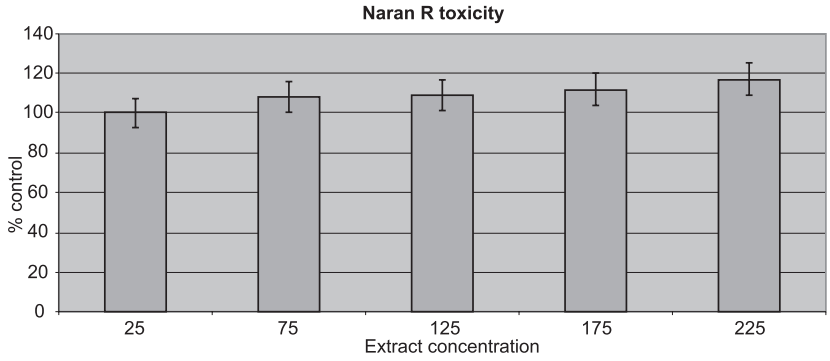


Figure 1. Toxicity analysis of Naran R ethanol-water extract. HSF cells (1 x 10^5 cells/mL) after 24 h incubation. MTT method. Extract concentration (μg/mL) (n = 4)

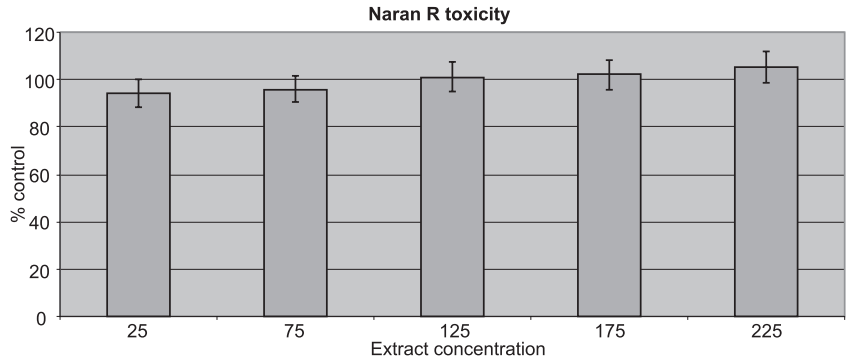


Figure 2. Toxicity analysis of Naran R ethanol-water extract after 24 h of incubation. HSF cells (1 x 10^5 cells/mL) incubation. Neutral red test. Extract concentration (μg/mL) (n = 4)

Table 1. Toxicity analysis of Naran R ethanol-water extract. HSF cells (1 × 10^5 cells/mL) after 24 h incubation. Trypan blue coloration.

	Extract concentration (μg/mL)	% viability
Ethanol-water extract of Naran R	25	96.3 ± 0.6
	75	95.7 ± 1.2
	125	94.3 ± 1.5
	175	91.7 ± 0.6
	225	85.7 ± 1.5
Control		98.3 ± 0.6

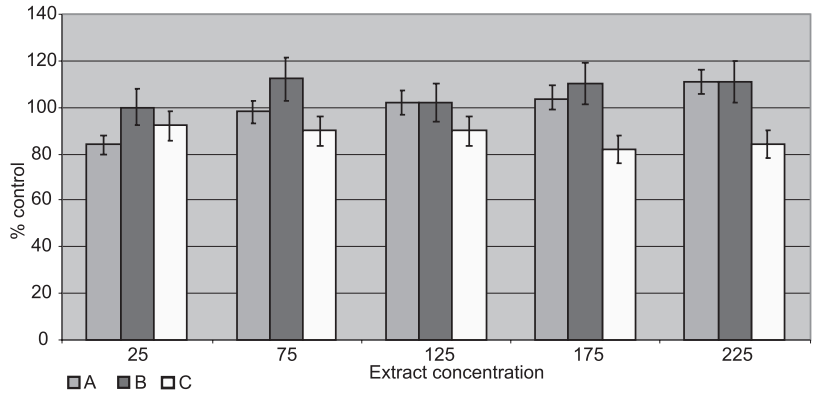


Figure 3. Activity analysis of Naran R ethanol-water extract after 24 (A), 48 (B) and 72 (C) h of incubation with HSF cells (density 2 × 10^4 cell/mL). MTT test. Extract concentration (mg/mL) (n = 4)

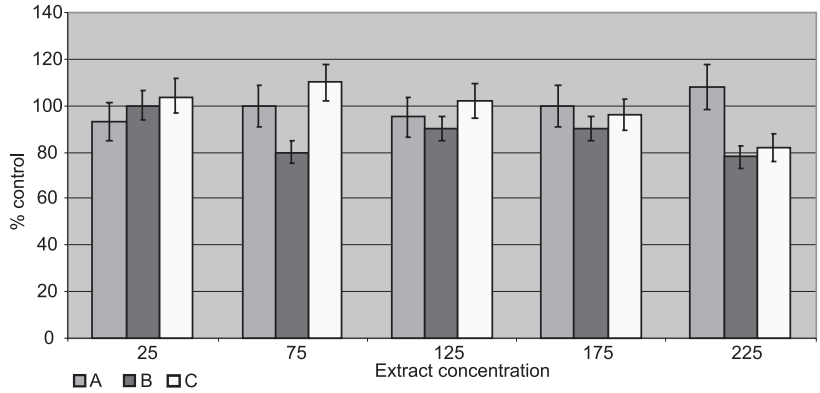


Figure 4. Activity analysis of Naran R ethanol-water extract. HSF cells (2 × 10^4 cell/mL) after 24 (A), 48 (B) and 72 (C) h of incubation. Neutral red test. Extract concentration (µg/mL) (n = 4)

influence on cytoskeleton structure (Fig. 6). At concentration 75 µg/mL the cytoskeleton was stable. It changed when extract at concentration 125 µg/mL was used.

Moreover, free radical scavenging ability of Naran R was analyzed. Tests were conducted using $DPPH^{?\cdot}$ reduction method. In this method, the measurement of antioxidant activity is determined by decrease of the oxidized form of $DPPH^{\cdot}$ in the sample. In comparison to the control, 8% reduction of free radical DPPH concentration in cultures with Naran R ethanol-water extract was found.

DISCUSSION

Investigations of plant composition for radiodermitis therapy was motivated by lack of preparations with confirmed efficacy in this type of ailment. If after irradiation erythema and dry epidermis peeling begins, powders (e.g., Alantan), aerosols (Panthenol) or creams (1% hydrocortisone) are administered. In damp epidermis peeling Silol, Panthenol or Linomag can be used. Chronic postradiation skin inflammation is difficult to cure. Hydrocolloid bandages, interferon a, pentoxiphylline are applied. In complicated cases even surgical skin removal and its grafting are performed (13, 14).

New formulation is planned to be applied on the skin, therefore, the extract was tested with use of cell cultures of HSF. In experiments with use of cell cultures, six methods of assay of biological activity of ethanol-water extract analysis were performed. Succinyl dehydrogenase activity (MTT method), membranes integrity (trypan blue staining), membranes integrity (neutral red test), cells proliferation (r-RNA coloration), cytoskeleton structure (rhodamine-phalloidin fluorescent staining) and antioxidant activity ($DPPH^{\cdot}$ method) were tested.

Incubation with the extract resulted in a gradual slight increase of mitochondrial dehydrogenase

activity (MTT) Cells disintegration took place only at higher concentrations. During 24 h investigations of the extract toxicity, no significant modification in cells viability was observed in trypan blue coloration test. In neutral red uptake test cells' membranes were stable and the extract did not disturb the functions of cells even when its high concentrations were used.

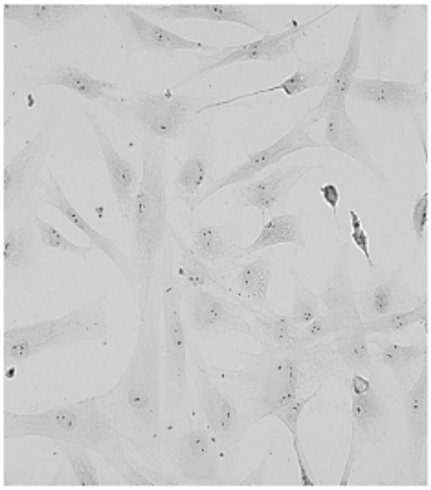
The activity of the extract was checked with use of the same methods but incubation was conducted for 72 h. The cells showed stability of cell membranes and intracellular structures. Naran R extract did not cause disorders of mitochondrial dehydrogenase (MTT test) over the incubation time (72 h). Membranes integrity in trypan blue staining after 72 h of incubation was lowered. In NR test, the influence of extract on cells' membranes stability was analyzed. Naran R extract did not damage them. Cell's ability to division and cytoskeleton structure were examined in extract concentrations determined earlier as safe. The extract had positive influence on rRNA synthesis, which is essential before cell division. After cells incubation with the extract, more (in comparision to control) small nucleoli with genetic material to mitotic cell division were observed. The cytoskeleton was steady with contact between cells. In the cells, the F-actin fibres were longitudinal, which is the proper structure for HSF cells.

One of the important analysis steps was the determination of antioxidant ability of plants' extract. Free radicals in oxygen breathing organisms arise all the time and take part in many physiologic and also pathologic processes. In the evolution process, a lot of mechanisms occurred to protect organism from the destructive action of free oxygen radicals. Under physiological conditions, without additional exposure on radiation risk, organisms have produced a set of enzymes (superoxide dismutase, catalase, glutathione peroxidase) and natural antioxidants (glutathione, bilirubin, glucose) for tissues protection (8, 15, 16). After X-ray therapy, organism's own mechanisms of prevention are not sufficient. In chain reactions, numerous free radicals are formed and this can lead to long lasting inflammations, and after a long time, to tumors (7).

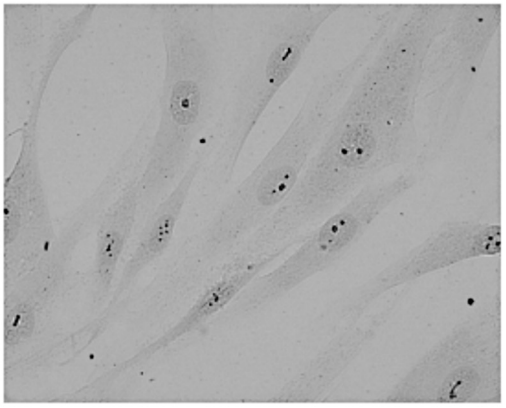
Application of preparation with scavenging free radicals activity should defend tissues against such complications (17, 18). The main source of such compounds are medical plants. In the proposed medicament, ethanol-water extract of five medicinal plants rich in flavonoids, polysaccharides, phenolic

Table 2. Etanol-water extract of Naran R activity after 24, 48 and 72 h of incubation with HSF cells (2×10^4 cells/mL). Trypane blue coloration.

	Culture time (h)	Extract concentration (mg/mL)	% of viability
Ethanol-water extract of Naran R plant composition	24	25	94.7 ± 1.2
		75	94.7 ± 2.1
		125	91.3 ± 1.5
		175	87.3 ± 0.6
		225	86.7 ± 2.1
	48	25	96.7 ± 0.6
		75	95.7 ± 0.6
		125	84.3 ± 2.5
		175	78.3 ± 2.1
		225	73.3 ± 2.1
	72	25	88.0 ± 1.7
		75	67.7 ± 3.2
		125	48.7 ± 4.2
		175	40.7 ± 4.7
		225	30.3 ± 3.5
Control	24		99.7 ± 0.6
	48		99.7 ± 0.6
	72		94.0 ± 1.0

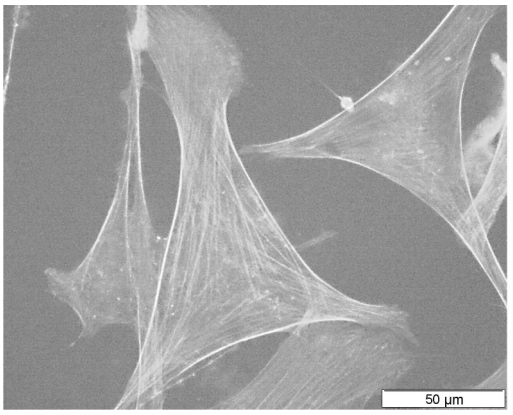


A



B

Figure 5. Photograph of AgNORs analysis after cells incubation with etanol-water extract of Naran R in concentration 75 µg/mL (A) and control image (B). Visual microscope, magnification 200×.



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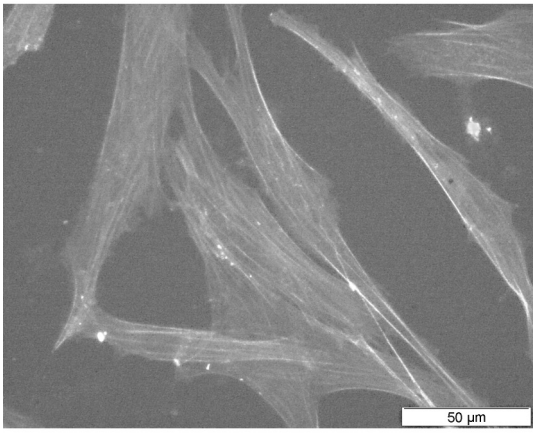

B

Figure 6. Photograph of cells' cytoskeleton staining with the use of rhodamine-phalloidin after their incubation with ethanol-water extract in 75 µg/mL concentration (A). Control image (B). Fluorescent microscope, magnification 400×.

acids, terpenoids and anthocyanins was used. These substances are known antioxidants and due to their presence in the extract the reduction of DPPH* concentration is observed (19–21).

To enable patients to apply the extract it is planned to prepare an ointment used in pre-clinical tests, containing the dry residue of ethanol-water extract. The dry residue will be dissolved in DMSO to obtain 75 mg/mL concentration, which was established in biological investigations as safe. This solution will be subjected to emulsifying process with an ointment base to obtain a preparation in the form of a W/O emulsion. This kind of emulsion is suitable for long lasting inflammations of skin, which occurs after radiation. To prepare the ointment as a base *Unguentum cholesteroli* (22) is recommended. Subsequently, the Naran R ointment is planned to be examined in clinical tests with oncological patients after radiotherapy.

CONCLUSIONS

Investigated herbal composition was prepared for traditional use in form of water infusions and compresses. Naran R was not previously tested in form of extracts with use of different solvents. Presented extract was selected among several others because it showed the best results in biological investigations and it gives the possibility to use it as a plant drug (23).

Six methods describing the structure, functions and ability to proliferation of cells confirmed the profitable influence of the extract on human skin. It is not toxic, its application will improve skin condition of patients and speed up the healing process. By lowering the free radicals concentration, the lowering of delayed radiation effect is expected as well. This should decrease the probability of postradiation skin damages.

The biological investigations of Naran R activity confirmed the safety of its application. The safe concentration of extract established in toxicity and activity investigations is 75 μg/mL. Good results in *in vitro* tests give possibility to carry out tests *in vivo* with oncological patients. Optimal extract concentration (non-toxic, with the best activity) will be employed to get an ointment, which is planned to be used on skin after radiation.

Free radicals reduction, good image of cell cultures, no toxic effect gives possibility of further clinical researches of Naran R ointment. Application of the investigated preparation should reduce the delayed radiation effect on patients' skin.

REFERENCES

1. Roszkowska-Jakimiec W., Wołoczyński S., Chlabicz M.: Rocz. Akad. Med. Bialymst. 49 (Suppl. 1), 234 (2004).
2. Paduch R, Matysik G., Wójciak-Kosior M., Niedziela P.: Rec. Progress Med. Plants 18, 127 (2007).
3. Matysik G., Wójciak-Kosior M., Paduch R., Soczewiński E.: Herba Polonica 49, 217 (2003).
4. Wójciak-Kosior M., Matysik G., Soczewiński E.: Herba Polonica 49, 195 (2003).
5. Jabłońska S., Chorzelski T. Skin diseases. (Polish). PZWL, Warszawa 1997.
6. Braun-Falco O., Plewig G., Wolff H. H., Burgdorf W. H. C. Dermatology (Polish). Publishing House Czelej, Lublin 2002.
7. Rassner G. Dermatology. (Polish) Urban and Partner, Wrocław 1994.
8. Bartosz G. Oxygen's second face (Polish). PWN, Warszawa 2003.
9. Krzakowski M. Clinical oncology (Polish). Borgis, Warszawa 2001.
10. Patent number: PL 208541 "Herbal composition for skin after radiotherapy treatment". Poland 2007.
11. Mosmann T.: J. Immunol. Meth. 65, 55 (1983).
12. Ganbold M., Barker J., Ma R., Jones L., Carew M.: J. Ethnopharmacol. 131, 396 (2010).
13. Gulcin I.: Toxicology 217, 213 (2006).
14. Paduch R., Matysik G., Wójciak-Kosior M., Kandefer-Szerszeń M., Skalska-Kamińska A., Nowak-Kryska M., Niedziela P.: Polish J. Environ. Stud. 17, 569 (2008).
15. Chang H-R., Huang H-P., Kao Y-L., Chen S-L., Wu S-W., Hung T-W., Lian J-D., Wang Ch-J.: Chem. Biol. Interact. 183, 172 (2010).
16. Paduch R., Wójciak-Kosior M., Matysik G.: J. Ethnopharmacol. 110, 69 (2007).
17. Janicki S. Applied pharmacy (Polish). PZWL, Warszawa 2006.
18. Jeziorski A. Oncology (Polish). PZWL, Warszawa 2005.
19. Lukivskaya O., Zavodnik L., Buko V.: Adv. Med. Sci. 51, 54 (2006).
20. Muller S., Millonig G., Waite G. N.: Adv. Med. Sci. 54, 121 (2009).
21. Aliyu A.B., Ibrahim M.A., Musa A.M., Musa A.O., Kiplimo J.J., Oyewale A.O.: Acta Pol. Pharm. Drug Res. 70, 115 (2013).
22. Malviya N., Jain S., Jain A., Jain S., Gurjar R.: Acta Pol. Pharm. Drug Res. 67, 391 (2010).
23. Harborne J.B., Williams Ch.A.: Phytochemistry 55, 481 (2000).
24. Kong J.-M.: Phytochemistry 64, 923 (2000).
25. Tarnawski M.: J. Pharm. Biomed. Anal. 41, 182 (2006).
26. Polish Pharmacopoeia IX. Polish Pharmaceutical Society Publishing House, Warszawa 2011.
27. Brodniewicz T., Grynkiewicz G.: Acta Pol. Pharm. Drug Res. 69, 1203 (2012).

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ANTIFUNGAL AND CYTOTOXIC ACTIVITIES OF *NANNORRHOPS RITCHIANA* ROOTS EXTRACT

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Abstract: This study was designed to evaluate the antifungal and cytotoxic activities of the *Nannorrhops ritchiana* (Mazari Palm) 80% methanol extract (NR-M) and its four crude extracts i.e., petroleum ether (NR-A), dichloromethane (NR-B), ethyl acetate (NR-C) and butanol (NR-D). The antifungal activity was determined by agar tube dilution method against nine fungal strains; *Aspergillus flavus, Trichophyton longifusis*, *Trichophyton mentagrophytes, Aspergillus flavus* and *Microsporum canis* were susceptible to the extracts with percentage inhibition of (70–80%). Extracts exhibited significant and good antifungal activity against various fungal strains. The results were deduced by comparing with those for miconazole, amphotericin B and ketoconazole as standard drugs. The fractions of methanolic extract were assayed for their brine shrimp cytotoxic activity. They exhibited low toxicity with LC_{50} values ranging from 285.7 to 4350.75 µg/mL at the concentration of obtained results warrant follow up through bioassay guided isolation of the active principles, future antiinfectious research.

Keywords: *Nannorrhops ritchiana*, antifungal activity, brine shrimp, cytotoxic activity

Natural products obtained from various sources like, plants, animals, and microorganisms are considered to be strong candidates of pharmaceutical drugs since long period of time and they constitute a vast majority of chemotherapeutic agents currently in use for various types of infectious diseases provoked by pathogenic bacteria and fungi, cardiovascular diseases, cancers and many others. The interest in using medicinal plant's extracts and their isolated components has increased not only in developing countries but also getting more popularity in developed countries. According to World Health Organization, medicinal plants would be the best source for variety of drugs (1) having less toxicity and being more economical. Therefore, medicinally important plants should be investigated for better understanding of the chemistry and chemical composition of their bioactive constituents, their efficacy and safety (2).

Pakistan occupies a unique position among developing countries due to widespread medicinal plants on account of its topology. It is noticeable that more than 50% of the population in Pakistan is still being treated with the herbal medicines by the traditional practitioners and over 350 herbal drugs have been reported. Pakistan flora is rich in variety of rare plants and offers a great chance to discover a lead molecule, that is a step forward towards the pharmaceutical drugs (3).

The compounds resulting from natural products are the most important biochemical tools that demonstrate the specific mode of action in various fungal pathogenic diseases, but there is still a great urgency of developing new antifungal agents that are more efficient, less toxic, having minimum side effects (4). Fungal pathogens exist in various forms and some of them cause severe infections of skin, hairs, nails, lungs, ears and joints in humans and animals (5) whereas others cause severe infections of central nervous system (CNS) in humans. At present, about 300 metabolites have been reported to be toxic to man and animals (6) and the main toxic effects are carcinogenicity, genotoxicity, teratogenicity, hepatotoxicity and immune suppression (7, 8). The human fungal diseases also include aspergillosis, actinomycosis, histoplasmosis and corcid-

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iomycosis. The discovery of antifungal drugs is the need of this era due to their versatility and the second most important factor is that they showed an enhanced degree of resistance to the existing antifungal drugs.

Nannorrhops ritchiana (Arecaceae) is the sole species of this genus (9). It is native to Southwestern Asia, from Southeast of the Arabian Peninsula to east through Iran and Afghanistan to Pakistan. It is widely distributed in the various areas of Balochistan province of Pakistan particularly in Harnai, Khuzdar and Barkhan at an altitude of 1600 m. It is one of the most versatile palms that can survive both in normal and under drastic weather conditions (10). The young leaves of plant with sweet astringent taste have been used as a purgative in livestock (11). The fruit is edible (12) and used by local communities for the treatment of diarrhea and dysentery (13). In Balochistan (14) it is used against various infectious diseases like gastrointestinal disorder (15). The constituents of plant ash have been quantitatively estimated in relation to biogeochemistry of the plant, the presence of minerals like calcium, magnesium, iron and nickel in soil and plant ash opened new avenue for the researchers (16). Therefore, research work is initiated on the roots of *N. ritchiana* to investigate antifungal and cytotoxic activities of various crude extracts.

In search of effective nontoxic oral drugs and topical applications, there is dare need of natural sources to be explored and exploited for relief.

EXPERIMENTAL

General

All reagents and solvents used were obtained from Sigma Aldrich (Shalimar Chemicals supplier, Pakistan). Commercial methanol used for extraction was purchased locally.

Plant material

The *Nannorrhops ritchiana* species was collected from Barkhan and Harnai areas in Balochistan, Pakistan. A voucher specimen No. UoB/230 has been deposited in the Herbarium of the Department of Botany, University of Balochistan, Quetta, Pakistan.

Preparation of extracts

The air-dried roots (15 kg) of *Nannorrhops ritchiana* were extracted with 80% aq. MeOH at room temperature for three times. The combined MeOH extracts were evaporated under vacuum to get crude extract (NR-M) 1.5 kg. Methanol crude extract was dissolved in cold distilled H_2O (10 L) and partitioned with petroleum ether (25 L) to afford after concentration the fraction NR-A. The aqueous layer was then extracted with CH_2Cl_2 (10 L) to obtain fraction NR-B, ethyl acetate (10 L) – fraction NR-C and butanol (10 L) – fraction NR-D, respectively. The fractionated extracts were tested for the antifungal activity and based on that planned for further phytochemistry studies.

Antifungal assay *in vitro*

Test organisms

Clinical isolates of human pathogens *Microsporum canis*, *Candida albicans*, *Candida glaberata* and *Aspergillus flavus*; animal pathogens, *Tricheophyton mentagrophytes*, *Fusarium monilifumus* and plant pathogens *Trichophyton longifusis*, *Fusarium solani lycopersici* and *Fusarium oxysporum lycopersici solanivar* were used in this study.

Agar tube dilution method

Agar tube dilution method (17) was used for the evaluation of antifungal activity. Sabouraud dextrose agar (SDA) was used for the growth of fungus and stock solution was prepared by dissolving 24 mg of each extract in 1 mL of sterile DMSO. Acidic medium (pH 5.5–5.6), containing high concentration of glucose or maltose prepared by mixing 32.5 g/500 mL of distilled water was put in the screw caped tubes and autoclaved at 121°C for 15 min. Tubes were then allowed to cool to 50°C and non-solidified SDA was loaded with 66.6 μL of extract, pipetted from the stock solution and allowed to solidify at room temperature. Then, tubes were inoculated with 4 mm piece of inoculums and incubated at 27–29°C for 7–10 days and relative humidity of incubation room was maintained at 40–50%. After this period, percentage growth inhibition was calculated with reference to the negative control by the formula:

$$\% \text{ inhibition} = \text{linear growth in test (mm)} / \text{linear growth in control (mm)} \times 100$$

Miconazole and amphotericin B were used as standard drugs, while miconazole, amphotericin B and DMSO were used as positive and negative controls (18–22).

Brine shrimp lethality assay

The brine shrimp lethality test (BST) was performed to predict the cytotoxic activity in the different crude extracts of *N. ritchiana*. The eggs were hatched in artificial sea water as per conventional method (23). Shrimp were transferred by pipette to each tube and sea water was added to make total

volume of 5 mL. The test tubes were kept under illumination so that the nauplii can easily be counted macroscopically in capillary. The survivors were counted with the help of magnifying glass (×3), the brine shrimp (LC_{50}) was determined.

RESULTS AND DISCUSSION

During this study, different fractions obtained from 80% methanol extract (NR-M) of roots of *Nannorrhops ritchiana* plant, petroleum ether (NR-A), dichloromethane (NR-B), ethyl acetate (NR-C), and butanol (NR-D) extracts were screened for antifungal activity (Fig. 1). *N. ritchiana* extracts showed the maximum inhibition of fungal growth and thus indicate the presence of interesting antifungal agents in the roots extract of this plant.

The antifungal activity resulting from crude methanol extract fraction (NR-M) exhibited good activity against *Tricheophyton mentagrophytes*, – 77.3 MIC µg/mL. Fraction NR-A showed significant antifungal activity against *Microsporum canis* 80%, *Trichophyton longifusis* 67%, good activity against *Tricheophyton mentagrophytes* 70%; extract NR-B showed good activity against *Trichophyton longifusis* 70% but moderate activity against *Microsporum canis* 60%, while extracts NR-C and NR-D exhibited medium activity against *Trichophyton longifusis* 60% and good against *Aspergillus flavus* 70%, respectively.

The brine shrimp lethality assay results presented in Table 2, show that the root extracts were found virtually non-toxic. They show negligible toxicity with $LC_{50} > 100$ µg/mL .

The young leaves of *Nannorrhops ritchiana* are used as a purgative in livestock (11) and fruit (12) is used in traditional medicine by local communities for the treatment of diarrhea, dysentery (13, 14) and against various infectious diseases like gastrointestinal disorder (15). These claims have been supported on the bases of the current bioassay findings, that showed the activity against fungal pathogens.

Table 1. Antifungal activities (MIC µg/mL) of crude fractions of *N. ritchiana* roots extract.

Fungal species*	% inhibition of standard drug[ii]	% inhibition of different fractions				
		A	B	C	D	M
M. canis	110.8[c]	80	60	–[i]	–[i]	–[i]
T. mentagrophytes	100[e]	67	–[i]	–[i]	–[i]	77.3
A. flavus	20[d]	–[i]	–[i]	–[i]	70	–[i]
T. longifusis	70[b]	70	70	62	–[i]	–[i]

[i] – not active. Reference standard drug: [b]miconazole (MIC µg/mL). [c]miconazole (MIC µg/mL), [d]amphotericin B (MIC µg/mL), [e]ketoconazole (µg/mL). Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol. M: methanol crude extract. (*only those fungi, which were significantly inhibited by respective sample have been included).

Table 2. Brine shrimp lethality test of *Nannorrhops ritchiana* roots extracts.

No.	Extracts	LC_{50} µg/mL	95% CI
1.	Petroleum ether (NR-A)	285.7	189.2–431.4
2.	Dichloromethane (NR-B)	1462.2	685.55–3123.3
3.	Ethyl acetate (NR-C)	4357.77	1274.1–14901.10
4.	Methanol (NR-M)	409.84	253.07–664.10
5.	Cyclophosphamide	16.32	10.60–25.14

The results are presented as LC_{50} values (µg/mL) and 95% confidence intervals (Cl). Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol. M: methanol crude extract. NR = *Nannorrhops ritchiana*

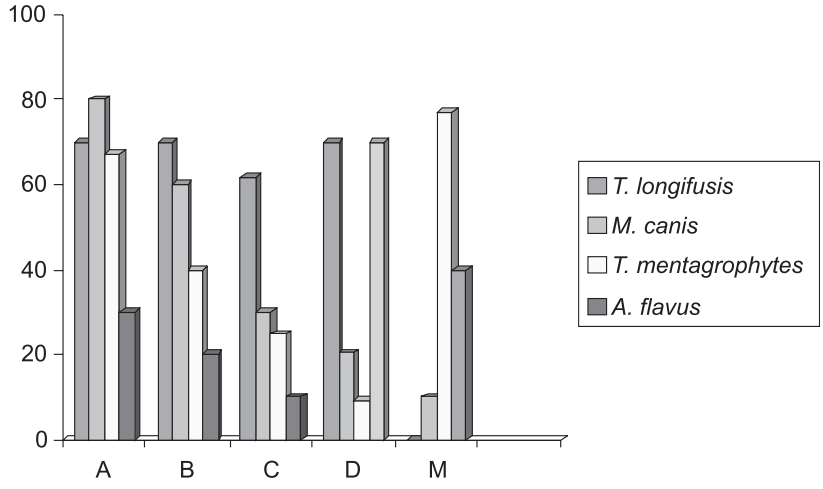

Figure 1. Antifungal activities of fractions of *N. ritchiana*. Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol. M: methanol crude extract

The crude methanol roots extract (NR-M) of *Nannorrhops ritchiana* and four fractions i.e., petroleum ether (NR-A), dichloromethane (NA-B), ethyl acetate (NR-C) and butanol (NR-D) extracts exhibited an overall significant antifungal activity (70–80%) against different pathogens but few fungi have low activity against different fractions.

Extracts of the roots exhibited mild cytotoxic activity against brine shrimp larvae with LC_{50} value ranging from 285.75 to 4357.77 µg/mL, whereas that of the standard anticancer drug was 16.32 (10.50–25.16) µg/mL. It is worth mentioning that the extract with very low toxicity on brine shrimp in particular are those active against antifungal pathogens.

CONCLUSION

This study has shown significant fungicidal activity in the roots of *Nannorrhops ritchiana* and it can be exploited as an ideal future drug as an anti dermatophyte. This activity may be attributed to the presence of alkaloids, phenols, polyphenols, saponins, tannins, anthraquinones, sterols and especially alkaloids, found in the crude extract and fractions thereof. These phytochemical classes of natural products are known to display antifungal activities. This also indicates that the root extracts were virtually non toxic on shrimps. They exhibited very low toxicity, giving LC_{50} values greater than 100 µg/mL. Selectivity of petroleum ether extract with LC_{50} values 285.77 µg/mL with very low toxicity is not appreciated to be used in anticancer and antitumor activities. It may be speculated here that the extracts would be useful for the treatment of diarrhea caused by the gastrointestinal infection. Further research is planned to isolate and identify the constituents present in these fractions. The characterization of the structures of bioactive constituents of these fractions present in this plant should be done in order to determine their full spectrum of efficacy.

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REFERENCES

1. Santos P.R.V., Oliveira A.C.X., Tomassini, T.C.B.: Rev. Farm. Bioquim. 31, 35 (1995).
2. Ellof J.N.: J. Ethnopharmacol. 60, 1 (1998).
3. Gibbons M., Spanner T.W.: Principes 39, 177 (1995).
4. Tasleem A., Mandal T.K., Rajesh D.: Natural products: Anti-fungal agents derived from plants, in Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry. Tivari V.K., Mishra B.B. Eds., pp. 283–311, Research Signpost, Trivandrum, Kerala, India 2011.
5. Haq I.: Report of Ministry of Health, Government of Pakistan. Hamdard Foundation Press, Karachi 1983.

6. Galvano F., Piva A., Riteieni A., Galvano G.: J. Food Prot. 64, 120 (2001).
7. Lacey J.: J. Stored Prod. Res. 24, 39 (1998).
8. Desjardins A.E., Manandhar G., Platter R.D., Margos C.M., Shrstha K., McCormick S. P.: J. Agric. Food Chem. 48, 1377 (2000).
9. Blatter E.: Palms of British India and Ceylon. Oxford University Press, London, New York 1926.
10. Gupta,S.C.: J. Res. Agra University 9, 103 (1960).
11. Sarfaraz K.M., Fazal R., Khalid U., Abdul A.K., Said. G., Naveed A., Muhammad S., ShahJehan K.: J. Med. Plant. Res. 5, 3679 (2011).
12. Abbas S.Q.: Ethnobotany Project WWF for Nature Pakistan, 1–23 (2003).
13. Bielski J.: Nannorrohps ritchiana. Floridata. http://www.floridata.com/ref./n/nann_rit.cfm. 2000.
14. Waheed M., Ashfaq A., Syed A.G., Mir A.K.: J. Med. Plants Res. 5, 1072 (2011).
15. Sadaf N., Shahid N., Erum B., Khuala S., Shamim S.A.: Chinese J. Geochem. 24, 327 (2005).
16. Plotkin M.J., Balick M.J.: J. Ethnopharmacol. 10, 157 (1984).
17. Rahman A., Choudhary M.I., Thomsen J.: Manual of Bioassay Techniques for Natural Product Research, pp. 82–84. Harward Academic Press, Amsterdam 1991.
18. de Almeida Alves T.M., Fonseca Silva A., Brandăo M., Mesquita Grandi T.S., Smânia E.F., Smânia A. Jr., Zani C.L.: Mem. Inst. Oswaldo Cruz 95, 367 (2000).
19. Vanden-Berghe D.A., Vlientinck A.J.: Screening Methods for Antibacterial and Antiviral Agents from Higher Plants. in Hostettmann K, Ed., Methods in Plant Biochemistry VI. Assays for Bioactivity, pp. 47–69, Academic Press, London 1991.
20. Choudhary M.I., Dur-e-Shahwar Z., Perveen Z., Abdul Jabbar I.A., Atta-ur-Rahman H.F.J.: Phytochemistry 40, 1243 (1995).
21. Janaki S., Vijayasekaran V.: Biomedicine 18, 86 (1998).
22. Peters W., Gilles H.M.: Color Atlas of Tropical Medicine and Parasitology, Wolfe Medical Publications, London 1989.
23. Meyer B.N., Ferrign R.N., Putnam J.E., Jacobson L.B., Nicholas D.E., McLaughlin J.L.: Planta Med. 45, 31 (1982).

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ANTIMICROBIAL ACTIVITY OF PREPARATION BIOARON C®

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Abstract: The antimicrobial activity of sirupus Bioaron C®, a preparation, whose main ingredient is an extract from the leaves of *Aloe arborescens,* was tested against different microorganisms isolated from patients with upper respiratory tract infections. The experiments were performed on 40 strains: 20 strains of anaerobic bacteria, 13 strains of aerobic bacteria and 7 strains of yeast-like fungi from the genus *Candida* and on 18 reference strains (ATCC). The antimicrobial activity of Bioaron C (MBC and MFC) was determined at undiluted concentration. Bioaron C proved to be very effective against the microorganisms causing infections. At the concentration recommended by the producer, the preparation showed biocidal activity (MBC, MFC) against the strains of the pathogenic microorganisms, which cause respiratory infections most frequently, including, among others, *Peptostreptococcus anaerobius, Parvimonas micra, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus anginosus, Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa* and *Candida albicans*, already after 15 min. The MIC of Bioaron C against most of the tested microorganisms was 5 to 100 times lower than the usually applied concentration. The great antimicrobial activity means that the preparation may be used in the prevention and treatment of infections of the upper respiratory tract. Bioaron C® may be an alternative or complement to classical therapy, especially in children.

Keywords: antimicrobial activity, Bioaron C®, *Aloe arborescens*

Infections of the upper respiratory tract are quite frequent, especially in children. Susceptibility to the infections does not only depend on the infectious agents (viruses, bacteria), but also on the system's immunity. The human immune system, similarly to the respiratory system, reaches maturity at the age of 10–12 years. The most common primary cause of the upper respiratory tract catarrhs are viruses, which damage the epithelium of the respiratory tract, thus making it easier for bacterial pathogens to enter. Inflammation is a process during which the cells of the immune system, i.e., monocytes (macrophages), B, T, NK and cytotoxic lymphocytes and granulocytes are activated to protect the body against harmful stimuli. Treatment of a cold should, then, consist in alleviating the ailments and strengthening the immune system. A hasty use of antibiotic therapy damages the natural bacterial flora, impairing the functioning of the immune system, and often contributes to bacterial resistance to antibiotics, which, in consequence, leads to infection relapses. In addition, upper respiratory tract catarrh in children is often accompanied by appetite disorders, chronic diarrhea, oral candidiasis, or even inhibition of weight and height gain (1–5). Therefore, it seems necessary to search for ways of improving immunity, especially in individuals with its deficiencies, i.e., children susceptible to infections, elderly people or ones with chronic diseases and children who suffered from infections.

There are not many medicines on the Polish pharmaceutical market whose usefulness in the prophylaxis of chronic respiratory infections has been documented. For over 20 years, syrup Bioaron C®, a preparation registered in Poland as a medicinal product, whose main ingredient is an extract from the leaves of candelabra aloe (*Aloe arborescens* Mill.), has been effectively used to prevent such infections. Moreover, Bioaron C® contains vitamin C and thickened juice from the fruit of black choke-

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berry (*Aronia melanocarpa* Elliot.). The ingredients of the preparation show synergism in the immunostimulant activity, contributing to an improvement in the body's resistance to infections, particularly those within the respiratory tract (2, 5–9). Furthermore, they inhibit infection development by modulating transcription of the enzymes participating in chronic inflammations (10). The preparation is intended for children in upper respiratory tract infections and lack of appetite in the course of or after prolonged illness. It is available in pharmacies in Poland, Russia and the Ukraine (4).

The main ingredient of Bioaron C® is an aqueous extract from the leaves of *Aloe arborescens* – one of about 400 species of aloe, from the family Asphodelaceae, which, being tropical plants, are endemic to southern and eastern Africa. The medicinal and cosmetic properties of aloe were already known in ancient Egypt. Nowadays, aloe is grown in greenhouses in many countries. In Poland, it is cultivated for medicinal purposes by Phytopharm Kleka SA only, where leaves of candelabra aloe are obtained from 3-year-old plants for production of medicines such as Bioaron C®. Fresh *Aloe arborescens* leaves are used to make juice or aqueous extracts, which, thanks to the content of numerous active compounds, are used to improve immunity, but also show antimicrobial activity. From the pharmacological viewpoint, the most important components of candelabra aloe are carbohydrates, both polysaccharides (up to 30% of mucilage) and monosaccharides, including glucans, mannans, glucomannans and galactourans. Another group of compounds are glycoproteins (aloectin A and B). Moreover, aloenins – glycoside derivatives of α-pirone, sterols (campesterol, ß-sitosterol), organic acids (salicylic, citric, malic and veratric acid), amino acids, enzymes, vitamins, microelements and a small concentration of aloin are present (4, 11–14). Numerous *in vitro* and *in vivo* studies have confirmed the immunostimulant, antiviral and antibacterial activity of the components found in *Aloe arborescens*. Strong immunomodulatory activity of low-mass polysaccharides (up to 400 KDa) isolated from aloe gel, which increase production of cytokines, nitrogen oxide release and expression of surface and phagocyte particles has been shown on mouse macrophages in *in vitro* studies. The same polysaccharides have also demonstrated anticancer activity *in vivo* (11). *In vitro* studies have confirmed immunomodulatory and hemagglutinating properties of some glycoproteins, which exhibit anti-inflammatory activity, too (12, 15). The antiviral activity of candelabra aloe proven in many *in vitro* studies results from the influence on replication of the human rhinovirus type 14 (5). The immunomodulatory activity, in turn, consists in normalization of T lymphocytes level and a decrease in the activity of proinflammatory cytokines. The leaf extract stimulates synthesis of the interferon responsible for the inhibition of virus replication, raises the level of antibodies in the organism and stimulates tissue regeneration processes. The results of clinical studies suggest that children over 3 years old who have taken 5 mL of an aloe extract twice a day for at least 3 weeks have suffered from upper respiratory tract infections much more seldom (1, 4). Candelabra aloe extracts also regulate digestive tract functions, protect the liver, lower blood glucose levels, accelerate wound healing, and even show anticancer activity (15–18). It has been shown, too, that *Aloe arborescens* extracts exhibit antimicrobial activity (17, 19–20).

Additionally, vitamin C present in Bioaron C® has strong antioxidant activity and contributes to regeneration of the organism. Anthocyanins from chokeberry demonstrate anti-free radical and detoxification activity. It has also been concluded that the ingredients of Bioaraon C promote appetite enhancement, which is particularly important in the case of pediatric and geriatric patients being weakened as a result of an ongoing infection. The preparation is well-tolerated by children (1, 4, 7).

The aim of this study was to evaluate the antimicrobial activity of Bioaron C® against anaerobic and aerobic bacteria as well as yeast-like fungi isolated from patients with upper respiratory tract infections.

MATERIALS AND METHODS

Material for the study

Material for the study was Bioaron C® (Phytopharm, Kleka). Concentration recommended by the producer (undiluted preparation) was used to evaluate the bactericidal (MBC) and fungicidal (MFC) activity. The sensitivity of the microorganisms tested (MIC) to Bioaron C® was determined by the serial dilution method, with the use of the following concentrations of the preparation on media appropriate for a given microorganism: 6.2; 12.5; 25.0; 50.0; 100.0 and 200.0 mg/mL.

Microorganisms

Strains of anaerobic and aerobic bacteria and yeast-like fungi cultured from material collected from patients with upper respiratory tract infections were examined. In addition, strains of reference

microorganisms from the microbiological collection ATCC (American Type Culture Collection) were used in the experiments.

Anaerobic bacteria

The materials collected from patients with upper respiratory tract infections were delivered to the laboratory in containers with transport liquid prepared by the PRAS method. Next, the samples were inoculated into media appropriate for anaerobic bacteria culture, including enriched and selective ones (21). Incubations was performed at 37°C for 14 days in anaerobic jar containing 10% of $C0_2$, 10% of H_2 and 80% of N_2 a palladium catalyst and anaerobiosis indicator. The anaerobic microorganisms cultured were identified in accordance with the present principles, on the basis of their morphological, physiological and biochemical properties, including API 20A tests (bioMerieux), their ability to produce C_1 to C_6 fatty acids and succinic, fumaric and lactic acids from glucose by the gas chromatography method, as well as the colony's UV natural fluorescence ability (22, 23). Twenty strains of anaerobic bacteria from the following genera were used in the study: *Peptostreptococcus* (2 strains), *Finegoldia* (2), *Parvimonas* (2), *Actinomyces* (1), *Bifidobacterium* (1), *Propionibacterium* (2), *Prevotella* (5), *Porphyromonas* (2), *Fusobacterium* (2) and *Bacteroides* (1) as well as 6 reference strains (Table 1).

Aerobic bacteria

The materials from patients were inoculated into enriched and selective media and incubated at 37°C for 24–48 h. Next, the cultured strains were

Table 1. The MBC of Bioaron C® against anaerobic bacteria at concentration recommended by producer and MIC of these bacteria.

Anaerobic bacteria	Number of strains	MBC of Bioaron C®		MIC (mg/mL)
		after 15 min	after 30 min	
Gram-positive cocci				
Peptostreptococcus anaerobius	2	1	2	≥ 200/100*
Finegoldia magna	2	1	2	≥ 200/100*
Parvimonas micra	2	1	2	≥ 200/100*
Gram-positive baccilli				
Actinomyces odontolyticus	1	1	1	≥ 200
Bifidobacterium breve	1	1	1	≥ 200
Propionibacterium acnes	1	0	0	≥ 200
Propionibacterium granulosum	1	0	1	≥ 200
Total Gram-positive anaerobic bacteria	10	5	9	
Gram-negative baccilli				
Prevotella intermedia	3	0	2	≥ 200
Prevotella levii	1	1	1	≥ 200
Prevotella loescheii	1	0	0	≥ 200
Porphyromonas asaccharolytica	1	0	1	≥ 200
Porohyromonas gingivalis	1	0	0	≥ 200
Fusobacterium nucleatum	1	0	0	≥ 200
Fusobacterium necrophorum	1	0	0	≥ 200
Bacteroides fragilis	1	0	0	≥ 200
Total Gram-negative baccilli	10	1	4	
TOTAL ANAEROBIC BACTERIA	20	6	13	
Reference strains				
Finegoldia magna ATCC 29328	1	1	1	≥ 200
Peptostreptococcus anaerobius ATCC 27337	1	0	0	≥ 200
Propionibacterium acnes ATCC 11827	1	0	0	≥ 200
Bifidobacterium breve ATCC 15700	1	1	1	≥ 200
Fusobacterium nucleatum ATCC 25586	1	0	0	≥ 200
Bacteroides fragilis ATCC 25585	1	0	0	≥ 200

0 = no activity of the preparation
≥ 200/100* = ≥ 200 (1 strain) and 100 (1 strain)

identified (with the use of appropriate tests, such as bioMerieux) in accordance with principles being now in force for these bacteria (22–24). The experiments were performed on 13 isolated strains of aerobic bacteria from the following genera: *Staphylococcus* (2 strains), *Enterococcus* (1), *Streptococcus* (3), *Acinetobacter* (1), *Citrobacter* (1), *Escherichia* (1), *Haemophilus* (1), *Klebsiella* (1), *Pseudomonas* (1), *Serratia* (1) and on 7 reference strains (Table 2).

Yeast-like fungi

The materials obtained from patients with upper respiratory tract infections were inoculated into Sabouraud medium and incubated at 37°C for 24–72 h. The strains of the yeast-like fungi cultured were identified on the basis of cell morphology in preparations stained by Gram's method, appearance of the colony on Sabouraud medium and CHROMagar Candida medium (BioRad), the germ tube test, the ability to produce chlamydospores and their biochemical features (20C AUX bioMerieux) (25). Seven strains of yeast-like fungi from the genus *Candida* isolated from infections and 5 reference strains were subjected to the study (Table 3).

Determination of antimicrobial activity

Bactericidal activity (MBC – minimal bactericidal concentration) of Bioaron C® was determined in relation to anaerobic and aerobic bacteria. Suspensions of bacterial strains containing 10^6 CFU (colony forming units) in 1 mL were added to 1 mL of undiluted Bioaron C®; then, after 15 and 30 min, 0.1 mL was taken and inoculated into 2 mL of appropriate broth. In the case of anaerobic bacteria strains, thioglycolate broth was used, while for aerobic bacteria strains BHI broth (Merck) was employed. A medium inoculated with 0.1 mL of the bacteria culture constituted a growth control of the tested strain. Incubation of the inoculations and con-

Table 2. The MBC of Bioaron C® against aerobic bacteria at concentration recommended by producer and MIC of these bacteria.

Anaerobic bacteria	Number of strains	MBC of Bioaron C®		MIC (mg/mL)
		after 15 min	after 30 min	
Gram-positive cocci				
Staphylococcus aureus	1	1	1	≥ 200
Staphylococcus epidermidis	1	1	1	100
Enterococcus faecalis	1	0	1	≥ 200
Streptococcus anginosus	1	0	1	100
Streptococcus pneumoniae	1	1	1	6.2
Streptococcus pyogenes	1	1	1	6.2
Total Gram-positive cocci	6	4	6	
Gram-negative baccilli				
Acinetobacter baumannii	1	0	1	≥ 200
Citrobacter freundii	1	0	1	≥ 200
Escherichia coli	1	1	1	100
Haemophilus influenzae	1	1	1	6.2
Klebsiella pneumoniae	1	1	1	≥ 200
Pseudomonas aeruginosa	1	1	1	≥ 200
Serratia marcescens	1	1	1	100
Total Gram-negative baccilli	7	5	7	
TOTAL ANAEROBIC BACTERIA	13	9	13	
Reference strains				
Staphylococcus aureus ATCC 25923	1	1	1	≥ 200
Streptococcus pneumoniae ATCC 49619	1	1	1	12.5
Streptococcus pyogenes ATCC 19615	1	1	1	6.2
Haemophilus influenzae ATCC 49274	1	1	1	12,5
Moraxella catarrhalis ATCC 25238	1	1	1	50
Klebsiella pneumoniae ATCC 13883	1	1	1	≥ 200
Pseudomonas aeruginosa ATCC 27853	1	1	1	≥ 200

0 = no activity of the preparation

Table 3. The MFC of Bioaron C® against yeast-like fungi at concentration recommended by producer and MIC of these microorganisms.

Yeast-like fungi Number	Number of strains	MFC of Bioaron C®		MIC (mg/mL)
		after 15 min	after 30 min	
Candida albicans	3	1	2	200/100*
Candida glabrata	1	0	0	≥ 200
Candida krusei	1	1	1	≥ 200
Candida parapsilosis	1	1	1	50
Candida tropicalis	1	1	1	200
TOTAL YEAST-LIKE FUNGI	7	4	5	
Reference strains				
Candida albicans ATCC 10231	1	1	1	100
Candida glabrata ATCC 66032	1	0	0	≥ 200
Candida krusei ATCC 14234	1	1	1	≥ 200
Candida parapsilosis ATCC 22019	1	1	1	100
Candida tropicalis ATCC 750	1	1	1	200

0 = no activity of the preparation; 200/100* = 200 (1 strain) and 100 (2 strains)

trol media was performed in anaerobic conditions at 37°C for 48 h (anaerobic strains) or in aerobic conditions at 37°C for 24 h (aerobic bacteria). A lack of any growth of the tested bacteria in an appropriate medium proved MBC of the preparation against the examined strain.

Fungicidal activity (MFC – minimal fungicidal concentration) was evaluated against strains of yeast-like fungi at concentration of Bioaron C® recommended by its producer. The determination of MFC was conducted in the same way as that of MBC in relation to aerobic bacteria – with the use of BHI broth. A lack of any growth of the yeast-like fungi suggested fungicidal (MFC) activity of the preparation.

Sensitivity of the examined microorganisms (MIC – minimal inhibitory concentration) to Bioaron C® was determined by the serial dilution method.

In the case of the MIC study for anaerobic bacteria, the preparation in question was diluted in Brucella agar, supplemented with 5% of defibrinated sheep blood, menadione and hemin (26). In order to test the sensitivity of aerobic bacteria, Bioaron C® was diluted in Mueller-Hinton agar (27), while the determination of MIC for yeast-like fungi was carried out on the preparation diluted in Sabouraud agar (28). Inoculum containing 10^6 CFU per drop was seeded upon the surface of the right medium. A medium without Bioaron C® was microorganisms growth control. Incubation of the media was conducted at 37°C for 48 h in anaerobic jars in anaerobic conditions (for anaerobes) or at 37°C for 24 h in aerobic conditions (for aerobes and fungi). The MIC was assumed to be such dilution of the preparation, which totally inhibited the growth of the tested strains of the investigated microorganisms.

RESULTS

The antimicrobial activity (MBC, MFC and MIC) of Bioaron C® was tested on 40 strains of microorganisms isolated from patients with upper respiratory tract infections. Twenty strains of isolated anaerobic bacteria, Gram-positive – 6 cocci and 4 rods, and 10 Gram-negative rods were used for the study. The next 13 isolated strains were aerobic bacteria (6 Gram-positive cocci and 7 Gram-negative rods). Apart from that, 7 strains of cultured yeast-like fungi from the genus *Candida* were used. Reference strains (6 anaerobes, 7 aerobes and 5 yeast-like fungi) were used as controls in each test. Tables 1–3 present the results.

Activity of Bioaron C® against anaerobic bacteria

Table 1 shows the results of the bactericidal activity (MBC) of Bioaron C® against the anaerobic bacteria isolated from patients with respiratory tract infections at the concentration recommended by the producer and against the reference strains. The preparation exhibited greater activity against Gram-positive anaerobic bacteria in comparison with Gram-negative bacteria. Fifteen min after application, half of the Gram-positive strains were killed, and 30 min after application, as many as 90% of these microorganisms were killed. In the case of Gram-negative anaerobic bacteria, the biocidal activity 15 min after application affected only 1

strain (10%), and 30 min after application – 4 strains of anaerobic bacteria (40%). All in all, 15 min after its application, Bioaron C® demonstrated biocidal activity against 6 out of 20 strains of anaerobic bacteria: *Peptostreptococcus anaerobius*, *Finegoldia magna, Parvimonas micra, Actinomyces odontolyticus, Bifidobacterium breve* and *Prevotella levii* (30%), and 30 min after application, it affected 13 out of 20 tested strains (61%).

Table 1 gives also the results of the investigation into the sensitivity (MIC) of the same strains of anaerobic microorganisms to Bioaron C®. Out of all the isolated anaerobic bacteria, only 3 (50%) of Gram-positive cocci: *Peptostreptococcus anaerobius*, *Finegoldia magna* and *Parvimonas micra* were sensitive to the preparation at concentration equal to 100.0 mg/mL. The other Gram-positive cocci, Gram-positive rods and Gram-negative anaerobic baccilli required concentration of 200.0 mg/mL or more for their growth to be inhibited.

Activity of Bioaron C® against aerobic bacteria

The results of the biocidal activity of Bioaron C® against the aerobic bacteria isolated from infections and the reference strains have been gathered in Table 2. The preparation was effective against Gram-positive aerobic cocci at the concentration recommended by its producer. It killed 4 out of 6: *Staphylococcus aureus* and *S. epidermidis*, *Streptococcus pneumoniae* and *S. pyogenes* (67%) strains as quickly as after 15 min, while after 30 min, all (100%) of the tested Gram-positive aerobic cocci were destroyed. The strains included: *Staphylococcus aureus*, *Enterococcus faecalis, Streptococcus anginosus, Streptococcus pneumoniae* and *Streptococcus pyogenes*, which often cause infections of the upper respiratory tract. Bioaron C® also showed great activity against Gram-negative aerobic rods. It exhibited biocidal activity against 5 strains (71%) already after 15 min, and against all the 7 Gram-negative aerobic baccilli tested after 30 min.

Table 2 contains also the results of the study on the sensitivity of the aerobic bacteria to Bioaron C®. Three out of the 13 tested bacteria (23%) were sensitive to very low concentration (MIC = 6.2 mg/mL), while 4 strains (31%) were sensitive to concentration equal to 100.0 mg/mL. Seven out of the 13 investigated strains (54%) turned out to be sensitive to concentration ranging from = 6.2 to 100.0 mg/mL. This means that more than half of the examined strains appeared to be sensitive to concentration of the preparation 10 to 100 times lower than the usually applied concentration. The rest of the tested aerobic bacteria required greater concentration of the preparation, i.e., = 200.0 mg/mL, for their growth to be inhibited.

Activity of Bioaron C® against yeast-like fungi

Table 3 presents the results of the fungicidal activity (MFC) of Bioaron C® against the yeast-like fungi from the genus *Candida*, isolated from patients as well as the reference strains. It was shown that after 15 min the preparation exhibited fungicidal activity against 4 out of 7 strains: *Candida albicans*, *C. krusei*, C. *parapsilosis* and *C. tropicalis* (57%), and after 30 min – against 5 of the tested fungi (71%) isolated from patients, including the genera *Candida krusei* and *Candida tropicalis*, which are highly resistant to antifungal medicines.

The results of the sensitivity (Table 3), of the same 7 (and 5 reference) strains to Bioaron C® have been gathered. Five (71%) of the strains isolated from patients were sensitive to the preparation when its concentration ranged from 50.0 to 200.0 mg/mL. The other 3 needed greater concentration (MIC > 200.0 mg/mL) for their growth to be inhibited. The concentration inhibiting growth of the fungal strains was 5 to 15 times lower than the concentration used in practice.

DISCUSSION

The results obtained from the study unambiguously suggest that, at the concentration recommended by the producer, Bioaron C® clearly showed biocidal activity against microorganisms isolated from patients with upper respiratory tract infections and analogous reference strains. The study demonstrated that the preparation was effective against half of the evaluated Gram-positive anaerobic bacteria and 10% of the Gram-negative anaerobic bacteria, as well as against 67% of the aerobic bacterial strains and 57% of the yeast-like fungi strains as quickly as after 15 min. Bioaron C® also demonstrated high MIC in relation to the tested microorganisms.

Bioaron C® is a preparation recommended not only prophylactically, but also in the treatment of upper respiratory tract infections. Numerous earlier studies of this preparation confirmed its effectiveness in such infections. Thanks to its ingredients, especially the aqueous extract from candelabra aloe, Bioaron C® is classed as a plant immunomodulator. The improvement of the body's immunity is also influenced by vitamin C and chokeberry juice present in the preparation (1, 2, 6–9, 13, 17).

The present research has confirmed the advisibility of the use of this preparation in upper respira-

tory tract infections caused by bacteria and yeast-like fungi, as well. Due to increased resistance to antibiotics and easy spread of the antibiotic-resistant bacterial strains, for example, *Staphylococcus aureus* or *Klebsiella pneumonia*, proving the activity of Bioaron C® against the strains of the above-mentioned bacteria, among others, offers a possibility to use the preparation in both viral and bacterial infections. Data from the literature on the subject confirm the antibacterial and antifungal activity of candelabra aloe (14, 18–20, 29–32).

The study showed that, out of the 40 strains isolated from patients with upper respiratory tract infections, 19 were killed 15 min after Bioaron C® application at the concentration recommended by its producer, whereas 31 were killed after half an hour. The activity (MIC) of Bioaron C® against most of the tested microorganisms was 5 to 100 times lower than the concentration used in practice. The preparation exhibited high activity against the bacteria, which cause respiratory infections most often, including *Peptostreptococcus anaerobius, Parvimonas micra, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus anginosus, Klebsiella pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa* and *Candida albicans*. Pellizzoni et al. (20) had also proven the bactericidal activity of the fractions from *Aloe arborescens* leaves against, among others, *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli*. The antibacterial activity of candelabra aloe against *E. coli* was also described by Bisi-Johnson et al. (30) and Coopoosamy and Naido (31). Studies of extracts from the leaves of several kinds of aloe, including *A. arborescens*, demonstrated activity against *E. coli*, *K. pneumoniae* and *C. albicans* (19), while Ghuman i Coopoosamy (17) confirmed high effectiveness of standardized extracts from candelabra aloe against five Gram-positive bacteria, for instance, *Staphylococcus aureus* and *S. epidermidis*, and four Gram-negative bacteria, including *E. coli* rods.

This research into Bioaron C®, similarly to reports in the literature, hereby proves that the preparation shows antimicrobial activity, probably thanks to the extract from aloe contained in it. The great antimicrobial activity allows it to be applied prophylactically as well as in the treatment of upper respiratory tract infections. Bioaron C® may be recommended especially in autumn and winter, or even be an alternative to hasty prescription of antibiotics in some cases. It may also be a valuable complement to classical therapy, particularly in pediatric medical care.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

REFERENCES

1. Alkiewicz J.: Postępy Fitoterapii 4, 18 (2000).
2. Demkow U., Skopińska-Różewska E.: Effect of Bioaron C preparation on immunity, in Role of immunomodulators of natural origin in prevention and treatment of deseases (Polish). Skopińska-Różewska E., Siwicki A.K. Eds., pp. 51-56, Medyk, Warszawa 2003.
3. Zeman K.: Alergia 17(2), 46 (2003).
4. Bastian P., Fal A.M., Jambor J., Michalak A., Noster B. et al.**:** Wien. Med. Wochenschr. 163, 73 (2013).
5. Glatthaar-Saalmüller B., Michalak A., Bastian P., Fal A.M.: Postępy Fitoterapii 3, 156 (2012).
6. Horoszkiewicz-Hassan M., Beuscher N., Lehnfeld R., Theiss U., Pfarr E.: Herba Pol. 51, 45 (2005).
7. Prokhorov E.V., Ostrowskiy I.M., Belskaya E.A., Tolstikova E.A., Hodanich N.A., Hilinskaya I.A.: Sowremiennaja piediatrija 3 (37), 81 (2011).
8. Skopińska-Różewska E., Pastewka K., Wasiutyński A., Skopinski P., Sommer E. et al.: Centr. Eur. J. Immunol. 35, 199 (2010).
9. Skopińska-Różewska E., Wasiutynski A., Skopinski P., Siwicka D., Zdanowski R. et al.: Centr. Eur. J. Immunol. 36, 139-144 (2011).
10. Cichocki M., Michalak A., Appel K.: Acta Biochim. Pol. 3, 196 (2012).
11. Im S.A., Oh S.T., Song S., Kim M.R., Kim D.S. et al.: Int. Immunopharmacol. 5, 271 (2005).
12. Kodym A.: Farm. Pol. 57 (19), 887 (2001).
13. Olennikov D.N., Ibragimov T.A., Chelombit'ko V.A., Nazarova A.V., Rokhin A.V. et al.: Chem. Nat. Comp. 45, 478 (2009).
14. Zapata P.J., Navarro D., Guillén F., Castillo S., Martínez-Romero D. et al.: Ind. Crops Prod. 42, 223 (2013).
15. Imanishi K.: Phythother. Res. 7, S20-S22 (1993).
16. Furukawa F., Nishikawa A., Chihara T., Shimpo K., Beppu H. et al.: Cancer Lett. 178, 117 (2002).
17. Ghuman S., Coopoosamy R.M.: J. Med. Plants Res. 5, 3572 (2011).
18. Jia Y., Zhao G., Jia J.: J. Ethnopharmacol. 120, 181 (2008).
19. Mbanga J., Mangoma N., Saidi B.: J. Anim. Vet. Adv. 9, 2918 (2010).

20. Pellizzoni M., Ruzickova G, KalhotkaL. Lucini L.: J. Med. Plants Res. 6, 1975 (2012).
21. Holdeman L.V., Cato E.P., Moore W.E.C.: Anaerobe Laboratory Manual, 4th edn., Virginia Polytechnic Institute and State University, Blacksburg, Virginia 1977.
22. Forbes B.A., Sahm D.F., Weissfeld A.S.: Bailey and Scott's Diagnostic Microbiology. 12th edn., Mosby Elsevier Press, St. Louis 2007.
23. Holt J.G.: Bergey's Manual of Determinative Bacteriology. 9th edn., Williams and Wilkins, Baltimore 1994.
24. National Committee for Clinical Laboratory Standards. Abbreviated Identification of Bacteria and Yeast; Approved Guideline. NCCLS document M35-A12. Wayne, Pa. 2002.
25. Krzyściak P, Skóra M, Macura AB.: Atlas of human pathogenic fungi (Polish), Med. Pharm, Wrocław 2011.
26. National Committee for Clinical Laboratory Standards. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard, 6th edn., NCCLS document M11-A6. Wayne, Pa. 2004.
27. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. 7th edn., CLSI document M7-A7: Wayne, Pa. 2006.
28. National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard. 2nd edn., NCCLS document M27-A2: Wayne, Pa. 2002.
29. Ali M.I., Shalaby N.M., Elgamal M.H., Mousa A.S.: Phytother. Res. 13, 401 (1999).
30. Bisi-Johnson M.A., Obi C.L., Hattori T., Oshima Y., Li S., Kambizi L. et al.: BMC Complement. Altern. Med. 11 (14), 1-5 (2011).
31. Coopoosamy R.M., Naidoo K.K.: J. Altern. Complement. Med. 19, 425 (2013).
32. El Fiki N.M., Shehata I.A., Ibrahim T.A., Sleem A.A., Shoukry M.A.: Planta Med. 77 (12), PL107 (2011).

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PHARMACEUTICAL TECHNOLOGY

EVALUATION OF COPROCESSED DISINTEGRANTS PRODUCED FROM TAPIOCA STARCH AND MANNITOL IN ORALLY DISINTEGRATING PARACETAMOL TABLET

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Abstract: The study evaluated two novel coprocessed excipients (with two methods) as disintegrants in an orally disintegrating paracetamol tablet formulation. The tablets produced were assessed for mechanical properties with the use of friability and tensile strength while the release properties were assessed with wetting time, water absorption ratio, disintegration time and dissolution profile. The results obtained showed that the methods of coprocessing and disintegrant incorporation influenced the activities of the disintegrants. The novel disintegrant enhanced the mechanical properties of the tablets containing them as shown by lower friability and higher tensile strength of the tablets. The result further showed that the rate and amount of water absorbed, type of disintegrant and the method of disintegrant incorporation influenced the total amount of paracetamol released. The study concluded that the novel disintegrants will be effective in the formulation of orally disintegrating paracetamol tablets.

Keywords: co-fusion, co-grinding, dissolution, tensile strength, friability, disintegration time, water absorption ratio, wetting time

Tablets are the most preferred oral dosage forms due to their numerous advantages, which includes: ease of manufacture and administration, high physical and chemical stability, high patient compliance, convenient packaging/storage and the ability to provide accurately measured dose of the drug (1–3). However, its use in therapy is still associated with some challenges most especially among the geriatric and pediatric populations. Over the past two decades, orally disintegrating tablets (ODTs), a patient friendly dosage form, which disintegrates rapidly in the mouth upon contact with saliva, have gained increased importance, focus and patient acceptance because of their ability to addresses some of the challenges facing conventional tablets (4–6). These ODTs obviate the need to swallow tablets thereby making drug usage more convenient for pediatric and geriatric patients who usually have compromised swallowing ability due to various physiological/psychological factors (7). The convenience of administration without water also enables "dosing on the go", which facilitates patient adherence to the dosing regimen or administration (8).

Various methods such as lyophilization, molding, cotton candy, direct compression or tableting after granulation have been used in the manufacture of ODTs (6). Coprocessed multifunctional excipients based on polyols, disintegrants and binders with improved physico-mechanical properties (e.g., pleasant mouth-feel, low hygroscopicity, better flow and compactability) have been used to conveniently manufacture ODTs with excellent disintegration properties without compromising the mechanical properties (8).

The objectives of this study were to coprocess native tapioca starch (an excipient with binding, disintegrating and diluent properties) (9) with mannitol (an excipient that aids dissolution; has diluent and sweetening properties; and has the ability to impart cooling and pleasant mouth feel into ODTs) (10); and to evaluate the novel excipients produced as disintegrants in an orally disintegrating paracetamol tablet formulation.

EXPERIMENTAL

Materials

The materials used were: paracetamol B.P. and corn starch B.P. (BDH Chemicals Ltd., Poole, UK), lactose B.P. (A.B. Knight and Co., London, UK),

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tapioca starch (prepared in our laboratory from tubers of *Mannihot utilisima* L.*)*, mannitol (BDH Chemicals Ltd., Poole, UK) and acetone (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany).

Extraction of tapioca starch

The tapioca starch was extracted from the root tubers of cassava (*Manihot utilisima* L.) using established procedures (11). The cassava tubers were peeled, washed and cut into small pieces, which were soaked in distilled water for 48 h for softening. The softened tubers were milled to a pulp, and distilled water was added to dilute the slurry, which was then sieved using a 100 µm mesh. The procedure was repeated three times until starch was fully extracted from the tubers as confirmed by iodine test on the remaining chaff, which was negative. The extracted starch was dried at 50°C in hot air oven (Gallenkamp, Model OV-335, Vindon Scientific Ltd., Oldham, UK) for 72 h. The dried mass was powdered in a laboratory mill (Christy and Norris Ltd., Chelmsford, UK) and stored in a screw capped bottle until needed.

Coprocessing by co-fusion

Fifty grams each of dried mannitol (MNT) and tapioca starch (TPS) were fused together by dispersing the TPS in distilled water. The dispersion was then stirred for 5 min at 50°C to form a paste. The dry MNT powder was then added to the TPS paste and mixed together for 10 min. The resulting paste (fused MNT and TPS) was dried at 50°C in a hot air oven for 24 h before it was milled and sieved using a 250 µm sieve. The coprocessed product (FTM) was stored in a screw capped bottle until needed

Coprocessing by co-grinding

Fifty grams each of mannitol (MNT) and tapioca starch (TPS) was used. The MNT and dry TPS were triturated together using a porcelain mortar and pestle for 10 min to ensure uniform size reduction and mixing of the two powders (12). The resulting product (GTM) was then sieved using a 250 µm sieve and stored in a screw capped bottle until needed.

Swelling capacity test

The swelling capacity (SC) test was carried out according to the method described by Bowen and Vadino (13).

Preparation of binder mucilage

Corn starch mucilage was prepared by weighing the amount required (Table 1). The starch powder was suspended in the required amount of distilled water in a beaker and heated with continuous stirring until mucilage was formed. The mucilage was then used while hot to facilitate an effective binding of the powder mass.

Table 1. Basic formulation table.

	Formula				
Ingredients	I	II	III	IV	V
Paracetamol	90%	90%	90%	90%	90%
Corn starch (Binder)	3%	3%	3%	3%	3%
Lactose (Diluent)	6%	5%	4%	3%	2%
Disintegrant	1%	2%	3%	4%	5%

Table 2. Physicochemical properties of excipients.

Excipients	Particle density (g/cm^{-3})	Bulk density (g/cm^{-3})	Swelling index	Moisture content (%)
MNT	1.517 ± 0.011	0.349 ± 0.007	0.00 ± 0.00	1.62 ± 0.08
TPS	1.455 ± 0.004	0.546 ± 0.008	1.04 ± 0.06	13.43 ± 0.11
FTM	1.389 ± 0.018	0.500 ± 0.012	1.57 ± 0.20	8.51 ± 0.09
GTM	1.431 ± 0.026	0.512 ± 0.018	0.34 ± 0.04	8.76 ± 0.07

Table 3. Physical properties of tablets compressed at 121.38 MNm^{-2}.

Disintegrants	Disintegrant concentration (% $^{w}/_{w}$)	Tensile strength (MNm^{-2})		Friability (%)		Disintegration time (min)	
		Intragranular	Extragranular	Intragranular	Extragranular	Intragranular	Extragranular
TPS	1	0.54 ± 0.010	0.33 ± 0.040	6.40 ± 0.020	10.09 ± 0.010	3.12 ± 0.026	3.14 ± 0.061
	2	0.56 ± 0.012	0.42 ± 0.021	5.54 ± 0.015	7.13 ± 0.041	2.24 ± 0.091	2.34 ± 0.007
	3	0.56 ± 0.031	0.52 ± 0.013	4.19 ± 0.014	3.87 ± 0.031	2.20 ± 0.014	2.20 ± 0.042
	4	0.57 ± 0.009	0.60 ± 0.031	3.15 ± 0.010	2.24 ± 0.051	1.58 ± 0.031	2.12 ± 0.050
	5	0.62 ± 0.011	0.63 ± 0.079	1.89 ± 0.012	1.06 ± 0.017	1.01 ± 0.022	1.00 ± 0.001
FTM	1	0.63 ± 0.021	0.78 ± 0.029	0.94 ± 0.019	0.68 ± 0.012	16.31 ± 0.045	6.53 ± 0.044
	2	0.64 ± 0.007	0.79 ± 0.017	1.76 ± 0.011	0.49 ± 0.030	6.56 ± 0.026	4.32 ± 0.041FTMFTM
	3	0.76 ± 0.031	0.81 ± 0.045	0.79 ± 0.031	0.38 ± 0.051	4.22 ± 0.016	3.51 ± 0.033
	4	0.76 ± 0.042	0.87 ± 0.016	0.54 ± 0.051	0.36 ± 0.020	2.45 ± 0.021	1.52 ± 0.011
	5	0.81 ± 0.008	0.89 ± 0.003	0.47 ± 0.010	0.31 ± 0.010	2.37 ± 0.015	1.34 ± 0.020
GTM	1	0.46 ± 0.014	0.74 ± 0.004	7.20 ± 0.021	0.83 ± 0.010	9.40 ± 0.018	4.36 ± 0.022
	2	0.52 ± 0.002	0.86 ± 0.013	5.18 ± 0.005	0.90 ± 0.041	4.45 ± 0.032	4.12 ± 0.021
	3	0.55 ± 0.014	0.92 ± 0.046	3.87 ± 0.010	0.65 ± 0.012	2.55 ± 0.026	2.35 ± 0.012
	4	0.59 ± 0.019	0.93 ± 0.020	1.25 ± 0.016	0.58 ± 0.001	2.23 ± 0.021	1.20 ± 0.032
	5	0.71 ± 0.007	0.94 ± 0.043	1.08 ± 0.022	0.44 ± 0.020	1.30 ± 0.013	0.59 ± 0.014

*The mean ± SD, n = 3.

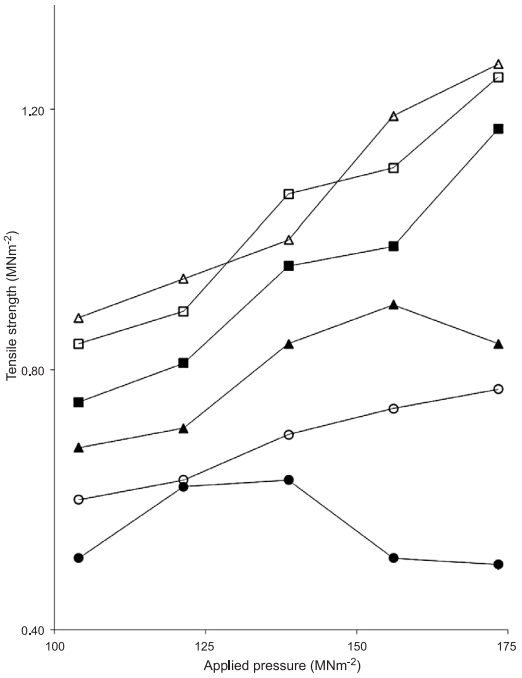

Figure 1. Plots of tensile strength against applied pressure for paracetamol formulations containing 5% w/w disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

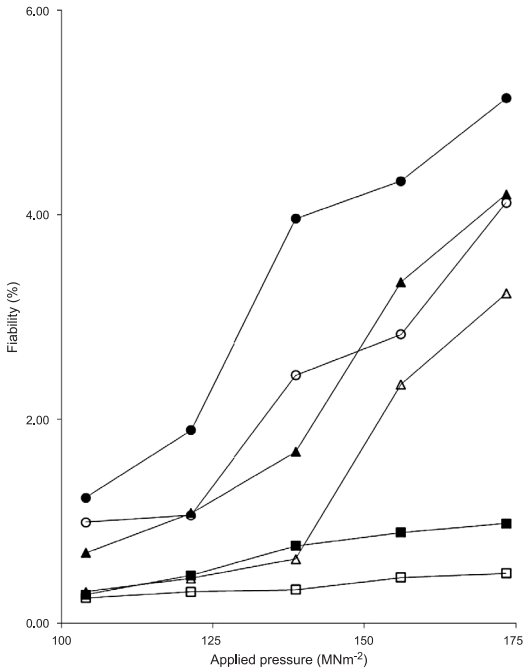

Figure 2. Plots of friability against applied pressure for paracetamol formulations containing 5% $^{w}/_{w}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

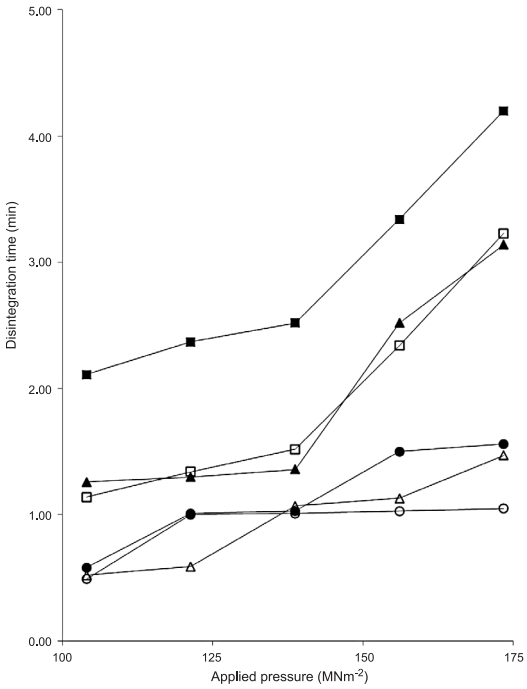

Figure 3. Plots of disintegration time againts applied pressure for paracetamol formulations containing 5% $^{w}/_{w}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

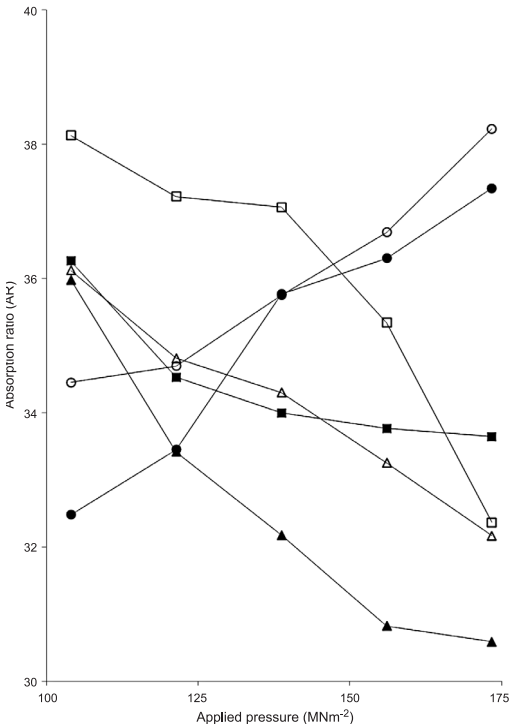

Figure 4. Plots of absorption ratio against applied pressure for paracetamol formulations containing 5% $^{w}/_{w}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

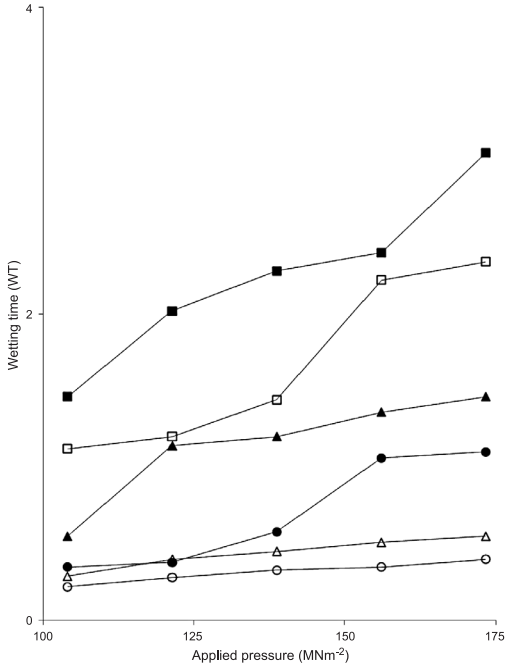

Figure 5. Plots of wetting time against applied pressure for paracetamol formulations containing 5% $^{W}/_{W}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

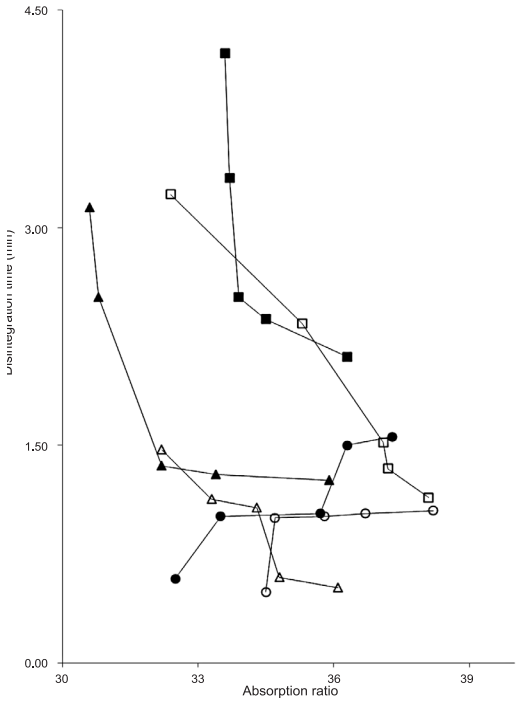

Figure 6. Plots of absorption ratio against disintegration time for paracetamol formulations containing 5% $^{W}/_{W}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

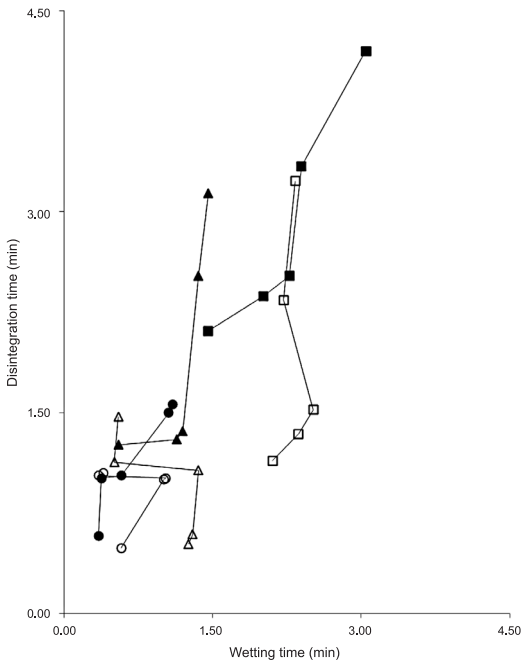

Figure 7. Plots of wetting time against disintegration time for paracetamol formulations containing 5% w/w disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

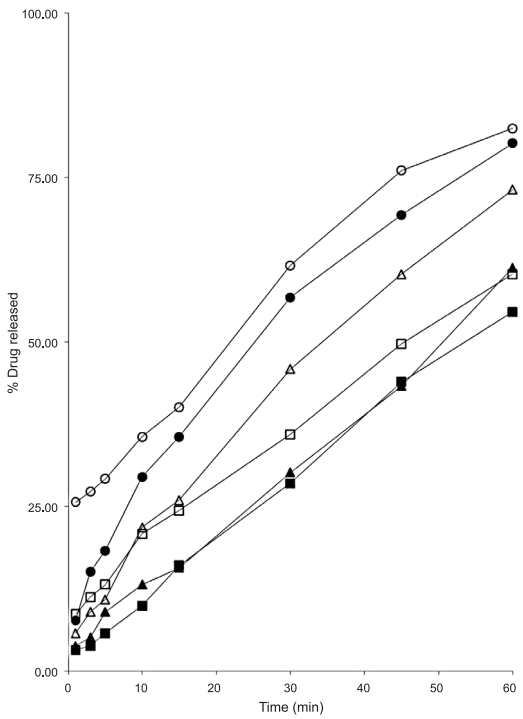

Figure 8. Plots of % drug released against time for paracetamol formulations containing 3% $^{W}/_{W}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

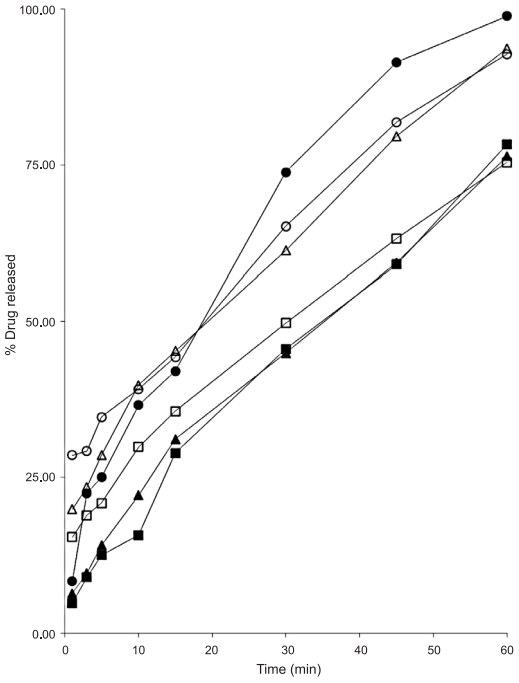

Figure 9. Plots of % drug released against time for paracetamol formulations containing 5% $^{w}/_{w}$ disintegrant. TPS (●), FTM (■), GTM (▲) for intragranular disintegrant and TPS (○), FTM (□), GTM (△) for extragranular disintegrant

Preparation of granules

Three hundred grams batches of paracetamol granules containing different concentrations of the disintegrants added as intragranular or extragranular disintegrants were prepared by the wet granulation method of massing and screening. The granules containing intragranular disintegrant were prepared by dry mixing the required quantities of paracetamol, lactose and the disintegrant for each batch for 5 min in a Hobart planetary mixer (Hobart Canada Inc., Don Mill, ON, Canada) and then moistening them with the corn starch binder mucilage. Wet massing continued for 5 min before the resulting wet masses were granulated by passing them through a sieve size 1400 µm, dried at 60°C for 2 h in a hot air oven. The dried granules were then re-sieved through a sieve size 1000 µm after which they were stored in airtight containers (14).

Granules containing extragranular disintegrants were prepared by dry mixing the required quantities of paracetamol and lactose for each batch for 5 min in a Hobart planetary mixer. The blend was then moistened with the corn starch binder mucilage. Wet massing was carried out for 5 min, after which the wet masses were granulated by passing them through a sieve size 1400 µm, dried at 60°C for 2 h in a hot air oven and then re-sieved through a sieve size 1000 µm. The required amount of disintegrant was added and adequately mixed with the granules before storage in an airtight container (14).

Preparation of tablets

Quantities (555 mg) of granules from each batch were compressed for 30 s into tablets with predetermined loads (104.04, 121.38, 138.72, 156.06 and 173. 40 MNm^{-2}) on a Carver hydraulic hand press (Carver, USA) using a 12 mm die and flat-faced punches lubricated with a 2% w/v dispersion of magnesium stearate and talc (1 : 1) in acetone before each compression. After ejection, the tablets were stored over silica gel for 24 h to allow for elastic recovery and hardening and to prevent falsely low yield values during analysis.

Determination of tablet crushing strength and friability

The tablet crushing strength was determined using the Erweka digital hardness tester (G.B. CALEVA, Dorset, England). The percent friability (FR) of the tablets was determined using the Roche friabilator (Erweka Apparatebau, Germany) operated at 25 rpm for 4 min.

Measurement of water absorption ratio and wetting time

The water absorption ratio was determined according to the method described by Battu et al. (15), while the wetting time (WT) was determined according to the method described by Bi et al. (16). All determinations were done in triplicate and the mean values were taken.

Disintegration test

Tablet disintegration time (DT) was determined in distilled water at 37 ± 0.5°C in a B.P. Manesty (Manesty Machines Ltd., Liverpool, UK) disintegration test unit. Six tablets at each compressional force were placed on the wire mesh just above the surface of the distilled water in the tube. The time taken for each tablet to disintegrate and all the granules to go through the wire mesh was recorded. Results were expressed as an average of three determinations.

Dissolution test

The dissolution rate of the relevant tablets was determined at 37 ± 0.5°C in 1 L of distilled water

using a Veego dissolution testing station (Veego Instruments Co., Mumbai, India) and a stirring speed of 100 rpm according to USP XXIII. The tablet was placed in the rotating basket and 5 mL of the medium was sampled with a pipette and filtered. The same quantity of the medium was added at the same temperature immediately after each sampling to keep the volume of the dissolution medium constant. The concentration of dissolved paracetamol in the medium was determined spectrophotometrically at 249 nm with a Unicam 8620 UV/Vis spectrophotometer (Pye Unicam, UK). All determinations were made in triplicate and the results are given as the mean values.

RESULTS AND DISCUSSION

Table 3 and Figures 1–3 show the mechanical and disintegration properties of formulations containing the native and novel disintegrants. A concentration and applied pressure dependent behavior was observed for all the parameters. An increase in disintegrant concentration in the formulations resulted in tablets with higher tensile strength, suggesting a positive disintegrant influence on the mechanical strength of the tablets. An increase in the tensile strength was also observed when the applied pressure was increased except for formulations containing intragranular TPS and GTM, where a decrease in tensile strength was observed after an initial period of increase. This decrease could be due to overcompaction at high compressional forces (17). A rank order of FTM > GTM > TPS and GTM > FTM > TPS were obtained for tensile strength for formulations containing intragranular and extragranular disintegrants, respectively. This suggests that the method of disintegrant incorporation into the formulation influenced the disintegrant's effect on mechanical strength of the tablets. The friability of the tablets was also observed to reduce with an increase in disintegrant (Table 3) with a rank order of FTM < GTM < TPS with formulations containing intragranular disintegrants being more friable that those with extragranular disintegrants. However, an increase in friability was observed with an increase in the applied pressure (Fig. 2) due to excessive expansion of the tablets after ejection from the die.
The disintegration time decreased as the concentration of the disintegrant in the formulation increased while it increased with increasing compressional force. The ability of the disintegrants to facilitate a disintegration time less than 1 min was observed

Table 4. Absorption ratio and wetting time values of tablets compressed at 121.38 MNm^{-2}.

Disintegrants	Disintegrant concentration (% w/w)	Absorption ratio (R)		Wetting time (min)	
		Intragranular	Extragranular	Intragranular	Extragranular
TPS	1	27.03 ± 0.132*	28.45 ± 0.310	1.52 ± 0.002	1.23 ± 0.001
	2	28.38 ± 0.072	31.42 ± 0.052	1.22 ± 0.006	1.08 ± 0.004
	3	31.85 ± 0.092	33.25 ± 0.112	1.08 ± 0.003	1.02 ± 0.002
	4	33.21 ± 0.102	34.19 ± 0.014	0.48 ± 0.002	0.39 ± 0.002
	5	33.45 ± 0.212	34.70 ± 0.116	0.38 ± 0.004	0.28 ± 0.003
FTM	1	22.68 ± 0.124	27.27 ± 0.011	4.12 ± 0.003	2.52 ± 0.004
	2	24.15 ± 0.047	29.20 ± 0.002	2.46 ± 0.005	2.30 ± 0.002
	3	32.16 ± 0.099	32.65 ± 0.010	2.34 ± 0.001	1.57 ± 0.003
	4	33.01 ± 0.082	34.75 ± 0.102	2.34 ± 0.001	1.44 ± 0.001
	5	34.53 ± 0.216	37.22 ± 0.073	2.02 ± 0.002	1.20 ± 0.003
GTM	1	25.33 ± 0.108	28.26 ± 0.427	2.58 ± 0.002	2.27 ± 0.001
	2	27.30 ± 0.106	28.99 ± 0.052	2.31 ± 0.001	1.53 ± 0.010
	3	29.50 ± 0.065	29.94 ± 0.061	1.47 ± 0.004	1.23 ± 0.007
	4	32.14 ± 0.081	34.28 ± 0.072	1.29 ± 0.003	0.57 ± 0.004
	5	33.42 ± 0.018	34.81 ± 0.013	1.14 ± 0.005	0.40 ± 0.002

*The mean ± SD, n = 3.

(Fig. 3) in formulations containing 5% w/w intragranular and extragranular TPS and extragranular GTM at applied compression force of = 121.38 MNm^{-2}. This suggests that the pressure applied in compressing the tablets would affect the activity of a disintegrant in an orally disintegrating tablet formulation. It is important to note that while the US FDA's (18) requirement for ODT is a disintegration time less than 30 s, scientific literature generally categorizes tablets with disintegration times of less than 1 min as ODT (10, 19–21). Furthermore, disintegration time less than 3 min in the mouth as observed in all formulations containing 5% w/w disintegrants except FTM at applied pressure = 156.06 MNm^{-2} and intragranular GTM at applied pressure = 173.40 MNm^{-2}, meets the requirements of the European Pharmacopoeia (22) for fast disintegrating tablets, which requires that fast disintegrating tablets should disintegrate in the mouth in less than three minutes. It was also observed that formulations containing extragranular disintegrants produced tablets with lower disintegration times (Table 3) when compared with their intragranular counterparts. This could be as a result of faster access to the disintegrating fluid, which would lead to a quicker wetting time and a subsequent faster initiation of the disintegration process in these formulations.

The absorption ratio (AR) and wetting time (WT) of the tablets containing the test disintegrants are shown in Table 4 and Figures 4 and 5. It was observed that the wetting time and absorption ratio were dependent on the type and concentration of the disintegrant used and the applied pressure. Generally, AR values increased with an increase in disintegrant concentration for native and novel disintegrants with formulations containing extragranular disintegrants absorbing more water that those containing intragranular disintegrants. This could be due the general ability of the disintegrant to facilitate water uptake into the tablet. The lower AR values of intragranular disintegrant could also be due to the presence of the disintegrant in the inner matrix of the tablets. A reduction in AR values with an increase in applied pressure (Fig. 4) was observed for formulations containing the novel disintegrants FTM and GTM, while the AR values of formulations containing TPS increased with applied pressure. The presence of water soluble mannitol in the novel disintegrants might have resulted in a reduced water requirement to overcome the stronger bonds formed at higher pressures. The rank order for water absorption is FTM > TPS > GTM. The tablet wetting time increased with increasing applied compression pressure. This could be due to a reduction in tablet porosity as the applied pressure increased. Formulations containing extragranular disintegrants had lower wetting times compared to their intragranular counterparts; probably due to a faster access of the disintegrant to the disintegrating fluid. A reduction in wetting time was also observed with an increase in disintegrant concentration with extragranular disintegrants facilitating faster wetting time. This could be due to the general ability of the disintegrant to facilitate a higher water absorption and the easy access of extragranular disintegrant to the disintegrating fluid.

Representative plots of disintegration time (at 5% w/w disintegrant concentration) against absorption ratio and wetting time are shown in Figures 6 and 7, respectively. It was observed that disintegration time reduced with increased water absorption for formulations containing FTM and GTM disintegrants while it increased in formulations containing TPS disintegrants. This suggests a different mechanism of disintegration in coprocessed disintegrants whereby the water soluble mannitol allows for a faster water absorption and subsequent swelling of the tapioca starch component. Thus, ODTs containing GTM and FTM disintegrants would have a reduced disintegrating fluid requirement and the ability to optimize the low volume of saliva (normal flow of about 0.1 to 0.2 mL/min, reaching 7 mL/min on stimulation) present in the oral cavity to facilitate their oral disintegration (22). On the other hand, formulations containing TPS, whose mechanism of disintegration was only by water uptake and swelling, will require a higher amount of water to facilitate disintegration since the dissolution effect of mannitol will be absent. It was also observed that an increase in wetting time increased the disintegration time for all formulations containing intragranular disintegrants while it had no influence on formulations containing extragranular disintegrant at applied compressional pressure greater than 138.72 MNm^{-2}.

Figures 8 and 9 show the dissolution behavior of the formulations (3 and 5% w/w, respectively) as percent drug release against time for 60 min. It was observed that tablets containing extra-granular disintegrants produced an initial burst effect, which resulted in the availability of a higher amount of the drug in the dissolution medium within 5 min. This could be as a result of the faster disintegration time of the tablets prepared with extra-granular disintegrants and the reduced access to water expected with intra-granular disintegrants located within the matrix of the tablets, which would result in reduced disintegration and drug release. It was also observed at disintegrant concentration of 5% w/w that the extra-granular addition of TPS and FTM disintegrants facilitated a faster

availability of the drug, while the total amount of drug released after 60 min was higher in tablets containing intragranular disintegrants. This could be due to the continued ability of the disintegrant present within the inner matrix of the tablet prepared with intragranular disintegrant to facilitate drug release from granules after the initial disintegration of the tablets into granules. The fact that this effect occurred at higher concentration of disintegrants with higher swelling index (i.e., FTM > TPS > GTM as presented in Table 2), implies the strong influence of the swelling ability of the disintegrant on the drug release pattern. It also suggests that there could be a change in the mechanism of drug release at high concentrations, when different methods of disintegrant inclusion into formulations are employed.

The type of disintegrants (i.e., co-ground, co-fused or native) also affected the dissolution profiles of the formulations. It was observed that tablets prepared with TPS facilitated the release of the highest amount of the drug from the tablets, followed by GTM and FTM, respectively. This ranking further supports that of the disintegration time obtained and suggests the strong influence of a fast disintegration time on drug release from the tablet matrix.

CONCLUSION

From the results obtained from the study, it can be concluded that:

The method employed in coprocessing excipients would affect the properties of the excipients and the effect they have on the formulations in which they are used.

The coprocessed disintegrants consisting of native tapioca starch and mannitol, when used in the formulation tablets would enhance the mechanical properties of the resulting tablets.

The rate/amount of water ingress into the tablets and disintegration time would be limiting factors in early onset of drug dissolution and bioavailability.

Coprocessed disintegrants consisting of native tapioca starch and mannitol would be effective disintegrants in the formulation of orally disintegrating tablets with disintegration times of less than 1 min without compromising the mechanical properties of the tablets.

REFERENCES

1. Rubinstein M.H.: in Pharmaceutics: the science of dosage form design. Aulton, M.E. Ed. ELBS 21st edn., p. 304, Longman group Ltd., UK 1988.
2. Patel S., Kaushal A.M., Bansal AK.: AAPS PharmSciTech. 8 (4), E110 (2007).
3. Saha S., Shahiwala A.F.: Expert Opin. Drug Deliv. 6, 197 (2009).
4. Pfister W., Gosh T.: Pharm. Technol. 29, 138 (2005).
5. Al-khattawi A., Mohammed A.R.: Expert Opin. Drug Deliv. 10, 651(2013).
6. Brniak W, Jachowicz R, Krupa A, Skorka T, Niwinski K.: Pharm. Dev. Technol. 18, 467 (2013).
7. Varia I., Venkataraman S., Hellegers C.: Psychopharmacol. Bull. 40, 47 (2007).
8. Soh J.L.P., Grachet M., Whitlock M., Lukas T.: Pharm. Dev. Technol.., 18, 172 (2013)
9. Alebiowu G., Osinoiki K.A.: Farmacia 58, 341 (2010).
10. Samprasit W., Opanasopit P., Akkaramongkolporn P., Ngawhirunpat T., Wongsermsin K., Panomsuk S.: Pharm. Dev. Technol. 17, 315 (2012).
11. Alebiowu G.: J. Pharm. Res. 6, 139 (2007).
12. Sharma V., Philip A.K., Pathak K.: AAPS PharmSciTech. 9, 87 (2008).
13. Bowen F.E., Vadino W.A.: Drug Dev. Ind. Pharm. 10, 505 (1984)
14. Alebiowu G., Itiola O.A.: Acta Pharm. 53, 1 (2003).
15. Battu S.K., Repka M.A., Majumda S., Madhusudan R.Y.: Drug Dev. Ind. Pharm. 33, 1225 (2007).
16. Bi H., Sunada H., Yonezawa Y., Danjo K., Otsuka A., Iida K.: Chem. Pharm. Bull. 44, 2121 (1996)
17. Ilic I., Kása Jr P., Dreu R., Pintye-Hódi K., Srcic S.: Drug Dev. Ind. Pharm. 35, 1271 (2009).
18. Guidance for Industry. Orally Disintegrating Tablets. US FDA 2008.
19. Habib W., Khankari R., Hontz J.: Crit. Rev. Ther. Drug Carrier Syst. 17, 61 (2000).
20. Shimizu T., Sugaya M., Nakano Y., Izutsu D., Mizukami Y., Okochi K., Tabata T. et al.: Chem. Pharm. Bull. 51, 1121 (2003).
21. Hirani J.J., Rathod D.A., Vadalia K.R.: Trop. J. Pharm. Res. 8, 161 (2009).
22. European Pharmacopoeia, 6th edn., Council of Europe, Strasbourg 2007.
23. Smart J.D.: Crit. Rev. Ther. Drug Carrier Syst. 21, 319 (2004).

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BIOPHARMACEUTICAL EVALUATION OF NEW SLOW RELEASE TABLETS OBTAINED BY HOT TABLETING OF COATED PELLETS WITH TRAMADOL HYDROCHLORIDE

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Abstract: This study was aimed at a biopharmaceutical evaluation of a new oral dosage form of tramadol hydrochloride (TH) – slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP), compared to the conventional slow release tablets, Tramal Retard®, 100 mg (TR). Both TP and TR formulations showed a similar release profile of TH (f_2 was 71) in *in vitro* release studies. The *in vivo* study was a two-treatment, two-period, two-sequence, single-oral dose 100 mg, crossover design using rabbit model with the phases separated by a washout period of 14 days. It was shown that the amount of TH absorbed into the systemic circulation is similar for TP and TR (the 90% confidence intervals for the $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$ and ratios were 85–122 and 92–107%, respectively). However, after administration of slow release tablets obtained by hot tableting of coated pellets, a prolonged absorption and elimination processes and a smoother and more extended plasma profile of TH were observed. It can be assumed that the use of a new oral dosage form of TH in patients affects the extension of analgesia after single administration of the drug, with its gradual absorption into the systemic circulation.

Keywords: tramadol, hot tableting, pellets, rabbits

Tramadol hydrochloride (TH) is well established in the treatment of acute and chronic pain. As it is effective and well-tolerated, the use of TH has increased substantially in a wide range of pain conditions (1, 2). Its usual dosage regimen is 50–100 mg every 4–6 h up to 400 mg, the mean absolute bioavailability is about 70% and the elimination half-life is 5.5 h (3–6).

Successful long-term treatment of patients with painful conditions requires an appropriate dosage form, optimal dosing, and patient compliance. Sustained-release formulations (SR) are very helpful in achieving treatment objectives. Stable serum levels without marked peak-to-trough fluctuations and reduced frequency of dosing improve patient compliance, patient satisfaction, and, ultimately, quality of life (7, 8). SR formulations are recommended in chronic pain treatment, especially in patients who require around-the-clock treatment of pain for an extended period of time (9–11). Many oral SR formulations of TH, including pellets, have been shown to improve patient compliance and chronic pain control (1, 10–20).

In our previous work, a newly developed method of hot tableting of pellets with TH was presented (21). Pellets were coated with an Aquacoat ECD aqueous dispersion. In the proposed hot tableting method, granulates containing PEG 3000 provide the tableted pellets sufficient protection from being destroyed. An evidence of such protection is confirmed by the fact that TH's slow release profile from the tableted pellets is comparable to that of uncompressed pellets (21). It is known that differences between oral formulations might represent the most important factor responsible for the differences in both rate and extent of absorption of the drug, reflected in the pharmacokinetic parameters (7, 22). Thus, our study was aimed at a biopharmaceutical

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evaluation of those newly developed tableted pellets with TH.

MATERIALS AND METHODS

Preparation of coating pellets with TH

Pellet cores were obtained by wet granulation of a powder mixture, and the extruded mass was spheronized afterwards. The initial experiments allowed us to establish the following composition of pellet cores: TH 60.0%, microcrystalline cellulose (MCC), PH101 35.0% and glyceryl behenate 5.0%. All substances were granulated with water (30 g/100 g of the powder mixture) in a high-shear mixer (Glatt VG 5, Dresden, Germany). The wet granulate was extruded in an extruder (Caleva model 25, Dorset, UK) through a 1.0 mm diameter sieve. The extrudate (about 30 g) was spheronized for 5 min in a 120 mm spheronizer (Caleva model 120, Dorset, UK) fitted with a cross-hatch plate rotating at 1800 rpm. The resulting pellet cores were dried at 45°C in a tray oven for 16 h. Pellet cores of 0.6–1.0 mm in diameter comprised the largest fraction (about 65%) in the given conditions of extrusion and spheronization.

Pellets cores were coated in a fluidized bed coater using the Würster bottom column with ethanol solution of ethylcellulose (EC/EtOH). The ethanol solution of ethylcellulose was plasticized with triethyl citrate 25%, w/w, based on the mass of the polymer. The coating conditions were as follows: inlet air temperature (45°C), outlet air temperature (35°C), atomization air pressure 2.0 bar), fluidizing air flow (180 m^3/h) and coating rate 10–13 g/min. The film coating resulted 27 µm thickness was sprayed onto drug-loaded pellets to achieve drug release over 8 h.

Hot TH pellets tableting

Tablet formulation was composed of a mixture of TH pellets (0.6–1.0 mm) in the ratio of 50.0% pellets and 50.0% granulate, which was obtained during wet granulation process conducted in a high-shear mixer using water as a binder (30 g/100 g of powder): PEG 3000 (24.3%) and microcrystalline cellulose (24.3%). Granulate was separately dried in a blow dryer (Venticell BMT, Brno, Czech Republic) at 45°C for 16 h. The dried granulates were then passed through a sieve with a mesh size of 1.0 mm. Then the pellets (100 mg pellets containing 45.8 mg TH), croscarmellose sodium (2.4%) and sodium stearyl fumarate (0.4 %) were added, and the mixture was mixed for 5 min. Croscarmellose sodium was added as a disintegrating substance to ensure disintegration of tablets into pellets. Sodium stearyl fumarate was used as a lubricant to prevent hot granulate from sticking to the punches.

A ratio of 50.0% pellets and 50.0% granulate ensured that the tablet formulation has adequate flow, which could otherwise be adversely affected by tackiness of heated tablet formulation.

The resulting tablet formulation was spread evenly on a paper tray and heated in a blow dryer to a temperature not greater than 56°C. A tablet press granulate feeder was heated in another blow dryer. The feeder was then immediately mounted onto a rotary tablet press (Korsch XL 100, Berlin, Germany) filled with the heated tablet formulation. The tableting parameters were as follows: spherical punches of 10.0 mm in diameter, curve radius – 9 mm, main compression force – 1.0 kN, precompression force – 0.1 kN, single tablet mass – 370.0 mg.

In vitro release studies

Dissolution test was performed in 1000 mL of water at 37°C (± 0.5°C). An automated Hansson Research Sr8+ basket apparatus dissolution tester (Hansson Research, Chatsworth, CA, USA) with an on-line UV/Vis spectrophotometer (Agilent 8453, Wilmington, USA) was used. At different time intervals (1, 2, 3, 4, 5, 6, 7 and 8 h) the concentration of TH in the samples was analyzed spectrophotometrically at 272 nm. Single tablet mass was 370.0 mg and contained 98.7 mg TH. The dissolution results were calculated with reference to SR tablets (Tramal Retard®, 100 mg) batch no AN043 (Grünenthal GmbH, Aachen, Germany). All dissolution profiles are the mean of 12 dissolution tests performed under sink conditions.

Similarity of dissolution profile of the tablets was compared using model-independent method by linear regression at specified time points, and calculating a similarity factor f_2. An f_2 value between 50 and 100 suggests that two dissolution profiles are similar.

$$f_2 = 50 \times \log\{[1 + (1/n)\sum_{t=1}^{n}(R_t - T_t)^2]^{-0.5} \times 100$$

In this equation, f_2 is the similarity factor, n is the number of time points, R_t is the mean percent drug dissolved of e.g., a reference product, and T_t is the mean percent drug dissolved of e.g., a test product (23).

In vivo studies

Ten adult healthy New Zealand White rabbits (mean weight ± SD, 3.45 ± 0.20 kg) were used in this study. Before the study, animals were housed individually in standard cages. Food was withheld from all animals for 12 h prior to and 12 h following

drug administration. Throughout the study, the rabbits had an unlimited access to water. The studies were carried out in accordance with the consent of the Local Ethics Committee at the University of Life Sciences in Poznan (No. 15/2008) and the "Guide for the Care and Use of Laboratory animals" (24).

A two-treatment, two-period, two-sequence, single-oral dose, randomized, crossover design was used with the phases separated by a washout period of 14 days.

SR formulations (a slow release tablets obtained by hot tableting of coated pellets, 100 mg) prepared in the Department of Pharmaceutical Technology, Medical University of Gdańsk, Gdańsk, Poland, and SR tablets (Tramal Retard® 100 mg tablets, batch no 292L01; Grünenthal, Aachen, Germany), were used for oral administration.

All animals received *per os* (*p.o.*) one tablet (100 mg of TH) of each formulation. Immediately after administration of TH, 20 mL of fresh water were given to the rabbits to ensure that the tablet was swallowed and entered the stomach.

Two weeks after oral administration, all animals were given TH intravenously (*i.v.*) at a dose 10 mg/kg (Poltram 100 mg/2 mL, batch No. 510804; Polpharma, Poland) with a view to calculating absolute bioavailability (F) and mean absorption time (MAT) of TH.

TH was always administered between 8 a.m. and 9 a.m. All blood samples (1.5 mL) were drawn from the catheter remaining in the marginal ear vein.

Blood samples were collected before administration of TH (sample 0) and then at 5, 15, 30, 45, 60, 120 min, and 4, 8, 24, 30 h after *p.o.* administration, or 1, 5, 10, 15, 30, 45, 60, 120 min, and 4, 6, 8 h following *i.v.* administration. Blood samples were transferred into labelled, heparinized test tubes and immediately centrifuged at 2880 × g for 10 min. Plasma samples were stored at –30°C until analysis.

Drug analysis

Chemicals and reagents

Tramadol hydrochloride, $C_{16}H_{25}O_2N{\cdot}HCl$, CAS: 27203-92-5; internal standard – phenacetin, CAS: 62-44-2 and triethylamine (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile, n-hexane, methanol, ethyl acetate (HPLC grade) were from Merck (Darmstadt, Germany). Sodium hydroxide, monopotassium phosphate, anhydrous potassium hydrogen phosphate (analytical grade) were from POCH (Gliwice, Poland).

Chromatographic system

Plasma concentrations of TH were analyzed by high-performance liquid chromatography with diode-array UV detection (isocratic analysis using HPLC Waters 2695 Separations Module with autosampler, Waters 2487 Dual λ Absorbance Detector, the wavelength 218 nm, an analytical column LiChrosorb RP-18, 250 mm × 4.6 mm, 5 µm from Waters; temperature of column 30°C, mobile phase: acetonitrile (300 mL) – 0.01 M phosphate buffer (700 mL) with addition of 0.05% triethylamine (0.5 mL) to achieve pH of mobile phase 3.0, flow rate of mobile phase 1.0 mL/min, volume of each injection 100 µL, retention time of TH and phenacetin, respectively, 5.64 and 8.19 min, total analysis time 12.0 min, according to methods developed by Gan et al. (25) and Szkutnik-Fiedler et al. (26). Data collection and processing were carried out using Empower Pro software, v. 1154. HPLC method was validated in accordance with the published EMA guideline (27).

Method validation

The HPLC-UV method was specific and selective. There were no interfering peaks in blank plasma at the retention times of TH and internal standard. The calibration for TH was linear in the range of 10–1000 ng/mL (n = 9, r = 0.9996 ± 0.0002, RSD 0.02%).

The recovery of TH was greater than 90% for all tested concentrations. Intra-day and inter-day precision of TH in rabbit plasma were less than 7%, and accuracy was less than 10%. The LLOQ of TH was 10 ng/mL, and LOD was 2.11 ng/mL. CV values of short-term stability of plasma samples were 4.39 and 6.64% for TH concentrations of 50 and 200 ng/mL, respectively. The recovery of TH in long-term freezer stability after storage at –30°C for 30 days was more than 88% for both tested concentrations.

Pharmacokinetic and statistical analysis

The pharmacokinetics of TH after oral and intravenous administration was determined by use of the noncompartmental approach based on the statistical moment theory. All parameters were calculated using WinNonlin® 5.3 Professional (Pharsight). The maximum drug plasma concentration (C_{max}) and the time at which C_{max} was achieved (t_{max}) after *p.o.* administration were determined directly from the concentration *vs.* time curve. The elimination rate constant (k_{el}) was calculated by a linear least squares regression analysis, using the last three plasma ln concentrations *vs.* time points.

Elimination half-life ($t_{1/2kel}$) was calculated according to equation $t_{1/2kel} = \ln 2/k_{el}$. The mean residence time (MRT) was equal to $AUMC_{0-\infty}$ /$AUC_{0-\infty}$. Area under the plasma curve from zero to the last measurable concentration (AUC_{0-t}) was calculated using the linear trapezoidal method. Area under the plasma curve from zero to infinity ($AUC_{0-\infty}$) and area under the first moment curve from zero to infinity ($AUMC_{0-\infty}$) were calculated by the linear trapezoidal method with extrapolation to infinity. The extrapo-

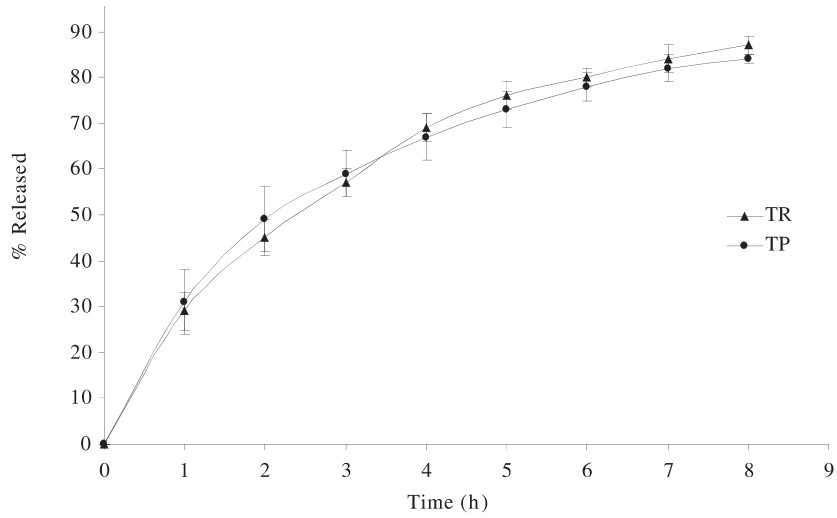

Figure 1. *In vitro* tramadol hydrochloride release (mean ± RSD) from slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP) compared to Tramal Retard® tablets, 100 mg (TR), n = 12

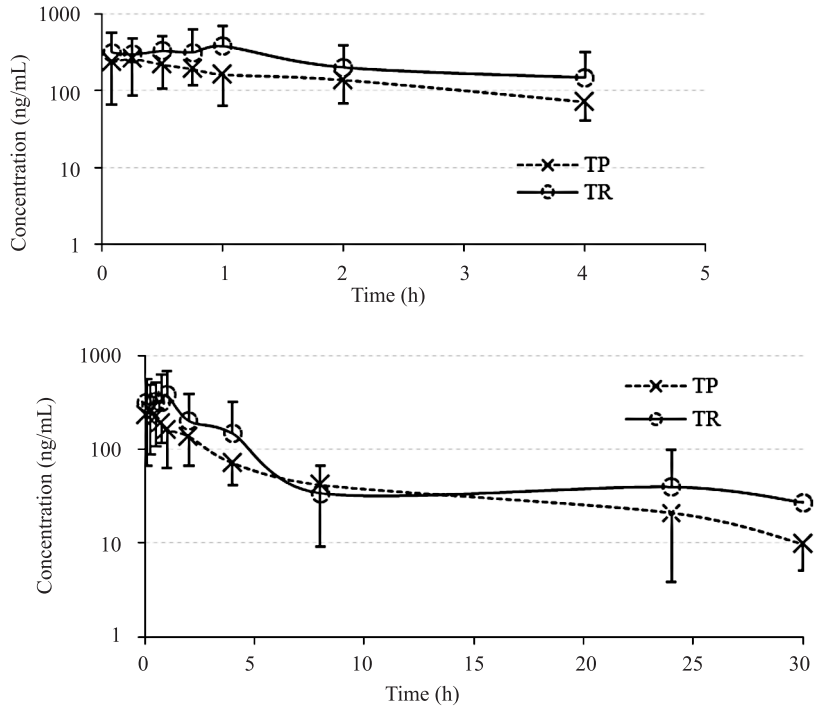

Figure 2. Plasma concentration-time profiles (mean ± SD) of tramadol hydrochloride after oral administration of slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP) and Tramal Retard® tablets, 100 mg (TR) in rabbits (n = 10). One hour after administration of tramadol hydrochloride a statistically significant difference was observed (p = 0.0276)

lated area was estimated by equations: $AUC_{last-\infty} = C_{last} / k_{el}$, $AUMC_{last-\infty} = (t_{last} \times C_{last} / {}_{kel} + C_{last} / k_{el}^2$, in which C_{last} is the last measured concentration and t is the time of C_{last}. $AUMC_{0-\infty}$ and $AUC_{0-\infty}$ values were dose normalized. The absolute bioavailability $F(\%)$ of TH was calculated using equation:

$$F(\%) = [AUC_{0-\infty p.o.} \times D_{i.v.} / AUC_{0-\infty i.v.} \times D_{p.o.}] \times 100$$

The relative bioavailability (RB (%) = $AUC_{0-\infty}$TP/$AUC_{0-\infty}$TR), mean absorption time ($MAT = MRT_{p.o.} - MRT_{i.v.}$), absorption rate constant ($k_a = 1/MAT$) and absorption half-life ($t_{1/2ka} = \ln 2 \times MAT$) were also determined.

Coefficients of variation (CV), defined as the ratio of the SD to the mean ($CV = (SD/mean) \times 100\%$) for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ and TH plasma concentrations at all evaluated time points after administration of TP and TR, were also calculated.

All statistical analyses were performed using Statistica PL 10 software (StatSoft, Inc.).

Shapiro-Wilk test was used to check if analyzed data follow normal distribution. Paired *t*-test or Wilcoxon test (in case data did not follow normal distribution, i.e., for plasma concentrations at time points: 30, 45, 60 min and 24 h and t_{max}) were used to compare plasma concentrations and pharmacokinetic parameters of TH. The arithmetic mean, standard deviation, median, range (minimum and maximum value) and geometric mean for pharmacokinetic parameters of TH are presented. The TP and TR bioequivalence was evaluated by means of statistical analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the geometric mean ratios (TP/TR) with logarithm (ln)-transformed AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}. The bioequivalence was considered when the ratio of averages of log-transformed data for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} was within 80–125%. All tests were considered statistically significant at p value less than 0.05.

RESULTS

In vitro release studies

Dissolution profiles of TP and TR proved to be similar (similarity factor f_2 for the TH release profiles was 71) (Fig. 1).

In vivo studies

Mean plasma concentration-time profiles of TH are shown in Figure 2. Statistical evaluation of plasma concentration-time profiles of TH after oral administration of TP and TR showed no significant differences between these two formulations at all evaluated times, except after 1 h (170.6 ± 101.5 ng/mL *vs.* 423.1 ± 315.4 ng/mL for TP and TR, respectively, p = 0.0276). CV values for TH plasma concentrations at all time points ranged from 43.0 to 72.2% and from 59.1 to 92.6% (after administration of TP and TR, respectively). CV values for TH plasma concentrations in the first two hours (time points: 5, 15, 30, 45, 60 and 120 min) were generally lower after TP administration (72.2, 61.5, 53.6, 43.0, 59.5 and 55.7%) compared with TR (66.1, 91.4, 59.1, 99.7, 74.5 and 99.0%). Pharmacokinetic

Table 1. Comparison of the bioavailability of tramadol hydrochloride (one-way analysis of variance ANOVA) after oral administration of a new slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP) and Tramal Retard® tablets, 100 mg (TR) in rabbits.

	AUC_{0-t} (ng × h/mL)		$AUC_{0-\infty}$ (ng × h/mL)		C_{max} (ng/mL)	
	TP	TR	TP	TR	TP	TR
Mean	962.22	1100.08	1060.93	1119.14	279.67	446.96
SD	309.30	673.51	548.14	656.68	109.47	151.60
Median	906.13	939.45	962.89	984.63	285.40	458.70
Min	452.26	424.99	464.69	457.23	146.10	220.90
Max	1403.20	2501.06	2299.14	2520.20	502.50	653.50
%CV	32.14	61.22	51.66	58.68	39.14	33.92
Geometric mean ratio TP/TR and 90% CI	98.42 (85 – 122)		98.47 (92 – 107)		61.98 (56 – 70)	

(n = 10).

TH – tramadol hydrochloride, C_{max} – maximum plasma concentration, AUC_{0-t} – area under the plasma curve from zero to the last measurable concentration, $AUC_{0-\infty}$ – area under the plasma curve from zero to infinity, Mean – arithmetic mean, SD – standard deviation, Min – minimum value, Max – maximum value, 90% CI – 90% confidence interval of the geometric mean ratio (TP/TR), CV – coefficient of variation defined as the ratio of the SD to the mean: $CV = (SD/mean) \times 100\%$.

Table 2. Descriptive statistics of the pharmacokinetic parameters of tramadol hydrochloride after oral administration of a new slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP) and Tramal Retard® tablets, 100 mg (TR) in rabbits (n = 10).

	k_{el} (h^{-1})		$AUMC_{0-\infty}$ ($ng \times h^2/mL$)		MRT (h)		$t_{1/2kel}$ (h)		t_{max} (h)		MAT (h)		k_a (h^{-1})		$t_{1/2ka}$ (h)	
	TP	TR	TP	TR	TP	TR	TP	TR	TP	TR	TP	TR	TP	TR	TP	TR
Mean	0.18	0.32	5994.64	12113.57	5.91	5.01	4.40	3.89	0.34	1.05	3.14	2.09	0.42	0.51	2.17	1.45
SD	0.16	0.24	3842.13	20797.35	4.14	3.47	2.88	2.65	0.25	1.29	1.52	0.49	0.26	0.13	1.06	0.35
Median	0.11	0.30	4799.50	4530.45	5.01	3.60	4.22	3.05	0.25	0.63	2.91	2.10	0.34	0.48	2.01	1.46
Min	0.03	0.06	678.38	720.40	1.46	1.60	1.01	1.10	0.08	0.08	0.96	1.26	0.19	0.36	0.67	0.87
Max	0.61	0.74	14626.90	78552.20	17.06	14.40	11.82	10.00	1.00	4.00	5.06	2.80	1.04	0.79	3.51	1.94
G mean	0.13	0.23	4703.36	5097.65	4.77	4.14	3.56	3.16	0.26	0.56	2.75	2.03	0.36	0.49	1.91	1.41
p value	p = 0.0303		p = 0.1559		p = 0.2769		p = 0.3129		p* = 0.0249		p = 0.0177		p = 0.1525		p = 0.0177	

* Wilcoxon test

Statistical analysis of the data was performed using the paired t-test and Wilcoxon test (t_{max}). All tests were considered statistically significant at $p < 0.05$.

TH – tramadol hydrochloride, k_{el} – elimination rate constant, $AUMC_{0-\infty}$ – area under the first moment curve from zero to infinity, MRT – mean residence time, $t_{1/2kel}$ – elimination half-life, t_{max} – time to reach maximum plasma concentration, MAT – mean absorption time, k_a – absorption rate constant, $t_{1/2ka}$ – absorption half-life, Mean – arithmetic mean, SD – standard deviation, Min – minimum value, Max – maximum value, G mean – geometric mean

parameters of TH and their statistical evaluation are summarized in Table 1 and Table 2. The mean C_{max} values (mean ± SD) of the TP and TR were: 279.67 ± 109.47 and 446.96 ± 151.60 ng/mL, respectively (statistically significant differences: p = 0.0003, *t*-test). The medians (range) of t_{max} values were also statistically different for TP and TR (Wilcoxon test, p = 0.0249) and were 0.25 (0.08–1.00) and 0.63 (0.08–4.0) hours, respectively.

However, comparison of the extent of the drug absorption (AUC_{0-t} and $AUC_{0-\infty}$ values) revealed no statistically significant differences. Moreover, 90% confidence intervals (CIs) for the ratios of geometric means for AUC_{0-t} and $AUC_{0-\infty}$ were within the range 80 to 125% proposed by EMA (23) and were 85 to 122%, and 92 to 107%, respectively. Ninety percent CIs for C_{max} (56 to 70%) were not included in this range (Table 1). Comparison of %CV for AUC_{0-t} and $AUC_{0-\infty}$ has shown generally lower values after TP administration (Table 1).

After administration of TP, higher values of: MAT (by 33.1%, p = 0.0177, *t*-test), MRT (by 15.2%, p > 0.05, *t*-test), $t_{1/2ka}$ (by 33.8%, p = 0.0177, *t*-test), $t_{1/2kel}$ (by 11.6%, p > 0.05, *t*-test) and lower values of: $k_{a,}$ (by 21.4%, p > 0.05, *t*-test) and k_{el} (by 77.8%, p = 0.0303, *t*-test) for TH were observed. It may indicate a prolonged absorption and elimination processes of TH (Table 2).

The absolute bioavailability $F(\%)$ of TH was 32.8 ± 7.4% and 45.8 ± 12.9 % after TP and TR administration. The relative bioavailability of TH was 83.97%.

DISCUSSION

A new oral dosage form of TH – slow release tablets obtained by hot tableting of coated pellets, 100 mg (TP), was compared in our study to the conventional slow release tablets – Tramal Retard®, 100 mg (TR). It is known that pellets constitute multiple-unit dosage forms, which have many advantages as compared to the traditional tablets (21). They are more evenly distributed in the stomach, which leads to a lower risk of high local concentration and of adverse effects. Any disturbances at the administration stage, for example crushing with teeth, or otherwise changed release rate of absorption may not apply to all pellets. Moreover, these forms are

characterized by a high reproducibility of release due to a relatively large surface and the short diffusion way of the drug (21, 28). Results of many animal studies (29–33) could indicate that different oral formulations of TH have a variable systemic availability and, consequently, could cause an unpredictable clinical response; this is why pharmacokinetic study of each new formulation of the drug is so important.

In our study, no statistically significant differences between these two formulations were observed when comparing the extent of the drug absorption (90% CIs values for AUC_{0-t} and $AUC_{0-\infty}$ were within the range of 80–125%) and concentration-time profiles (except TH concentrations 1h after administration, p = 0.0276). However, a comparison of TP's and TR's pharmacokinetics may indicate prolonged absorption and elimination processes of TH after TP administration (higher values of MAT, MRT, $t_{1/2ka}$ and $t_{1/2kel}$ and lower values of k_a and k_{el}). TP exhibited a significantly lower C_{max} and a shorter t_{max} of TH compared with TR. The C_{max} of TH, both after TP and TR administration, was higher than that reported e.g., in dogs (mean ± SD, 40 ± 110 ng/mL; SR tablet, dose of 4–6 mg/kg) (29), horses (mean ± SEM, 57 ± 0.07 ng/mL; SR tablet, dose of 5 mg/kg) (30), but lower than in cats (mean ± SEM, 914 ± 232 ng/mL; IR tablet dose of 5.2 mg/kg) (31) and dogs (mean ± SD, 1402.75 ± 695.52 ng/mL; IR tablet, dose of 100 mg, mean dose of 11.2 mg/kg) (32).

Values of plasma concentrations and hence pharmacokinetic parameters of TH after TP and TR administration were considerably variable among rabbits. Although all of the rabbits had similar body weight and fasted 12 h prior to drug administration and 12 h afterwards to minimize the variability caused by food, differences in bioavailability, gastrointestinal transit time, disintegration of the oral dose form, and rate of absorption can affect plasma concentrations of TH. However, TP formulation produced a smaller inter-subject variability in AUC_{0-t} and $AUC_{0-\infty}$ and plasma concentrations of TH at all evaluated time points. Inter-animal variability (related to differences in absorption rate or gastrointestinal transit time, etc.) was also described by e.g., Pypendop and Ilkiw (31) or Cox et al. (22) in most of the pharmacokinetic parameters and plasma concentrations of TH after oral administration.

The mean absolute bioavailability of TH after TP and TR administration was relatively low (about 40%). This may be related to the poor absorption and strong metabolism of TH in rabbits. It is known that administration of controlled release drugs does not always entail increased bioavailability (28). Generally, biological availability of TH in animals after administration of SR tablets is usually lower than after IR tablets (29, 30, 33).

CONCLUSIONS

After administration of slow release tablets obtained by hot tableting of coated pellets, a prolonged absorption and elimination processes and a smoother and more extended plasma profile of TH in rabbits were observed. Using animal model, both TP and TR tablets have a similar *in vitro* release profile and similar values. It can be expected that the use of a new oral dosage form of TH in patients affects the extension of analgesia after single administration of the drug, with its gradual absorption into the systemic circulation.

Conflict of interest

The authors state no conflict of interest.

REFERENCES

1. Klotz U.: Arzneimittelforschung 53, 681 (2003).
2. Chaurasia D., Kaushik K., Bhardwaj P., Chaurasia H., Jain S.K., Shobhna S.: Acta Pol. Pharm. Drug Res. 68, 795 (2011).
3. Cepeda M., Camargo F., Zea C., Valencia L.: J. Rheumatol. 34, 543 (2007).
4. Sudha B.S., Sridhar B.K., Srinatha A.: AAPS PharmSciTech. 11, 433 (2010).
5. Basu S.K., Kavitha K., Rupeshkumar M.: AAPS PharmSciTech. 12, 28 (2011).
6. Tiwari S.B., Murthy T.K., Pai M.R., Mehta P.R., Chowdar P.B.: AAPS PharmSciTech. 4, E31 (2003).
7. Nicholson B.: Pain Practice 9, 71 (2009).
8. Khandave S.S., Satish V.S., Joshi S.S.: Drug. Des. Devel. Ther. 4, 367 (2010).
9. Coluzzi F., Mattia C.: Ther. Clin. Risk Manag. 3, 819 (2007).
10. Curković B., Bernik N.: Arzneimittelforschung 53, 503 (2003).
11. Raber M., Schulz H.U., Schürer M., Krupp S., Momberger H.: Arzneimittelforschung 49, 594 (1999).
12. Barkin R.L.: Am. J. Ther. 15, 157 (2008).
13. NaeemAamir M., Ahmad M., Akhtar N., Murtaza G., Khan S.A., Shahig-uz-Zaman, Nokhodchi A.: Int. J. Pharm. 407, 38 (2011).

14. Beaulieu A.D., Peloso P.M., Haraoui B., Bensen W., Thomson G., Wade J., Quigley P. et al.: Pain Res. Manag. 13, 103 (2008).
15. Mariconti P., Collini R.: Minerva Anestesiol. 74, 63 (2008).
16. Keating G.M.: Drugs 66, 223 (2006).
17. Likar R., Wittels M., Molnar M., Kager I., Ziervogel G., Sittl R.: Clin. Ther. 28, 2022, (2006).
18. Frank M., Sturm M., Arnau H., Achenbach U.: Fortschr. Med. 117, 38 (1999), (German).
19. Sorge J., Stadler T.: Clin. Drug Invest. 14, 157 (1997).
20. Kumar P., Singh S., Mishra B.: Acta Pharm. 59, 15 (2009).
21. Sawicki W., Mazgalski J.: Drug Dev. Ind. Pharm. 35, 857 (2009).
22. Cox S., Villarino N., Doherty T.: Res. Vet. Sci. 89, 236 (2010).
23. European Medicines Agency. Guideline of the investigation of bioequivalence. The European Agency for the Evaluation of Medicinal Products CPMP/EWP/QWP/1401/98 Rev. 1/ Corr **, London, 20 January 2010.
24. Guide for the care and use of laboratory animals; Eighth edn. The National Academies Press Washington, D.C. 2011, http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-Laboratory-animals.pdf. Accessed 5 December 2011.
25. Gan S.H., Ismail R., Wan Adnan W.A., Wan Z.: J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 772, 123 (2002).
26. Szkutnik-Fiedler D., Grześkowiak E., Madziała J.: Farmacja Współczesna 6, 3 (2013).
27. European Medicines Agency. Guideline on bioanalytical method validation. Committee for Medicinal Products for Human Use (CHMP) EMEA/CHMP/EWP/192217/2009, 21 July 2011.
28. Sawicki W.: Eur. J. Pharm. Biopharm. 53, 29 (2002).
29. Giorgi M., Saccomanni G., Łebkowska-Wieruszewska B., Kowalski C.: Vet. J. 180, 253 (2009).
30. Giorgi M., Soldani G., Manera C., Ferrarini P., Sgorbini M., Saccomanni G.: JEVS 27, 481 (2007).
31. Pypendop B.H., Ilkiw J.E.: J. Vet. Pharmacol. Ther. 31, 52 (2008).
32. KuKanich B., Papich M.G.: J. Vet. Pharmacol. Ther. 27, 239 (2004).
33. de Sousa A.B., Santos A.C., Schramm S.G., Porta V., Górniak S.L., Florio J.C., de Souza Spinoza H.: J. Vet. Pharmacol. Ther. 31, 45 (2008).

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EVALUATION OF DICLOFENAC SODIUM SUSTAINED RELEASE MATRIX PELLETS: IMPACT OF POLYETHYLENE GLYCOLS MOLECULAR WEIGHT

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Abstract: Sustained release matrix pellets loaded with 5% w/w diclofenac sodium (DS) were prepared using extrusion/spheronization technique. Different polyethylene glycols (PEGs) of different molecular weight, namely PEG 2000, PEG 4000 and PEG 6000, were mixed with avicel PH 101® in different weight ratios to manufacture the pellet formulations and water was used as a binder. Mix torque rheometer was used to characterize the pellets' wet mass. Also, the prepared pellets were characterized for their particle sizes, DS content, shape and morphology as well as the *in vitro* drug release. The results showed increasing PEG weight ratio resulted in a reduction of wet mass torque as well as binder ratio, especially at PEG high weight ratios (30% and 50%) and the extent of lowering wet mass peak torque was inversely proportional to PEG molecular weight. The manufactured pellets exhibited size range of 993 µm to 1085 µm with small span values. The drug release from pellets was governed by the molecular weight of PEG used, since increasing PEG molecular weight resulted in slowing the drug release rate from pellets, but increasing its level resulted in enhancing release rate. This was attributed to increasing pellet wet mass peak torque by increasing PEG molecular weight and lowering it by increasing PEG level. The prepared pellets showed non-Fickian or anomalous drug release or the coupled diffusion/polymer relaxation.

Keywords: matrix pellets, diclofenac sodium, mix torque rheometry extrusion/spheronization, *in vitro* release

Multiple-unit dosage forms have gained much attention, with single-unit dosage forms, regarding both therapeutic and formulation benefits. Among the various types of multiple-unit dosage forms, pellets have attracted more attention due to their unique clinical and technical advantages. Pellets or spherical granules are produced by agglomerating fine powders with a binder solution. Pellets are defined as spherical, free-flowing granules with a narrow size distribution, typically varying between 500 and 1500 µm for pharmaceutical applications (1). The interest in pellets as dosage forms (filled into hard gelatin capsules or compressed into disintegrating tablets) has been increasing continuously. Several therapeutic advantages could be achieved using pellets as drug delivery system, over the single-unit regimen, such as less irritation of the gastrointestinal tract and a lowered risk of side effects due to dose dumping (2). In addition, formulation advantages such as the better flow properties, less friable dosage form, narrow particle size distribution, ease of coating and uniform packing can be gained with pellets. It was shown that multi-unit dosage forms have gained considerable popularity over conventional single units for controlled release technology. This is due to the rapid dispersion of pellets in the gastrointestinal tract; they maximize drug absorption, reduce peak plasma fluctuations and minimize potential side effects without lowering drug bioavailability (3). Pellets also reduce variations in gastric emptying rates and overall transit times. Thus, intra and intersubject variability of plasma profiles, which are common with single-unit regimens, are minimized.

Different authors formulated matrix pellets for controlled drug delivery systems techniques, which avoid the use of organic solvents during coating procedures, due to stringent global requirements of product safety. Also, by formulating sustained release matrix pellets, time and money could be saved by omitting the coating operation. As the level of understanding regarding the toxic effects of these

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solvents is increasing, industrial hygiene rules and FDA regulations are being tightened world over, limiting the use of these solvents and exposure of workers to these solvents. Therefore, several reports have been published on alternative techniques such as melt granulation (4), melt extrusion (5, 6), melt dispersion (7), and melt solidification (8) for controlled drug delivery systems. In addition, several attempts have been made to modify drug release from multiparticulate oral dosage forms by incorporating various hydrophobic materials into a basic formulation for pellets (9). Such systems retard the penetration of aqueous fluids into the formulation and hence slow the rate of drug release.

The rheological properties of wet masses can be monitored successfully using a mixer torque rheometer (10, 11) so as to formulate pellets of tailored pharmaceutical characteristics. It was shown that the rheological properties of wet mass could affect the release patterns from pellet formulations. Ibrahim (12) showed that mefenamic acid matrix pellets could be successfully correlated with the wet mass characteristic using mixer rheometry. This will help to obtain a controlled release dosage form capable of lowering the risk of side effects and improving patient convenience as an advantage of pellets as a drug delivery system. Also, Mahrous et al. (13) observed that an inverse relationship exists between indomethacin release from the pellets and the peak torque values of the used polymer mixture.

Diclofenac sodium (DS) is a non-steroidal anti-inflammatory drug (NSAID) belonging to the group of aryl acetic acid derivatives. It is widely used in treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (14). Because of its short biological half-life (2 h), it is eliminated from plasma compartments of the body within few hours, so frequent administration is necessary to maintain its therapeutic concentration. Thus, DS is an ideal candidate for sustained release purposes (15, 16). Therefore, the formulation of DS as a sustained release dosage form matrix pellets could be an alternative approach to overcome the potential problems in the gastrointestinal tract, in addition to minimizing dosing frequency (17, 18).

The objectives of the present study were to formulate sustained release matrix pellets loaded with diclofenac sodium using extrusion/spheronization technique as an alternative method to coating technique. Different grades of polyethylene glycols (PEG 2000, PEG 4000 and PEG 6000) were used in combination with avicel. The effect of polyethylene glycol molecular weight on the wet mass peak torque, pellets' shapes and sizes will be characterized using mixer torque rheometry and the *in vitro* release rate of the drug loaded pellets will be assessed as well.

EXPERIMENTAL

Materials

Diclofenac sodium (DS) was kindly supplied by Al-Jazeera Pharmaceutical Industries (Riyadh, KSA). Polyethylene glycols (PEG 6000, PEG 4000 and PEG 2000) were purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, UK). Microcrystalline cellulose, (MCC – Avicel® PH101) was purchased from Serva Feinbiochemica (Heidelberg, Germany). All other materials and solvents used were of reagent or analytical grade and used without further purification.

Methodology

Characterization of pellets wet masses using a mixer torque rheometer

The mixer torque rheometer used in the present study consists of a 135-mL capacity stainless steel bowl equipped with two mixing blades with rotational speed ranging between 20 and 150 rpm (MTR-3, Caleva, Dorset, England). Depending on the bulk density, a sample of 15–30 g of dry powder material is sufficient to cover the mixer blades. The torque is measured directly on the mixer bowl with the help of a torque arm connected from the main body of the mixer to a calibrated load transducer. The following equipment setting was used for all the studies: mixer speed, 50 rpm. The data acquisition and analyses were carried out by a personal computer using data acquisition system and software package supplied by the equipment manufacturer.

Powders were mixed in turbula mixer (type S27, Erweka, Apparatebau, Germany) and 15 grams sample of this dry blend was utilized in the wet massing studies. Two milliliters of granulating fluid were added in multiply additions over 10 wet massing intervals. Each wet massing interval consisted of a one minute mixing period and a 20-second torque data logging (collection) period with the MTR operating at 50 rpm. Mean torque was monitored during the granulation process.

Manufacture of pellets

Water was used as a granulating liquid in the manufacture of matrix pellets loaded with 5% w/w DS. The water volume required for wet massing was selected according to the highest torque value measured by the rheometer. The compositions of the studied pellet formulations are shown in Table 1. DS

and pellets excipients were mixed in turbula mixer at certain weight and the powder mixture was wetted with water. Next, the resulting wet mass was extruded at a speed of 90 rpm with a screen pore size of 1 mm ∅ (Mini Screw Extruder, Model MSE1014, Caleva, Dorset, England). Spheronization was performed in a spheronizer (Model 120, Caleva, Dorset, England) with a rotating plate of regular cross-hatch geometry, at a speed of 700 rpm, for 5 min. Pellets were then dried on a tray in a hot oven at 50–60°C for 6 h.

Drug content

Diclofenac sodium content of the manufactured pellets was determined spectrophotometrically at 285 nm in triplicate. Pellets were crushed in a porcelain mortar and about 20 mg of the crushed pellets were dispersed in 250 mL of phosphate buffer (pH 6.8) under sonication for 5 min. The supernatant was filtered through a cellulose nitrate filter with pores of 0.2 µm in diameter (Sartorius, Göttingen, Germany) and measured spectrophotometrically (UV-2800 spectrophotometer, Labomed Inc., USA), then MA content was calculated using a preconstructed calibration curve.

Morphological analysis

The morphological characteristics of particles were observed by scanning electron microscopy (SEM). The samples were sputter-coated with thin gold palladium layer under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then scanned and photomicrographs were taken with SEM (Jeol JSM-1600, Tokyo, Japan).

Particle size analysis

The size distribution of the manufactured pellets was investigated using laser light diffraction (Mastersizer Scirocco 2000, Malvern Instruments, Grovewood Road, UK). For a typical experiment, about 300 mg of pellets were fed in the sample micro feeder. All samples were analyzed 5 times and average results were taken. The pellets 10th (d(0.1)), 50th (d(0.5)) and 90th (d(0.9)) percentiles were used to characterize the pellets size distribution. The approximate mean diameter was taken as the average of d(0.1), d(0.5), and d(0.9) values.

The span value was employed to characterize the pellet size distribution, since a small span value indicates a narrow particle size distribution. It was calculated from the following formula (19):

$$\text{Span} = \frac{D_{90}-D_{10}}{D_{50}}$$

***In vitro* dissolution studies**

The dissolution measurements were performed using an automated dissolution tester (LOGAN Instrument Corp, Somerset, NJ, USA) coupled to an automated sample collector (SP-100 peristaltic pump, Somerset, NJ, USA). The USP dissolution basket method (apparatus 1) was used. MA loaded pellets equivalent to 20 mg DS were added to 500 mL of dissolution medium (phosphate buffer, pH 7.4). The temperature was maintained at 37 ± 0.5°C. An accurately weighed amount of the prepared pellets was added to each flask. For each sample formula, drug dissolution was run in triplicate and absorbance was recorded automatically at 285 nm up to 8 h. The percentage of drug dissolved was determined as a function of time.

Statistical analysis

The results were analyzed using the software GraphPad Prism5 (GraphPad Software, La Jolla, USA) applying one-way ANOVA. Differences between formulations were considered to be significant at $p \leq 0.05$.

Table 1. Composition of different pellets formulations loaded with diclofenac sodium.

Formula→ ↓Ingredients %	1	2	3	4	5	6	7	8	9	10
Avicel PH 101	85	75	45	43	85	75	45	85	75	45
PEG 2000	10	20	50	50	–	–	–	–	–	–
PEG 4000	–	–	–	2	10	20	50	–	–	–
PEG 6000	–	–	–	–	–	–	–	10	20	50
Diclofenac sodium	5%									
Water (binder)	Q. S.									

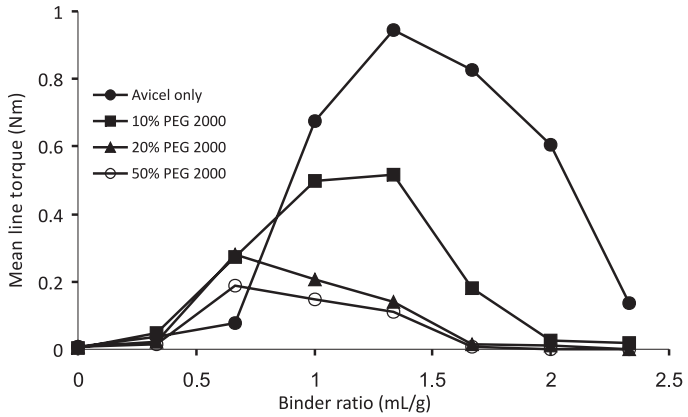

Figure 1. Effect of different concentrations of PEG 2000 on mean torque of Avicel PH101

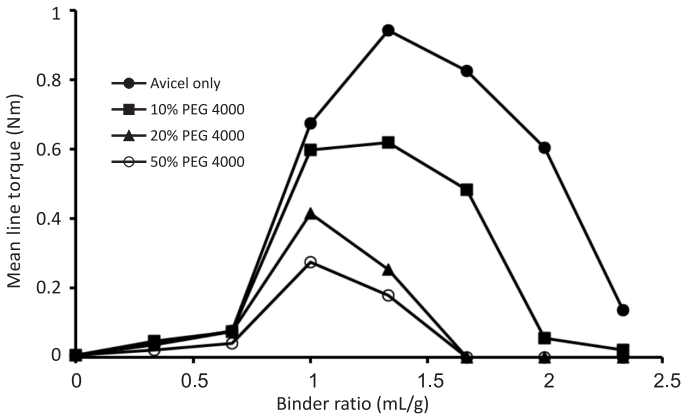

Figure 2. Effect of different concentrations of PEG 4000 on mean torque of Avicel PH101

RESULTS AND DISCUSSION

Wet massing studies

The experiments of wet massing studies were conducted for avicel-PEGs systems in order to establish the water/powder ratio needed to reach a maximum torque response and the effect of PEG grade and level on the pellet wet mass characteristics. Regarding Avicel-PEG 2000 systems (Fig. 1) different liquid saturation phases (pendular, funicular and capillary, respectively) were passed through by increasing binder level, with the maximum torque occurring at the capillary state. Avicel alone exhibited a typical progression of liquid saturation phases. The mean torque value was found to increase with an increase in the wet massing liquid (water) ratio. However, different profiles were detected regarding Avicel-PEG 2000 systems, increasing PEG 2000 weight ratio resulted in a severe reduction of the area of MTR curve, i.e., progression of liquid saturation phases occurs at lower water/powder ratio. In addition, reductions of peak torque water/powder ratios (mL/g) and peak torque magnitudes were recorded, which reached the lowest value (0.208 Nm) at 50% w/w PEG 2000 level (Fig. 1). The rheological behaviors of Avicel-PEG 4000 systems (Fig. 2) are quite the same as those recorded in case of Avicel-PEG 2000. However, there is an increased peak torque in case of Avicel-PEG 4000 levels compared to the use of corresponding levels of PEG 2000. For example,

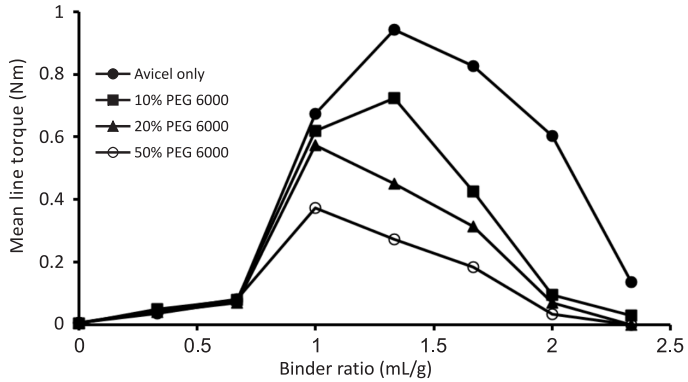

Figure 3. Effect of different concentrations of PEG 6000 on mean torque of Avicel PH101

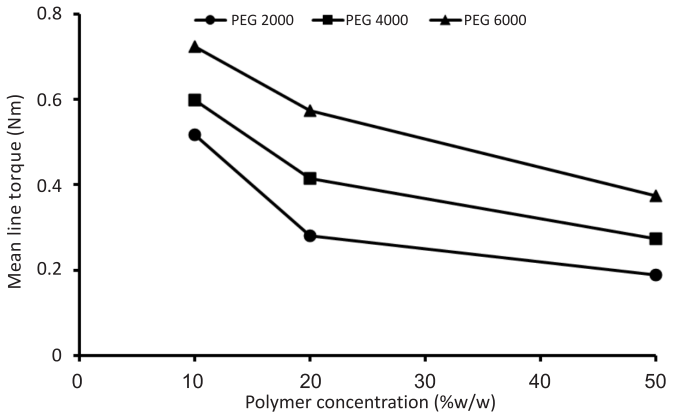

Figure 4. Effect of PEGs molecular weight and concentration of on mean line torque of Avicel PH101

upon mixing 50% level of PEG 2000 and PEG 4000 with avicel, the recorded peak torque values were 0.189 Nm and 0.247 Nm, respectively. Similarly, mixing PEG 6000 with avicel for wet massing resulted in increasing the wet mass consistency higher than that measured in case of PEG 2000 or PEG 4000 (Fig. 3). On the other hand, pendular, funicular and capillary phases in case of Avicel-PEG 6000 systems were reached at higher peak torque values than those observed in case of the other PEG polymer grades, and the peak torque values were found to decrease by increasing the PEG level. The impact of PEG molecular weight and concentration on the properties of DS pellets wet masses is displayed and summarized in Figure 4. It is clearly evident that high molecular weight grades showed an increase in the mean line torque of the wet mass at all the concentrations studied (10, 20 and 50%) and the mean torque value was found to be decreased by raising polymer level. According to Parker and Rowe (20), the degree of liquid spreading and wetting as well as the substrate binder interaction will determine the relative positions of the peak values of mean line torque. For each polymer concentration, an increase in the mean torque with the increase in the polymer molecular weight at different concentrations resulted in either a sharp or an extended peak followed by a drop in the torque as over-wetting of the powder mass occurred. In addition, the pendular and funicular states are characterized by a progressively increasing network

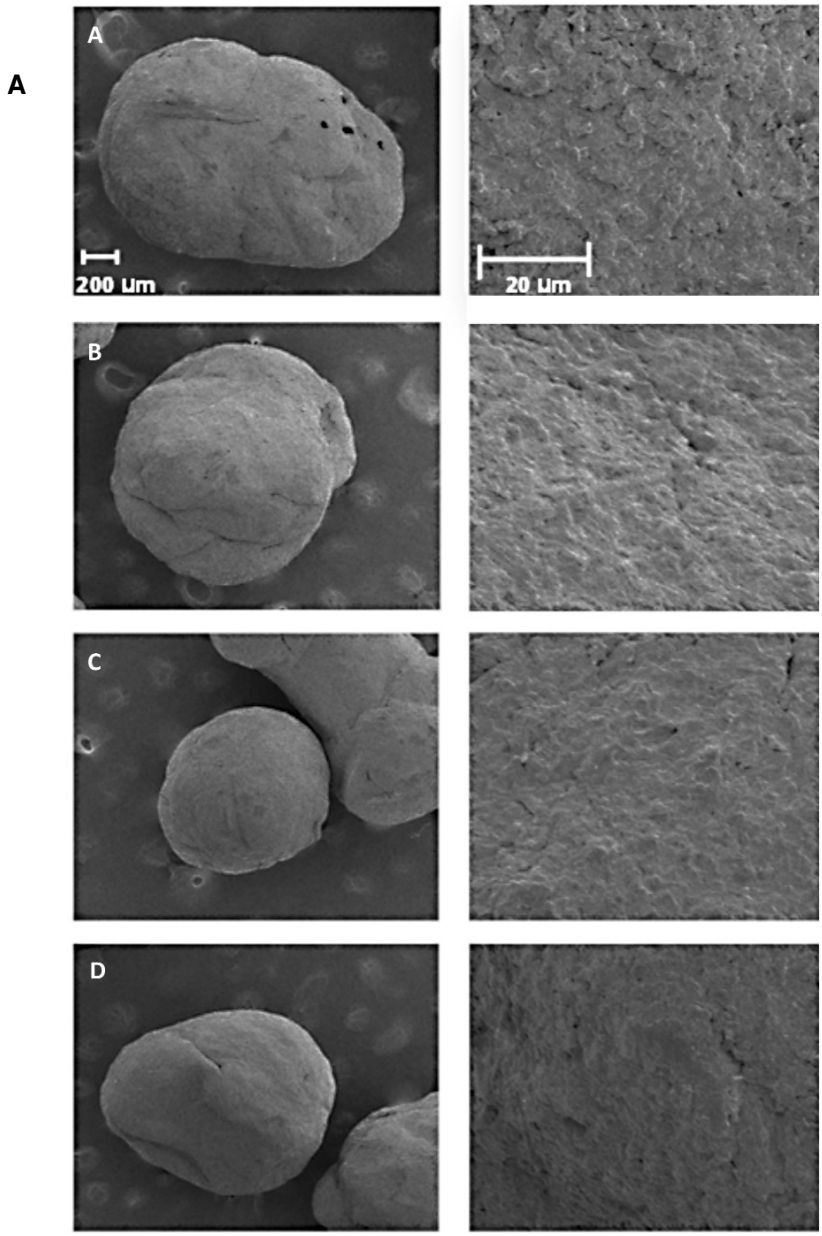

Figure 5. (**A**) Scanning electron micrographs of the pellets. (**B**) Scanning electron micrographs of the surface of pellets with A – avicel only; B – 50% PEG2000; C – 50% PEG4000; D – 50% PEG6000.

of liquid bridges. Both of these stages will cause an increase in cohesiveness of the powder mass and hence an increased torque on the mixer (21). The capillary state, which was reached when all the air spaces in the granular material were filled with liquid, occurs at the maximum on the curve. With further addition of liquid the torque decreases as slurry of particles dispersed in liquid is formed.

Drug content

The obtained results showed DS content ranged from 90–110% of the theoretical content, which revealed a homogenous drug distribution in the prepared pellets.

Pellets sizes and shapes

The calculated values of volume weighted mean particle size and the d (0.1), d (0.5) and d (0.9)

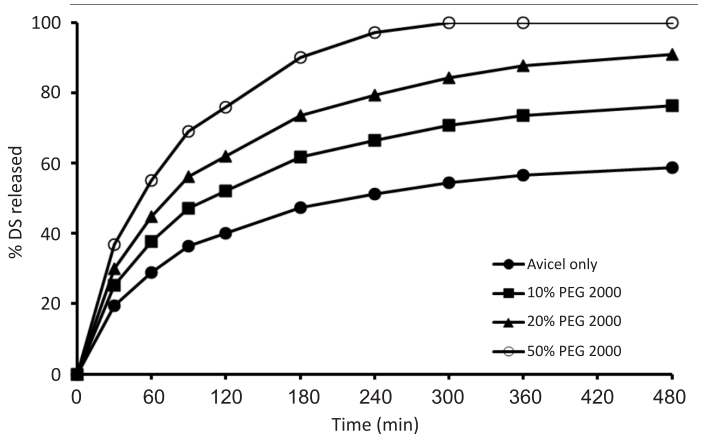

Figure 6. Effect of PEG 2000 concentration on the *in vitro* release profiles of DS from matrix pellets

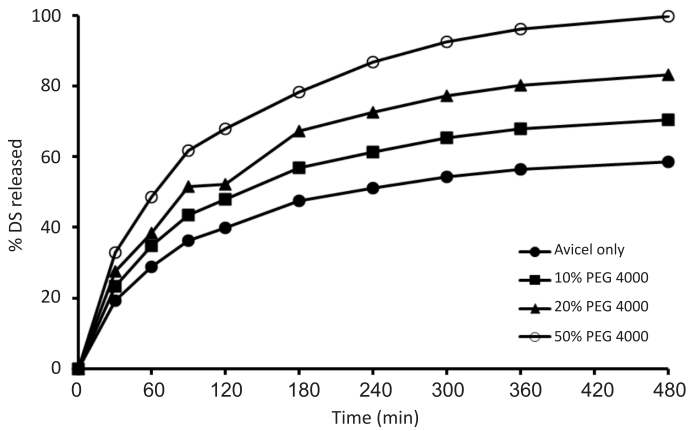

Figure 7. Effect of PEG 4000 concentration on the *in vitro* release profiles of DS from matrix pellets

different pellet formulae loaded with DS as determined by laser diffractometry are tabulated in Table 2. One can observed that the volume weighted mean of the manufactured pellets was found to be in the range 993 to 1085 μm. Also, the particle size distribution of DS loaded matrix pellets was characterized by small span values, as these calculated values were found to be 0.64–0.72 indicating a narrow particle size distribution (22). Moreover, for each polymer grade, increasing the polymer concentration resulted in a decrease in the calculated volume weighted mean as well as the span value of particle size distribution. This is in accordance with the data obtained from wet massing studies, which showed a decrease in the wet mass by increasing polymer concentration, which in turn, reduced torque values. Kristensen and Schæfer (23) found a linear correlation between the torque value and pellet size for formulations containing 80% (w/w) MCC.

Scanning electron micrographs of matrix pellets formulations containing 50% of each PEG grade mixed with avicel are compared with those prepared using avicel only are displayed in Figure 5. Most of the prepared pellets formulas were seen almost rounded and intact in shape, while pellets from avicel only (A) were not completely spherical. The higher torque value of this pellet wet mass formula (943) may be contributed to its irregular shape. 195,704 Also, pellet formula prepared using 50% PEG 2000 (B) showed smooth surface compared to

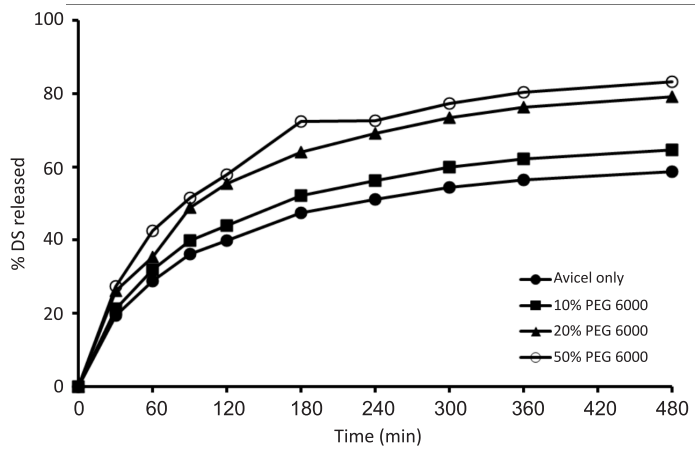

Figure 8. Effect of PEG 6000 concentration on the *in vitro* release profiles of DS from matrix pellets

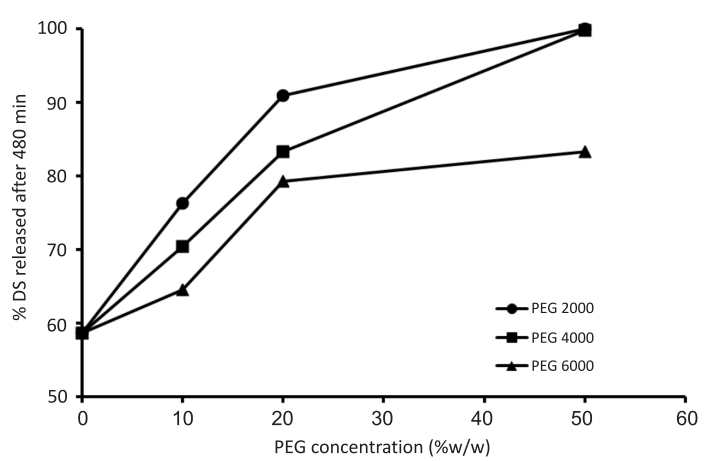

Figure 9. Effect of PEGs molecular weight and concentration of on the release rate of DS from matrix pellets after 480 min

those prepared from 50% PEG 4000 (C) and PEG 6000 (D). Increasing PEG molecular weight caused increased roughness of the pellet surface, which might be due to increasing pellet wet mass mean torque as previously described. These results are in accordance with the data obtained by Mahrous et al. (13), who showed that the more hydrophilic polymer (PEG 4000), when mixed with MCC, produced a wet mass having the lowest mean torque value compared to that recorded with the same weight ratio of PVP and HPMC. This in turn, reflects on the easy extrusion of PEG wet mass resulting in pellets with smooth less rough surfaces. In addition, Law and Deasy (24) showed that the use of hydrophilic polymers with MCC favored more spherical and smooth pellets.

***In vitro* release studies**

It was shown by Law and Deasy (24) that mixing various hydrophilic polymers with MCC may aid extrusion-spheronization and, at the same time, to enhance the dissolution of indomethacin. Therefore, the aim of studying DS *in vitro* release from matrix pellets is to investigate the effect of different PEGs on the drug release patterns. Incorporation of the drug in pellet formulations composed of MCC only resulted in slowing its release rate. Only 59% of the loaded DS was release

Table 2. Volume weighted mean particle size and the d(0.1), d(0.5), d(0.9) and span values of different pellet formulae loaded with 5%w/w diclofenac sodium (as determined by laser diffractometry).

Pellet formulations	Mean (μm)	d (0.1) μm	d (0.5) μm	d (0.9) μm	Span value
Avicel only	1065.74	741.52	1110.21	1541.87	0.72
Avicel + 10% PEG 2000	1030.24	734.21	1084.21	1498.21	0.71
Avicel + 20% PEG 2000	1000.21	711.51	1051.21	1421.84	0.68
Avicel + 50% PEG 2000	993.21	684.21	1000.10	1327.21	0.64
Avicel + 10% PEG 4000	1075.11	721.45	1121.11	1524.32	0.72
Avicel + 20% PEG 4000	1063.21	711.25	1101.01	1499.17	0.72
Avicel + 50% PEG 4000	1033.45	700.14	1042.11	1418.71	0.69
Avicel + 10% PEG 6000	1120.04	765.21	1132.10	1548.15	0.69
Avicel + 20% PEG 6000	1086.21	751.78	1123.34	1513.01	0.68
Avicel + 50% PEG 6000	1084.51	738.41	1108.91	1465.87	0.66

Table 3. Kinetic modeling of DS release from different sustained release matrix pellet formulations.

Formula	Zero order model	First order model	Higuchi diffusion model	Peppas model	n*
	r	r	r	r	
Avicel only	0.869	0.922	0.974	0.981	0.398
Avicel + 10% PEG 2000	0.957	0.970	0.999	0.999	0.50
Avicel + 20% PEG 2000	0.869	0.981	0.974	0.981	0.398
Avicel +50% PEG 2000	0.819	–	0.948	0.960	0.366
Avicel + 10% PEG 4000	0.869	0.938	0.975	0.981	0.398
Avicel + 20% PEG 4000	0.879	0.965	0.978	0.984	0.41
Avicel + 50% PEG 4000	0.872	0.967	0.976	0.982	0.399
Avicel + 10% PEG 6000	0.869	0.930	0.974	0.981	0.398
Avicel + 20% PEG 6000	0.870	0.952	0.973	0.978	0.41
Avicel + 50% PEG 6000	0.855	0.948	0.966	0.972	0.394

r = correlation coefficient, and n is the release exponent. * obtained from Korsmeyer-Peppas equation.

from avicel matrix pellets after 8 h (Figs. 6–8). The effect of PEG 2000 concentration on the *in vitro* release profile of DS from matrix pellets is illustrated in Figure 6. The drug release rate was found to be enhanced by increasing PEG 2000 level in the pellets. For example, complete drug release was observed after 4 h in case of pellet formulation containing 50% PEG 2000, while only 66 and 79% of the loaded drug were released from the formulae containing 10 and 20% of such polymer at the same time, respectively. In case of pellet formulations containing PEG 4000 (Fig. 7) similar finding were recorded as by increasing PEG 4000 level in the pellet formulation, a pronounced rapid release rate was observed. However, the enhancement of DS release was in case of using different PEG 4000 concentrations less that that exhibited by PEG 2000. Only 71, 83 and 99% of the loaded DS were released after 8 h from pellet formulations manufactured by using PEG 4000 concentrations of 10, 20 and 50% of the pellets' weight. Moreover, the addition of PEG 6000 in different levels caused an increase in the drug release rate by increasing PEG 6000 level (Fig. 8) but the enhancement is rather smaller than that was seen in case of PEG 2000 and PEG 4000. For example, pellet formulations containing PEG 6000 concentrations of 10, 20 and 50% of the pellets' weight released 66, 79 and 83% of the loaded DS after 8 h. Figure 9 correlates the effect of PEG molecular weight and level on the percentage of DS released after 480 min. It is clearly evident that increasing PEG level in the pellet formula caused a decrease in

the peal torque of wet mass, which in turn, enhanced DS release rate from pellet formulations. In addition, the effect of PEG 2000 and PEG 4000 on the drug release rate from pellet formulas is more noticeable than that exhibited by blending PEG 6000, especially at higher concentrations (20 and 50%).

In another study, Ibrahim (12) revealed that increasing lactose weight ratio was accompanied by enhancing the mefenamic acid release rate from matrix pellets by reducing pellet wet mass peak torque. He showed that lactose enhances the drug release rate by forming pores and it also promotes water penetration into the formulation core. In addition, increasing lactose concentration caused a pronounced lowering of the mean torque of pellet wet mass before extrusion/spheronization procedures. Also, Ibrahim et al. (25) found an inverse relationship between indomethacin release from its loaded pellets and the peak torque values of the polymer mixed with co-solvents.

Kinetic modeling of the *in vitro* release of MA from the matrix pellets

The *in vitro* release data of DS from different sustained release matrix pellets were fitted using zero order, first order and Higuchi diffusion models as well as Korsmeyer-Peppas equation to determine the best model that fits/describes drug release from pellet formulations. Preference of the best release mechanism is based on the correlation coefficient value. The data revealed a good fit to Higuchi diffusion model. Successive evidence of the relative validity of diffusion model was obtained by analyzing the data using the equation of Korsmeyer et al., and the release exponent (n) was calculated from Korsmeyr equation (26) :

$$Mt/M_8 = K \times t^{n}$$

where Mt/M_8 is the fraction released by the drug at time t, K is a constant incorporating structural and geometric characteristic and n is the release exponent characteristic for the drug transport mechanism. For spherical samples, when n = 0.43 Fickian diffusion is observed and the release rate is dependent on t, while $0.43 < n < 1.0$ this indicates anomalous (non Fickian) transport and when n = 1, the release is zero order.

The release kinetic parameters listed in Table 3 indicated that the calculated n values were found mostly less than 0.45, indicating the so called non-Fickian or anomalous drug release or the coupled diffusion/polymer relaxation. Other investigators showed that when liquid diffusion rate and polymer relaxation rate (erosion) are of equal magnitude, anomalous or non Fickian diffusion is observed (27, 28).

CONCLUSION

Diclofenac sodium was successfully prepared as sustained release matrix pellets using extrusion/spheronization technique. The results showed that the release of DS from matrix pellets can be tuned by controlling PEG molecular weight, which affects the rheological properties of pellets' wet masses. Mix torque rheometry was found to be a good tool for characterizing pellets' wet mass prior to extrusion/spheronization procedures. In addition, formulation of drug-loaded matrix pellets might be an alternative approach for pellet coating to avoid coating procedures' drawbacks.

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REFERENCES

1. Chambliss W.G.: in Pharmaceutical Pelletization. Technology, Ghebre-Sellassie I. Ed., p. 15, Marcel Dekker, New York 1998.
2. Sandberg A., Ragnarsson G., Jonsson U.E., Sjogren J.: Eur. J. Clin. Pharmacol. 33, S3 (1998).
3. Mehta K.A., Kislalioglu M.S., Phuapradit W., Malick A.W., Shah N.H.: Int. J. Pharm. 213, 7 (2001).
4. Schaefer T., Holm P., Kristensen H.G.: Drug Dev. Ind. Pharm. 19, 1249 (1990).
5. Sprockel O.L., Sen M., Shivanand P., Prapaitrakul W.: Int. J. Pharm. 155, 199 (1997).
6. De Brabander C., Vervae C.T., Remon J.P.: J. Control. Release 89, 235 (2003).
7. Follonier N., Doelker E., Cole T.: Drug Dev. Ind. Pharm. 20, 1323 (1994).
8. Siepmann F., Muschert S., Flament M.P., Leterme P., Gayot A., Siepmann J.: Int. J. Pharm. 317, 136 (2006).
9. Ghali E.S., Klinger G.H., Schwartz J.B.: Drug Dev. Ind. Pharm. 15, 1311 (1989).
10. Chatlapalli R., Rohera B.D.: Int. J. Pharm. 238, 139 (2002).
11. Soh J.L.P., Liew C.W., Heng P.W.S.: Int. J. Pharm. 315, 99 (2006).
12. Ibrahim M.A.: Acta Pharm. 63, 85 (2013).
13. Mahrous G.M., Ibarhim M.A., El-Badry M., Al-Anazi F.K.: J. Drug Deliv. Sci. Technol. 20, 119 (2010).

14. Goodman, L.S., Gilman, A.: The Pharmacological Basis of Therapeutics, McGraw-Hill, New York 1997.
15. Sivakumar T., Manna P.K., Sundar Rajan T., Ahmed M., Manavalan R.: Iranian J. Pharm. Sci. 3, 1 (2007).
16. Brogden R.N., Heel R.C., Pakes G.E., Speight T.M., Avery G.S.: Drugs 20, 24 (1980).
17. Khan S.Y., Akhter M.: Pharmazie 60, 110 (2005).
18. Sevgi F., Kaynarsoy B., Ozyazici M., Pekcetin Ç., Özyurt D.: Pharm. Dev. Technol. 13, 387 (2008).
19. Chen P.C., Park Y.J., Chang L.C., Kohane D.S., Bartlett R.R., Langer R. et al.: J. Biomed. Mater. Res. A 70, 412 (2004).
20. Parker M.D., Rowe R.C., Upjohn N.G.: Pharm. Tech. Int. 2, 50 (1990).
21. Luukkonen P., Schæfer T., Hellén L., Juppo A.M., Yliruusi J.: Int. J. Pharm. 188, 181 (1999).
22. SinhaV.R., Aggarwal A., Srivastava S., Goel H.: Asian J. Pharm. 4, 102 (2010).
23. Kristensen J., Schaefer T., Kleinebudde P.: Pharm. Dev. Technol. 5, 247 (2000).
24. Law M.F.L., Deasy P.B.: Int. J. Pharm. 146, 1 (1997).
25. Ibrahim M.A., Mahrous G.M., El-Badry M., Al-Anazi F.K.: Farmacia 59, 483 (2011).
26. Korsmeyer R.W., Gurny R., Docler E., Buri P., Peppas N.A.: Int. J. Pharm. 15, 25 (1983).
27. Korsmeyer R.W., Peppas N.A.: in Controlled Release Delivery Systems, Roseman T.J., Mansdorf S.Z. Eds., p. 77, Marcel Dekker, New York 1983.
28. Ritger P.L., Peppas N.A.: J. Control. Release 5, 37 (1987).

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INFLUENCE OF SPRAY DRYING MANUFACTURING PARAMETERS ON QUALITY OF LOSARTAN POTASSIUM MICROSPHERES

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Abstract: A general aim of the research was to develop a technology of manufacturing microspheres with losartan potassium as an active substance, and Eudragit L30D55 as a matrix with the use of spray drying technique. During the first step of the study, optimal values of parameters in spray drying process were established (i.e., operating temperature, peristaltic pump performance, aspiration value). Those values have a crucial effect on morphological parameters, and the size and homogeneity of received particles. In the obtained microspheres, the activity of excipients, which modify morphological properties of microspheres, were tested. Additionally, we studied the impact of the type and amount of plasticizer, as well as the amount of an adopted polymer in proportion to dry matter of losartan potassium, on quality of final product. Triethyl citrate and citric acid, plasticizers tested in reported studies, were also verified. A detailed study of the influence of both plasticizers on the qualities of microspheres containing losartan potassium on Eudragit L30D55 matrix indicated a positive influence of triethyl citrate and a negative influence of citric acid on morphological properties, shape and size of particles. The application of optimal parameters of spray drying and triethyl citrate as a plasticizer in the amount of 10 to 15% allows to obtain microspheres from 1.27 to 7.24 μm.

Keywords: microspheres, losartan potassium, spray drying, spray drying parameters, influence of plasticizers

Microspheres belong to a modern multi-compartment drug formulation, used in contemporary therapy for a controlled and targeted action of medication (1). They may be treated as a drug formulation with a modified release, which should deliver the medication in a strictly specified concentration, and to a site within a given, fixed time (2). The advantages of microspheres are: biocompatibility, an extended release profile and ability to incorporate unstable substances, such as proteins and nucleic acids, into a polymer matrix (2–6). Due to the size and shape of microspheres, controlling of a polymer matrix decay rate and a medicine release rate is also possible (5, 7, 8). Morphological properties of microspheres should fulfill published parameters, which means that the final product is monolithic, smooth and porous (1, 12), has spherical shape, and its size fits into the range from 1 to 500 μm (12).

Spray drying is a closed single phase process in which we can obtain a substance in a solid form from liquid output product (solution, suspension, and rarely emulsion) (2, 9, 10–12). This method enables to generate particles smaller than 10 μm (13).

In order to obtain the end product with required properties, the parameters of the process must be carefully selected. Almost each parameter, which is modified during the spray drying process, has a smaller or bigger effect on the obtained end product (2, 14, 15). The size of dried particles depends on the nozzle shape, on the indicator of feedstock, and conditions of the process (16). The humidity content is an indicator of the end product quality in a process of spray drying (17). The efficiency is very important for the progress of a total spray drying process, and it is determined as a proportion of the mass of a substance being dried to the end product mass (10).

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Active substances and excipients used in the study

Losartan potassium is an antagonist of a receptor to angiotensin II, which is applied mainly in a therapy of mild and moderate hypertension and cardiac failure (18, 19).

Polymer matrix Eudragit® and plasticizers were used as excipients, which have an influence on morphological properties, shape and size of particles, and flexibility of manufactured matrixes.

Eudragit L30D55 is a 30% hydrodispersion of polymer in water. The proportion of free ester groups to carboxylic groups is 1 : 1. It dissolves in pH over 5.5 creating salts with bases. This allows to obtain a coating which is not soluble in acidous gastric juice and it dissolves in alkaline intestinal juice (20).

Eudragit® copolymers function as polymer carriers because they are characterized by the following required properties: they are biocompatible, do not induce toxic effects, undergo degradation in physiological conditions, and reveal low index of polydispersion that indicates the homogenous length of polymer chains (21).

Plasticizers are a separate group of additives that were used in the study. Addition of plasticizer has an influence on flexibility, strength, and adhesive properties of received polymer coatings. Plasticizers affect also the penetration of active substances through a polymer coating. The type and quantity of plasticizer that is used allow to optimize the profile of release from the drug formulation (22–24).

The aim of this work was to establish the influence of spray drying parameters on a progress in drying process of losartan potassium hydrodispersion on Eudragit L30D55, and to establish parameters for obtaining microspheres of optimal size and homogeneity. We also evaluated the additives effect**,** that is the effect of the type and quantity of used plasticizer and polymer in proportion to dry substance of losartan potassium on a size and morphology of obtained microspheres.

EXPERIMENTAL

Materials and reagents

The substances that were used in study are: losartan potassium (Valeant, ICN Polfa Rzeszów S.A.), triethyl citrate (Sigma-Aldrich Chemie GmbH), citric acid (PPH „POCH" S.A.), Eudragit® L30D55 dispersion 30% w/w (Chemical/IUPAC name: Poly(methacrylic acid-co-ethyl acrylate) 1 : 1), (Degussa).

Technology of microspheres preparation

The method of spray drying was used to prepare microspheres with losartan potassium. Thirty percent w/w hydrodispersion of methacrylic acid copolymer – Eudragit® L30D55 with losartan potassium and/or plasticizer was subjected to drying. A spray drier Büchi Mini Spray Dryer B-191 was used. Spray drying was conducted by the use of a nozzle with a diameter of 0.7 mm, with an air flow rate of 600 L/h and pressure of 4 bar. Created microspheres were separated in a cyclone and collected in a collector.

In order to obtain a product with appropriate morphology, firstly, the parameters of spray drying were determined (spray drying was conducted using variable values of pump capacity, inlet temperature and aspirator capacity). Next, we studied the influence of added plasticizer to a polymer in terms of polymer dry matter.

Experimental selection of spray drying parameters

In order to establish optimal parameters for this process, spray drying involved the use of series of water suspension of losartan potassium and Eudragit L30D55 in proportion 1 : 1. To select optimal conditions of spray drying process, the following parameters were modified: input temperature of drying gas, aspirator capacity and peristaltic pump performance.

Spray drying process of losartan potassium on Eudragit L30D55 hydrodispersion was carried out in the following input temperatures: 120, 130, 140, 150, and 160^{o}C. The flow rate through the peristaltic pump was also modified and its performance was established at the level of: 10, 15, 20, 25, 30, 40, and 50%. Another parameter that was modified during our study was aspiration capacity. During the progress of a spray drying process, the aspirator capacity was equal to 60, 70, and 80%. Each spray drying process involved collection of a product, estimation of mass, size and morphology of particles as well as the evaluation of process efficiency.

On the basis of initial experiments, the following parameters were chosen: the peristaltic pump performance at the level of 10%, the inlet temperature of 150^{o}C, and the aspirator capacity of 80%.

Size measurement and determination of microspheres morphology

Observations of sample microstructure was performed by a high resolution scanning electron microscope (SEM), SUPRA 25, ZEISS. This microscope can analyze WDS and EDS systems at 20 kV.

The analyzed materials were observed with magnification of 1 kx, 5 kx, and 10 kx. A secondary electron detector was used to analyze the image.

The size of particles was assessed with the use of image analysis software provided by the microscope manufacturer (ZEISS). The photographs of microspheres structures were taken with magnification of 1 kx, 5 kx, 10 kx. Particle size measurements were made at the magnification of 10 kx in a number of visual fields, evaluating the size of any selected 20 microspheres (10 bigger and 10 smaller particles)

Study of the influence of the amount and type of plasticizer on the particles morphology

The study assessed the influence of the type and amount of added plasticizer on the morphology and size of obtained microspheres. For plasticizers, triethyl citrate and citric acid were selected for the study. Suspensions of losartan potassium on Eudragit L30D55 in 1 : 1 ratio and plasticizer were subjected to spray drying. Triethyl citrate was added in the proportion of 2, 5, 10, and 15% to dry matter. Citric acid was added in the proportion of 0.5, 1, and 2% to dry matter. For comparison, suspensions of losartan potassium on Eudragit L30D55 in 1 : 1, 1 : 2, and 1 : 3, without plasticizer were subjected to drying. The whole process was conducted at predetermined parameters, that is with the pump performance of 10%, inlet temperature of 150°C, and aspiration of 80%.

Study of the influence of the amount of applied polymer in proportion to losartan potassium dry matter on the size and morphology of particles

Four water solutions of losartan potassium, Eudragit L30D55 and triethyl citrate were used in spray drying. We observed a change in the amount of polymer, and the proportion of an active substance to a copolymer, namely 1 : 1, 1 : 2, 1 : 3, 1 : 4. To each solution, plasticizer was added, i.e., 10% of triethyl citrate, in relation to dry matter. The composition of formulations is presented in Table 1.

RESULTS

Optimization of spray drying parameters.

To select the optimal conditions of spray drying process, the following parameters were modified: input temperature of drying gas, aspirator capacity and peristaltic pump performance.

Each spray drying process involved collection of a product, estimation of mass, the size and morphology of particles as well as the evaluation of process efficiency. The selected results are presented in Table 2.

Figures 1 and 2 show the selected microspheres images made by a scanning microscope at magnification of 10000× dried at variable parameters of conducted process.

The experiments enabled the selection of the most optimal conditions of spray drying of losartan potassium with Eudragit L30D55. The aim of the selection was to obtain optimal efficiency of this process, but predominantly to obtain particles of optimal morphology. The following parameters were the most adequate, and were used in the further studies, input temperature of drying gas: 150°C, peristaltic pump performance: 10% and the capacity of aspirator: 80%.

Analysis of the influence of plasticizer addition

The analysis involved the verification of the influence of plasticizer addition on morphology, shape of microspheres, and efficiency of spray drying process. Triethyl citrate and citric acid were used as plasticizers for comparison. The spray drying process was conducted with addition of plasticizer (samples F4–F10) and without plasticizer addition (samples F1–F3) at previously established parameters of drying.

Table 1. The quantitative composition of the substances used in particular formulations.

Proportion drug : polymer	Losartan potassium amount [mg]	Water volume [mL]	30% w/w dispersion of Eudragit L30D55 amount [mg]	Dry matter of Eudragit L30D55 amount [mg]	Triethyl citrate volume [mL]*
1 : 1	200.00	100	666.67	200.00	35.09
1 : 2	200.00	100	1333.33	400.00	52.63
1 : 3	200.00	100	2000.00	600.00	70.18
1 : 4	200.00	100	2666.67	800.00	87.72

*Triethyl citrate density is 1.04 g/cm^3, in this part of study triethyl citrate was used in proportion of 10% in relation to the dry matter of losartan potassium and Eudragit L30D55.

Table 2. The selected, experimental parameters of spray drying of losartan potassium with Eudragit L30D55 (the proportion of active substance to copolymer is 1 : 1 in the relation to dry matter) and characteristic of the obtained product.

Input temperature [°C]	Pump efficiency [%]	Aspirator efficiency [%]	Product mass [mg]	Product efficiency [%]*	Particle size [mm]	Morphology
120	10	70	75	18.75	1.18 – 11.42	irregular particles, very few spherical structures, the size of particles very diversified
130	10	70	93	23.25	1.62 – 8.77	the presence of spherical, oval and irregular particles, size of microspheres diversified
130	20	80	123	30.75	1.58 – 9.24	presence of oval structures and particles that resemble in structure a truncated cone, size diversified, few spherical forms
140	10	70	99	24.75	1.54 – 9.14	particle structure is close to spherical with irregular edges, presence of many square forms
150	**10**	**70**	**172**	**43**	**1.52 – 7.91**	**many spherical forms and with a shape close to spherical, bigger particles with irregular shape**
150	10	80	167	41.75	1.27 – 7.24	predominance of spherical forms and forms similar in shape to spherical with slightly smudgy edge, very few forms of other shape
150	20	80	151	37.75	1.94 – 8.75	small spherical particles, bigger one with clearly irregular shapes, small number of square and truncated cone particles
160	10	80	155	38.75	1.45 – 13.86	many spherical forms and forms of shape close to spherical, bigger particles with irregular shape

*product efficienty [%] = (End product mass / (mass of losartan potassium [mg] + mass of dry matter of Eudragit L30D55) × 100%

Table 3. Drying results of losartan potassium on Eudragit L30D55 in proportion 1 : 1 with addition of plasticizer (triethyl citrate) and characteristics of generated microspheres.

Triethylcitrate relation to the dry matter [%]	Product mass [mg]	Process efficiency [%]	Average size of particles [mm]	Microspheres morphology	Sample symbol
2	163	40.75	1,17 – 8,06	spherical forms are dominant, vary few oval forms and those with irregular shapes	F4
5	181	45.25	0.95 – 7.95	clear spherical particles, presence of structure with almost spherical shape, singular oval particle	F5
10	179	44.75	0.82 – 6.97	clearly spherical particles, very few oval forms	F6
15	172	43.00	0.75 – 7.22	clearly spherical forms, few oval forms and those with irregular shape	F7

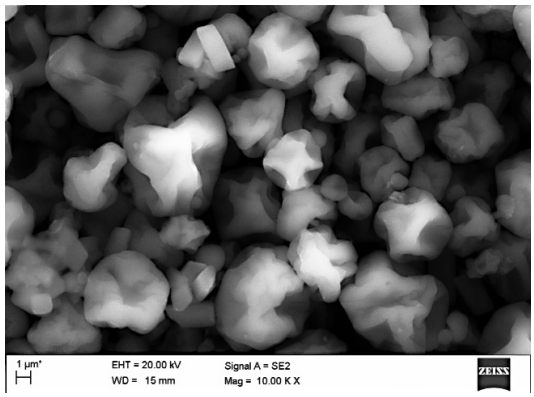

Figure 1. Photo, made by the scanning electron microscope (SEM), of microspheres in proportion losartan potassium : Eudragit L30D55 1 : 1 dried in parameters: inlet temperature 150°C, delivery of a pump 20%, aspirator capacity 70% (magnification 1000×)

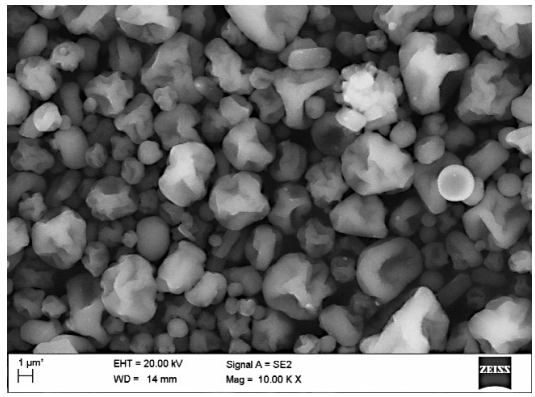

Figure 2. Photo, made by the scanning electron microscope (SEM), of microspheres in proportion losartan potassium : Eudragit L30D55 1 : 1 dried in parameters: inlet temperature 150°C, delivery of a pump 10%, aspirator capacity 80% (magnification 1000×)

Microspheres dried with addition of triethyl citrate had an optimal size and shape, which is especially clear in the case of samples where the plasticizer addition was 10% and 15%, respectively.

Triethyl citrate had also a beneficial effect on the size of microspheres, there was only a slight reduction of average particle size and improvement in a quality of microspheres. The increase of efficiency of the process was noticed in samples to which plasticizer was added in the amount of 5, 10, and 15%. The drying efficiency was improved at a level of 5%. The best effect on both, the process efficiency and morphology of achieved microspheres had an addition of plasticizer in the amount of 10% in terms of dry matter of losartan and Eudragit L30D55. The same amount of triethyl citrate was used in further studies. The detailed data on efficiency as well as the average size of particles, and a detailed description of morphological parameters of obtained microspheres after addition of triethyl citrate are shown in Table 3.

Citric acid had a negative effect on all parameters of spray drying process of losartan potassium with Eudragit L30D55. Each addition of plasticizer caused deterioration of particle morphology, and had a negative effect on process efficiency. The average size of particles increased but the quality of obtained microspheres deteriorated. The microscope image presents a great number of irregular particles and forms of non-spherical structure. The efficiency of the process after adding citric acid decreased to 25%. The detailed data on the efficiency of the process, size and morphology of particles are presented in Table 4.

In case of microspheres obtained without addition of triethyl citrate, we can observe the Eudragit L30D55 crystals ("broom shaped" structures that are visible on the image of sample F9), which cannot be seen in a microscope image of microspheres dried with addition of triethyl citrate.

Increased amount of polymer negatively affected the quality of obtained microspheres: the size of particle increased and the morphology deteriorated. Particular data on efficiency of the process, size and morphology of particles are shown in Table 5.

Figures 3 and 4 present the comparison of microspheres dried with addition of triethyl citrate in amount of 10% in terms of dry matter of losartan potassium and Eudragit L30D55 in proportion 1 : 1 in comparison to microspheres dried without addition of plasticizer.

Figures 3A, 4A, 3B and 4B illustrate respective microspheres with and without addition of plasticizer.

Influence of the amount of applied Eudragit on size and morphology of achieved microspheres

The process involved drying of losartan potassium suspensions on Eudragit L30D55 in the proportion of 1 : 1 to 1 : 4; to each formulation triethyl citrate was added in the amount of 10% in terms of dry matter. The obtained product was collected in a collector, weighed and its size and morphology were determined by a scanning microscopy technique. The results are presented in Table 6.

Figures 5 and 6 show selected scanning electron microscope images of microspheres, for samples presented in Table 6.

Table 4. Drying results of losartan potassium on Eudragit L30D55 in proportion 1:1 with addition of plasticizer (citric acid) and characteristics of generated microspheres.

Citric acid relation to the dry matter [%]	Product mass [mg]	Process efficiency [%]	Average size of particles [mm]	Microspheres morphology	Sample symbol
0.5	122	30.50	2.87 – 9.24	Disturbed structure of particle, many forms with irregular shape	F8
1	120	30.00	2.17 – 10.15	Presence of structures with irregular shapes, small number of spherical and oval forms, particle size diversified	F9
2	117	29.25	2.29 – 11.47	Clear irregular morphology of particles, no spherical and oval particles	F10

Table 5. Characteristic of microspheres dried with a method of spray drying without addition of plasticizer.

Proportion losartan potassium : Eudragit L30D55	Product mass [mg]	Process efficiency [%]	Average size of particles [mm]	Microspheres morphology	Sample symbol
1 : 1	165	41.25	1.27 – 7.24	Predominance of spherical forms and forms similar in shape to spherical with slightly smudgy edge, very few forms of other shape	F1
1 : 2	294	49.00	1.32 – 8.57	Domination of oval forms, very few spherical forms, presence of forms with irregular shape	F2
1 : 3	354	44.25	1.95 – 12.96	Clearly visible forms with very irregular shape, no spherical forms, big disproportion in particle size	F3

Table 6. Characteristics of microspheres spray dried of losartan potassium on Eudragit L30D55 in proportion from 1 : 1 to 1 : 4 with addition of triethyl citrate in amount of 10% in calculation to dry matter drug:polymer.

Proportion losartan potassium : Eudragit L30D55	Product mass [mg]	Process efficiency [%]	Size of particles [mm]	Morphology	Sample symbol
1 : 1	172	43.00	0.82 – 6.97	Clearly spherical particles, very few oval forms	W1
1 : 2	292	48.67	0.98 – 7.04	Spherical particles, some of them contain a pit in central part of coat, very few particles of irregular shape	W2
1 : 3	415	51.88	1.06 – 7.71	Spherical particles, some of microspheres contain a recess in central part of coat, occurrence of irregular shape particles and particles containing recess in part of coat	W3
1 : 4	523	52.30	1.26 – 10.12	Presence of spherical particles with pits in part of coat, occurrence of more numerous particles of irregular shape, presence of particles with shape resembling cone	W4

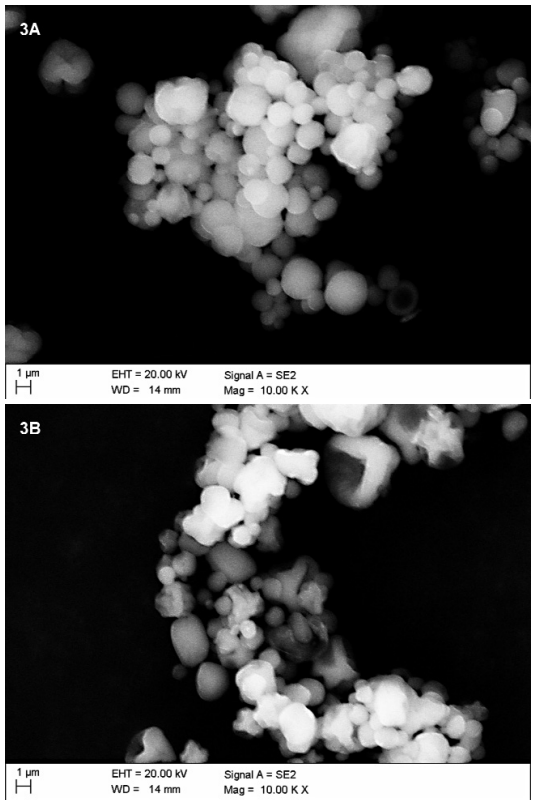

Figure 3. Photo, made by the scanning electron microscope (SEM), of microspheres in proportion losartan potassium : Eudragit L30D55 1 : 1. (3A contains the addition of triethyl citrate (sample F6 from Table 4), 3B without plasticizer (sample F1 from Table 1)

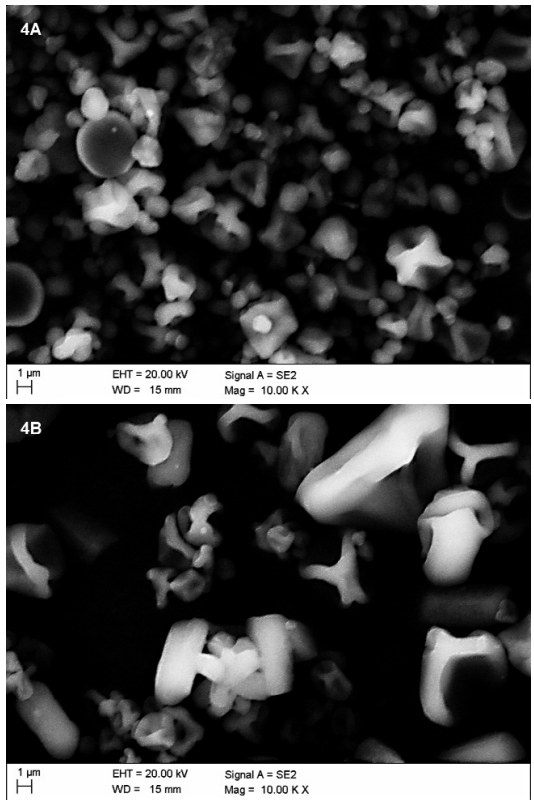

Figure 4. Photo, made by the scanning electron microscope (SEM), of losartan potassium microspheres : Eudragit L30D55. On the left side of microsphere in proportion losartan potassium : Eudragit L30D55 1 : 1 with addition of citric acid in amount of 10% of dry matter drug : polymer (sample F9 from Table 5 – Fig. 4A), on the right side without addition of plasticizer, proportion drug : polymer 1 : 3 (sample F3 from Table 6 – Fig. 4B)

DISCUSSION

The method of spray drying was used in this study to obtain microspheres with losartan potassium. Losartan potassium, the antagonist of an angiotensin II receptor, was used as a standard active substance (model substance). The medication was incorporated into the polymer matrix containing Eudragit L30D55 and plasticizer, i.e., triethyl citrate with established experimental content of 10%. The optimization of the spray drying process and determination of the optimal amount and type of used plasticizer were tested.

The research enables to evaluate the influence of peristaltic pump performance on morphology of the received end product. In all temperatures under investigation (140–160^{o}C), a disturbance in particle morphology together with the increase in peristaltic pump performance can be observed. The most spherical forms were obtained by peristaltic pump performance at 10%. The increase of the delivery of a peristaltic pump, was accompanied by the increased size of microspheres. The efficiency of this process deteriorated when values of pump performance increased.

Similar results were obtained by Esposito et al. (25). They observed that application of a flow rate of 0.5 mL/min led to creation of spherical forms with smooth surfaces, whereas, the increase of a flow rate to 5 mL/min disturbs particle morphology, resulting in the formation of particles with more irregular surfaces. At the same time, they drew the attention to depositing large quantities of the end product on the cyclone walls. Billancetti et al. (26), by decreasing the flow rate of liquid through the pump, reached a slight improvement of the pump performance, which is compatible with the experimental results of losartan potassium drying.

The study allows to select the optimal conditions for spray drying of losartan potassium with

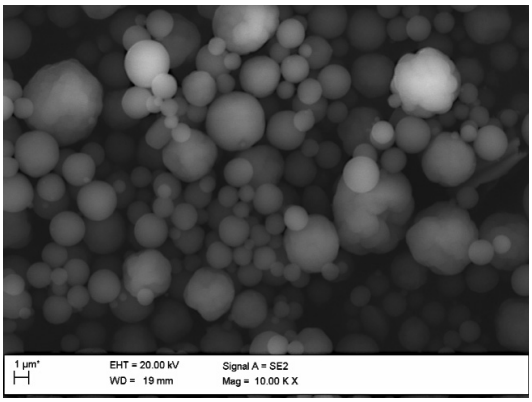

Figure 5. Photo, made by the scanning electron microscope (SEM), of microspheres in proportion losartan potassium : Eudragit L30D55 1 : 1 with addition of triethyl citrate in amount of 10% of dry matter drug : polymer (sample W1 magnification 10000×)

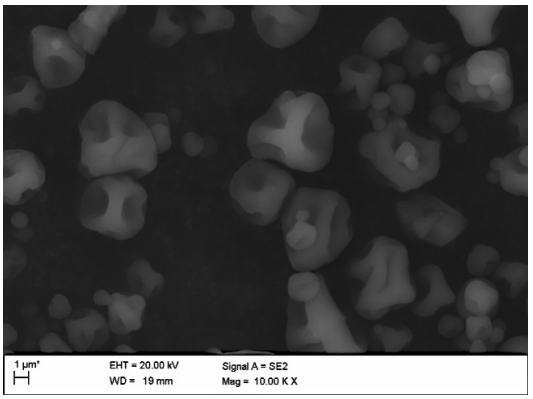

Figure 6. Photo, made by the scanning electron microscope (SEM), of microspheres in proportion losartan potassium : Eudragit L30D55 1 : 3 with addition of triethyl citrate in amount of 10% of dry matter drug : polymer (sample W3 magnification 10000×)

Eudragit L30D55. The most adequate parameters that were used for further study are: input temperature of drying gas of 150°C, peristaltic pump performance: 10%, and aspirator capacity of 80%. Application of these values enables to obtain almost 42% of end product efficiency, and ensures spherical morphology of particles.

The addition of plasticizer was also estimated in the study. The research of Rujivipat and Bodomier (27) proved that matrixes containing copolymer of metacrylic acid were not flexible enough and require the presence of plasticizer. Correspondingly to the study of Sawicki and Makulec (24), triethyl citrate was used, whereas citric acid as a plasticizer was tested in the study of Szymańska and Winnicka (2). Triethyl citrate was added in the proportion of 2, 5, 10, and 15% to dry matter of losartan potassium formulation: Eudragit L30D55 in proportion of 1 : 1. Citric acid was added in proportions of 0.5, 1, and 2% to dry matter of losartan potassium formulation: Eudragit L30D55 in proportion of 1 : 1. The morphological properties of the end product, and efficiency of reaction were accepted as the criterion of assessment.

The research has shown a positive influence of triethyl citrate on morphology and efficiency of microspheres, and a negative influence of citric acid on both parameters. The obtained microspheres dried with addition of triethyl citrate were characterized by an expected size and shape, correspondingly to the published standards. Triethyl citrate should be used in the quantity of 10 to 15%. We observed an increase in efficiency of the process in samples with added plasticizer in the amount of 5, 10, and 15%, and at these values the efficiency of drying improved approximately by 5%. Snejdowa et al. (28) suggest that added plasticizer could constitute as much as 30% of formulation. In their work, Sawicki and Makulec (24) claimed that triethyl citrate had a positive influence not only on particle morphology but also it improved the adhesive properties, and increased the flexibility of polymer matrixes.

Citric acid had a negative influence on all parameters of spray drying process of losartan potassium on Eudragit L30D55. Addition of this plasticizer resulted in deterioration of particle morphology, and had a negative influence on the efficiency of the process. The average size of particles increased but the quality of microspheres deteriorated.

CONCLUSIONS

1. Application of spray drying technique to produce microspheres with losartan potassium as an active substance on the Eudragit L30D55® matrix, allows to obtain particles with beneficial morphological parameters and a particle size.
2. The most beneficial morphological parameters of microspheres were obtained with the following parameters of spray drying: inlet temperature of 150°C, delivery of a peristaltic pump of 10%, aspiration value of 80%.
3. In order to improve the morphological properties of microspheres, plasticizers such as triethyl cit-

rate and citric acid, whose tests were reported in the literature, were verified.

A detailed study of the influence of both plasticizers on the properties of microspheres with losartan potassium on Eudragit L30D55 matrix indicated a positive effect of triethyl citrate and a negative effect of citric acid on morphological properties, shape and particle size.

4. Application of optimal parameters of spray drying and triethyl citrate as plasticizer in amount of 10 to 15% enables to obtain microspheres from 1.27 μm to 7.24 μm.

REFERENCES

1. Jachowicz R., Czech A., Jamróz W.: Farm. Pol. 65, 285 (2009).
2. Szymańska E., Winnicka K.: Farm. Pol. 65, 378 (2009).
3. Li X., Anton N., Ta T.M., Zhao M., Messaddeq N., Vandamme T.F.: Int. J. Nanomedicine 6, 1313 (2011).
4. Vasir J.K., Tambwekar K., Garg S.: Int. J. Pharm. 255, 13 (2003).
5. Sam M.T., Gayathri D.S., Prasanth V., Vinod B.: Internet J. Pharmacol. 6, 1 (2008).
6. Halkiewicz A., Janicki S.: Farm. Pol. 19, 836 (1995).
7. Szymańska E., Winnicka K.: Gazeta Farm. 10, 40 (2009).
8. Szymańska E., Winnicka K., Muśko M.: Farm. Pol. 66, 238 (2010).
9. Pattabhi K., Chowdary R., Srinivasa Rao Y.: Biol. Pharm. Bull. 27, 1717 (2004).
10. Bowey K., Neufeld R.J.: Biodrugs 24, 359 (2010).
11. Gac J., Gradoń L.: Inż. Ap. Chem. 50, 32 (2011).
12. Cal K., Sollohub K.: J. Pharm. Sci. 99, 576 (2010).
13. Sznitowska M.: Farm. Pol. 57, 962 (2001).
14. Samborska K.: Post. Tech. Przetw. Spoż. 1, 63 (2008).
15. Rizi K., Green R.J., Donaldson M., Williams A.C.: J. Pharm. Sci. 100, 566 (2011).
16. Raffin R.P., Jornada D.S., Ré M.I., Pohlmann A.R., Guterres S.S.: Int. J. Pharm. 324, 10 (2006).
17. Rattes A., Oliveira W.: Powder Tech. 171, 7 (2007).
18. Kawecka-Jaszcz K., Czarnecka D., Wojciechowska W.: Nadciśnienie Tętnicze A1 (2006).
19. Prejbisz A., Januszewicz A.: Choroby Serca i Naczyń 3, 195 (2006).
20. Ishtiaq A., Muhammad I., Habibur R.: Bangladesh Pharm. J. 13, 9 (2010).
21. Musiał W., Kubis A.: Polim. Med. 35, 39 (2005).
22. Tang E., Chan L., Heng C.: Am. J. Drug Deliv. 3, 17 (2005).
23. Csóka G., Gelencsér A., Kiss D., Pásztor E., Klebovich I., Zelkó R.: J. Term. Anal. Cal. 87, 469 (2007).
24. Sawicki W., Makulec A.: Farm. Pol. 65, 311 (2009).
25. Esposito E., Roncarati R., Cortesi R., Cervellati F., Nastruzzi C.: Pharm. Dev. Technol. 5, 267 (2000).
26. Bilancetti L., Poncelet D., Loisel C., Mazzitelli S., Nastruzzi C.: AAPS PharmSciTech. 11, 1257 (2010).
27. Rujivipat S., Bodmeier R.: Eur. J. Pharm. Biopharm. 81, 1 (2012).
28. Snejdowa E., Dittrich M.: Pharmaceutically used plasticizers. in: Recent Advances In Plasticizers. Luqman M. Ed., pp. 45–68, InTech, Rijeka 2012.

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PHARMACOLOGY

BIOEQUIVALENCE STUDY OF 400 AND 100 MG IMATINIB FILM-COATED TABLETS IN HEALTHY VOLUNTEERS

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Abstract: The aim of the study was to investigate the bioavailability of a generic product of 100 mg and 400 mg imatinib film-coated tablets (test) as compared to that of a branded product (reference) at the same strength to determine bioequivalence. The secondary objective of the study was to evaluate tolerability of both products. An open-label, randomized, crossover, two-period, single-dose, comparative study was conducted in 43 (Imatynib-Biofarm 100 mg film-coated tablet) and in 42 (Imatynib-Biofarm 400 mg film-coated tablet), brand name Imatenil, Caucasian healthy volunteers in fed conditions. A single oral dose administration of the test or reference product was separated by 14-day washout period. The imatinib and its metabolite N-desmethyl imatinib concentrations were determined using a validated LC MS/MS method. The results of the single-dose study in healthy volunteers indicated that the film-coated tablets of Imatynib-Biofarm 100 mg and 400 mg film-coated tablets manufactured by Biofarm Sp. z o.o. (test products) are bioequivalent to those of Glivec 100 mg and 400 mg film-coated tablets manufactured by Novartis Pharma GmbH (reference products). Both products in the two doses of imatinib were well tolerated.

Keywords: imatinib, bioequivalence, relative bioavailability, tolerability

Imatinib mesylate is potent revolutionary antineoplastic medication of high specificity (1–3). It functions at the molecular level inhibiting activity of particular tyrosine kinase enzymes, namely bcr-abl kinase, platelet-derived growth factor (PDGF) receptor and receptor for stem cell factor (c-kit receptor) at submicromolar concentrations. In consequence, the signal transduction *via* ligand-stimulated receptor autophosphorylation, inositol phosphate formation and mitogen-activated protein kinase (MAP kinase) activation is selectively inhibited with resultant cell proliferation arrest (4, 5). This abnormal enzyme is associated with the Philadelphia chromosome (Ph^+), which is a consequence of reciprocal translocation of genetic material between chromosomes 9 and 22 with generation of the chimeric BCR-ABL fusion gene. The highly elevated catalytic activity of the enzyme encoded by BCR-ABL fusion gene leads to a resistance to apoptosis, cell transformation and malignancy (including chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL)), and altered cell adhesion. Protein product of BCR-ABL fusion gene is present in 95% of CML and 10–25% of adult ALL cases. Imatinib mesylate competitively blocks the binding of ATP to the activation domain of bcr-abl, which modulates phosphorylation status of the kinase and diminishes its activity (3, 6). In consequence, imatinib mesylate suppresses the proliferation of bcr-abl+ cells (3). The efficacy of imatinib mesylate (STI571) in newly diagnosed chronic Ph^+ CML was evaluated in an open-label, multicentre, international phase III study referred as International Randomized Study of Interferon and STI571 (IRIS)

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(7–9). Moreover, given the literature data, combining imatinib mesylate with conventional chemotherapy for the treatment of newly diagnosed or minimally treated Ph^+ ALL may yield complete remission rates approaching 95%. The agent may be administered either concurrently or sequentially with chemotherapy (10).

Nowadays, it is known that certain other human tumors are considered to be associated with deregulation of c-kit receptor. These are gastrointestinal stromal tumors (GIST), germ cell tumors, mast cell tumors, small-cell lung cancers, melanoma, breast cancer, and neuroblastoma. Abnormal cell growth has been linked to mutation of c-kit, which leads to ligand-independent activation of the receptor (3, 5, 11–13). There are many studies from which it is well documented that imatinib mesylate has been shown to be highly effective in the treatment of unresectable and/or metastatic GIST, resulting in a substantial improvement in the survival rate. Imatinib has, therefore, become the standard of care in patients with advanced GIST (14–17).

Imatinib mesylate was also demonstrated to be effective in the treatment of adult patients with hypereosinophilic syndrome (HES) and/or chronic eosinophilic leukemia (CEL) who are Fip1-like1-platelet-derived growth factor receptor α chain (FIP1L1-PDGFRα) fusion kinase positive (18–21).

Imatinib mesylate has favorable pharmacokinetic profile. It is well absorbed form gastrointestinal tract, with bioavailability of almost 100% (22–24). At clinically relevant concentrations imatinib mesylate is highly bound to plasma protein (ca. 95%), primarily to a1-acid glycoprotein (22, 25). It undergoes a considerable metabolism in the liver, mainly *via* CYP3A4. The main metabolite, CGP 74588, is known to be active, and the elimination of this and other metabolites is more than 90% through the bile (22, 23). The elimination half-life of imatinib mesylate is approximately 18 h, with two- to three-fold accumulation at steady-state (22, 26).

The recommended dose of imatinib mesylate is 400 mg/day for adult patients in chronic phase CML and 100 mg/day with HES/CEL (27). The drug is a generally well tolerated drug, with chronic oral daily dosing.

The reference product in the present study was an already approved and commercially available Glivec (100, 400 mg), film-coated tablets (Novartis Pharma GmbH).

For the registration purposes, the efficacy and safety of this product has been proven already in clinical trials. This drug has therefore served as a reference and a basis for comparison to a imatinib mesylate test product: Imatynib-Biofarm (Imatenil). It is known that imatinib mesylate exists in two polymorphic forms α and β, which differ in their physicochemical properties i.e., melting points (t_{onset} 226°C and 217°C, respectively) or entalphy of thermal events (36). Moreover, the both polymorphs exhibit a tendency to generate amorphous form during some technological processes i.e., grinding (36). There are some suggestions that differences in the apparent solubilities of the various polymorphic forms can affect the drug bioavailability and bioequivalence (37). Since imatinib mesylate in the Imatynib-Biofarm (Imatenil) exists in α form only (38), whereas in Glivec the β form is postulated (39), therefore the differences in their bioavailability cannot be excluded. So, the aim of the study was to investigate the pharmacokinetic properties and the bioequivalence of imatinib from a new preparation: Imatynib-Biofarm 100 and 400 mg film-coated tablets Imatenil (Biofarm Sp. z o.o.) compared with the reference formulation Glivec (100, 400 mg), film-coated tablets (Novartis Pharma GmbH) following a single oral dose administration of 100 or 400 mg under fed conditions. Moreover, its active metabolite derivative – desmethyl imatinib (CGP74588) pharmacokinetics was planned to be evaluated as supportive information. The secondary objective of the study was to compare tolerability of the products.

EXPERIMENTAL

Subjects and Methods

Study products

The test products were: Imatynib-Biofarm 100 mg film-coated tablet (Imatenil) batch no: 030709 and Imatynib-Biofarm 400 mg film-coated tablet (Imatenil) batch no: 011210 manufactured by Biofarm Sp. z o.o. The reference product was Glivec (100 and 400 mg, film-coated tablets, batch no. S0041 and batch no. S0197, respectively, manufactured by Novartis Pharma GmbH.

Subjects

The study was performed as a single centre, open-labeled, randomized, two-period, 2-way crossover, single dose study under fed conditions with a washout period of 14 days between drug administrations in each treatment period. Subjects were selected according to the inclusion and exclusion criteria in order to obtain a low individual variability within the subject group. The demographic characteristics of the study population was shown in

Table 1. Healthy willing male and postmenopausal female subjects (male aged between 21 and 58 years and female aged between 42 and 58 years), able to communicate clearly with the study personnel and able to give written consent for participation in the study, who were non-smokers or no-users of tobacco products for at least ninety (90) days before screening, having body mass index (BMI) between 18.50 kg/m^2 and 26.99 kg/m^2 (the minimum body weight for males was not less than 60 kg, for females – not less than 50 kg), having no significant diseases (current or past), no clinically significant abnormal laboratory values or clinically significant abnormal results of 12-lead ECG, vital signs and chest X-ray, with physical examination without any clinically relevant abnormality were randomized and included to the study.

A single dose of test drug (Imatynib-Biofarm, 100 or 400 mg film-coated tablet) or a single dose of reference drug (Glivec, 100 or 400 mg film-coated tablet) were administered by the oral route for subsequent subjects in the morning on days 1 and 15 (treatment period I and II) in the sitting position, according to the randomization list and under open-label conditions. The tablet was administered, 30 min after standardized breakfast, with 240 mL of boiled water (at room temperature). No other food was allowed until 4 h after drug administration. No fluid intake apart from the fluid given at the time of drug intake was allowed from 2 h before until 2 h after dosing.

Study design

This study was prepared according to the Note for Guidance on the Investigation of Bioavailability and Bioequivalence, CPMP/EWP/QWP/1401/98 (28) and the Note for Guidance on the Investigation of Bioavailability and Bioequivalence, CPMP. EWP/QWP/1401/987/Rev 1 (29). The use of a generic preparation of a therapeutically well-established active drug principle has to be justified by an appropriate bioequivalence study, because the proof of bioequivalence of the test and reference products assures equal therapeutic efficacy. The study was conducted between January 2010 and June 2010 (for the dose of 100 mg) and December 2010 and April 2011 (for the dose of 400 mg) by Lambda Therapeutic Research Sp. z o.o., Centrum Badań Klinicznych NZOZ in Warszawa in compliance with the approved Protocols in adherence to Good Clinical Practices and Ethical Principles, as described in: ICH harmonized Tripartitate Guidelines for Good Clinical Practice and World Medical Association Declaration of Helsinki and its amendments (30, 31). Ethical approval was received from the Independent Ethics Committee (IEC) in Warszawa. The clinical trial registration numbers of the study were KB/693/09 for Imatynib-Biofarm 100 mg film-coated tablet and KB/733/10 for Imatynib-Biofarm 400 mg film-coated tablet. The Ministry of Health approval was obtained on January 21, 2010 for Imatynib-Biofarm 100 mg film-coated tablets (approval number CEBK/0034/10) for Imatynib-Biofarm 400 mg film-coated tablet was obtained on January 11, 2011 (approval number CEBK/0007/11). The studies were registered in EudraCT and obtained the numbers: EudraCT 2009-016180-10 and 2010-021028-91 for the dose of 100 mg and 400 mg, respectively. All eligible subjects provided written informed consent to participate and were free to withdraw from the study at any time without any obligation.

Blood sample collection

Blood samples for determination of imatinib and it's metabolite concentrations were collected up to 96 h after the drug administration in 19 time points in each treatment period.

Just before the first blood sampling (the morning of a day of drug administration), a cannula was introduced into a vein and blood samples were collected during the study by means of this cannual till pharmacokinetic blood sample at 72-h post dose in each treatment period. After the cannulation of the vein, a pre-dose PK sample of 7 mL was collected. Blood samples were obtained prior to dosing (baseline) and 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 5.0; 6.0; 8.0; 12.0; 16.0; 24.0; 36.0; 48.0; 72.0 and 96.0 h post-dose (19 samples per subject in each treatment period). Blood was collected from cubital vein or forearm vein or from veins into the tubes with anticoagulant. After collection, the blood samples were immediately cooled in an ice bath and then were centrifuged at 3000 ± 100 rcf or 4200 ± 100 rpm for 5 min below 10^{o}C to separate plasma. The separated plasma in the study for Imatynib-Biofarm 100 mg was transferred to pre-labelled polypropylene tubes in 2 aliquots (around 0.7 mL in case of pre dose sample and rest of the volume in second lot) and stored upright at a temperature –55^{o}C or colder for interim storage until shipment to bioanalytical laboratory, for analysis.

The separated plasma in the study for Imatynib-Biofarm 400 mg was transferred to pre-labelled polypropylene tubes in 2 aliquots (around 1.25 mL in first lot, 1.75 mL in case of pre dose sample and rest of the volume in second lot) and stored upright at a temperature –55^{o}C or colder for

interim storage until shipment to bioanalytical laboratory, for analysis.

Determination of imatinib and its metabolite N-desmethyl imatinib plasma concentrations

Plasma samples of subjects, were assayed for imatinib and its metabolite N-desmethyl imatinib (CGP74588) using a precise and accurate LC-MS/MS method (ACQUITY UPLC BSM binary solvent manager, Acquity UPLC SM sample manager with column heater and Waters Quattro Premier XE mass spectrometer) with imatinib-d_8 & N-desmethyl imatinib-d_8 as the internal standards (ISTD), which is validated according to the international guidelines at Lambda Therapeutic Ltd., UK. The method has been developed and validated for calibration curve ranging from 5.012 to 2999.700 ng/mL for imatinib and from 1.999 to 400.660 ng/mL for N-desmethyl imatinib. Briefly, the analytes and internal standards were extracted from plasma using liquid-liquid extraction method. It means that the frozen calibration curve standards, quality control samples and the study samples were thawed in a water bath maintained at room temperature and vortexed to ensure complete mixing of contents. Two hundred fifty microliters of each of the calibration curve standards, quality control samples and the subject samples were aliquoted into pre-labeled tubes. Fifty microliters of the ISTD dilution mixture (about 200 ng/mL of imatinib-d_8 and about 375 ng/mL of N-desmethyl imatinib-d_8) was added to each tube except for standard blank, Subject blank and blank QC tubes and vortexed for 1 min. Thereafter, 100 µL of 100 mM ammonium formate buffer (pH 7.0) was added to each tube and vortexed for 1 min followed by the addition of 4 mL of extraction solution and vortexed for 10 min. The samples were centrifuged at 3345 ± 150 rcf for 5 min at 10°C using imatinib-d_8 and N-desmethyl imatinib-d_8 as the internal standards. The plasma layer was flash-frozen in alcohol freezing bath and organic layer was transferred into pre-labeled tubes. The contents were evaporated to dryness at room temperature under nitrogen stream and reconstituted with 250 µL of the reconstitution solution and vortexed. Next, the contents were transferred into appropriate autosampler vials for analysis. Ten microliters of each sample was chromatographed on Hypersil GOLD, 150 × 4.6 mm, 5 µm column maintained at 40°C using an binary mode of mobile phase system composed of 70% of acetonitrile and 30% of 2 mM ammonium formate buffer (pH 3.5). Imatinib and ISTD-1 and N-desmethyl imatinib and ISTD-2 were monitored in the positive ion mode using the MRM transitions (m/z 494.22 > 394.10 for imatinib, m/z 502.00 > 394.10 for ISTD-1, m/z 480.20 > 394.10 for N-desmethyl imatinib, m/z 488.44 > 394.15 for ISTD-2) and retention times (imatinib and ISTD-1 – 1.74 min; N-desmethyl imatinib and ISTD-2 – 1.70 min). MassLynx Software Version 4.1 was used for the evaluation of chromatographic data. A linear equation was judged to produce the best fit for the concentration *vs.* area response relationship. The regression type was 1/concentration and peak area ratio for an 8-point calibration curve was found to be linear from 5.012 (lower limit of quantification) to 2999.700 ng/mL for imatinib and from 1.999 (lower limit of quantification) to 400.660 ng/mL for N-desmethyl imatinib with correlation coefficient (r) greater than 0.99 for imatinib and N-desmethyl imatinib during the course of validation. For imatinib, the range of precision and accuracy of the back-calculated concentrations of the standard curve points was from

Table 1. The demographic characteristics of the study population.

Parameter (units)	Imatynib-Biofarm, 100 mg and Glivec, 100 mg		Imatynib-Biofarm, 400 mg and Glivec, 400 mg	
	Mean ± SD			
	n = 43# (Subjects who were enrolled into the study)	n = 37 (Subjects who completed all phases of the study)	n = 42* (Subjects who were enrolled into the study)	n = 37 (Subjects who completed all phases of the study
Age (years)	32.1 ± 9.87	32.4 ± 9.84	33.9 ± 11,78	34.1 ± 11.59
Height (cm)	177.1 ± 7.62	177.4 ± 7.12	177.1 ± 8.70	177.4 ± 9.11
Weight (kg)	74.2 ± 8.10	75.0 ± 7.63	75.6 ± 7.28	75.6 ± 7.16
BMI (kg/m^2)	23.63 ± 1.89	23.83 ± 1.84	24.2 ± 1.79	24.1 ± 1.86

Arithmetic mean ± SD. # – 4 females; * – 4 females

1.0 to 5.5% and from 97.0 to 102.7%, respectively, whereas for N-desmethyl imatinib these parameters were from 1.9 to 4.2% and from 96.5 to 102.2%, respectively. Precision and accuracy of imatinib and N-desmethyl imatinib was determined for limit of quantification, low, medium and high concentrations of quality control samples in the biological matrix, based on the expected range. Accuracy (% nominal) for inter-day and intra-day was within 85–115% of the nominal value for all quality control samples except for LOQ QC, which was within 80–120%. For precision, the % CV was ≥ 15% for all quality control samples, except for LOQ QC, which was ≥ 20%.

Pharmacokinetic and statistical analyses

The pharmacokinetic parameters for imatinib and its metabolite N-desmethyl imatinib were determined from the plasma concentration *vs.* time curve with the aid of the WinNonlin Professional Software version 5.3. The parameters selected as primary endpoints of the study were: the area under the plasma concentration *vs.* time curve (AUC_{0-t}), and the maximum plasma concentration of the drug (C_{max}). The time to reach maximum plasma concentration of the drug (T_{max}), the elimination half-life ($t_{1/2}$) and lz as a first order rate constant associated with the terminal (log-linear) portion of the curve. C_{max} and T_{max} were obtained directly from the experimental data. The elimination rate constant (lz) was estimated by linear least squares regression analysis using at least last three or more non-zero plasma concentration values. The $t_{1/2}$ was calculated as ln 2/lz. The AUC_{0-t} was calculated as the area under the plasma concentration *versus* time curve from time zero to the last measurable concentration as calculated by linear trapezoidal method. The $AUC_{0-\infty}$ was calculated where $AUC_{0-\infty} = AUC_{0-t} + C_t/z$, where C_t is the last measurable concentration and z is the terminal elimination rate constant. For metabolite N-desmethyl imatinib: $AUC_{0-t} = AUC_{0-96}$ (truncated at 96 h). The residual area in percentage (AUC_%Extrap_obs) was determined by the formula $[(AUC_{0-\infty} - AUC_{0-t})/ AUC_{0-\infty}] \times 100$. The statistical calculations were performed using the SAS (Version 9.2 or higher) software. The tests for normality of ln-transformed pharmacokinetic parameters were performed with proper tests. The analysis of variance (ANOVA) was performed on the ln-transformed data. The statistical significance of effects was determined on basis of the calculated p-values with value larger than 0.05 meaning no statistical significance.

Based on the ANOVA results, 90% confidence interval (CI) for the μT/μR (ratio of geometric means for the test and the reference product) of the analyzed pharmacokinetic parameters was constructed. Bioequivalence was assumed when 90%

Table 2. Plasma pharmacokinetic parameters of imitanib after a single dose administration of the Imatynib-Biofarm 100 mg (test product) and the Glivec 100 mg (reference product).

Imatynib-Biofarm 100 mg (test product)					
Pharmacokinetic parameter	Arithmetic mean	Median	Geometric mean	SD	CV [%]
T_{max} (h)	2.9	2.5	2.5	1.5	51.7
C_{max} (ng/mL)	427	428	405	149	35.0
AUC_{0-t} (ng × h/mL)	6582	6510	6252	2169	32.9
$AUC_{0-\infty}$ (ng × h/mL)	67009	6558	6365	2244	33.4
λz (1/h)	0.041	0.040	0.041	0.0083	20.1
$t_{1/2}$ (h)	17.4	17.5	17.066	3.2	18.7
AUC_%Extrap_obs (%)	2.036	1.690	1.831	1.04	51.1
Glivec 100 mg (reference product)					
T_{max} (h)	3.2	3.0	2.8	1.5	47.8
C_{max} (ng / mL)	410	370	388	146	35.6
AUC_{0-t} (ng × h / mL)	6418	5999	6012	2344	36.5
$AUC_{0-\infty}$ (ng × h / mL)	6554	6104	6126	2440	37.2
λz (1 / h)	0.041	0.041	0.040	0.0088	21.3
$t_{1/2}$ (h)	17.5	16.9	17.2	3.8	22.0
AUC_%Extrap_obs (%)	2.141	1.715	1.871	1.45	67.7

Table 3. Plasma pharmacokinetic parameters of N-desmethyl imatinib after a single dose administration of the Imatynib-Biofarm 100 mg (test product) and the Glivec 100 mg (reference product).

Imatynib-Biofarm 100 mg (test product)					
Pharmacokinetic parameter	Arithmetic mean	Median	Geometric mean	SD	CV [%]
T_{max} (h)	3.2	3.0	2.7	1.8	57.0
C_{max} (ng/mL)	40.6	37.1	38.4	14.9	36.6
AUC_{0-t} (ng × h/mL)	937	861	898	282	30.1
$AUC_{0-\infty}$ (ng × h/mL)	1136	1016	1088	355	31.3
λz (1/h)	0.017	0.017	0.017	0.0035	20.6
$t_{1/2}$ (h)	17.3	16.0	16.6	5.2	30.0
AUC_%Extrap_obs (%)	17.3	16.0	16.6	5.2	30.0
Glivec 100 mg (reference product)					
T_{max} (h)	3.3	3.0	2.9	1.7	52.1
C_{max} (ng / mL)	39.0	38.2	37	12.8	32.9
AUC_{0-t} (ng × h / mL)	9434	833	889	335	35.5
$AUC_{0-\infty}$ (ng × h / mL)	1153	1018	1084	418	36.2
λz (1 / h)	0.016	0.016	0.016	0.0033	20.0
$t_{1/2}$ (h)	44.0	44.0	43.0	10.2	23.2
AUC_%Extrap_obs (%)	17.8	16.5	17.1	5.6	31.3

CI of the point estimate (test over reference products) for AUC_{0-t}, $AUC_{0-\neq}$ and for C_{max} falls within the 80.00–125.00% range and when the Schuirmannis TOST test (two one-sided *t*-test) was complied ($p < 0.05$) (28, 29, 32, 33). The statistical analysis for T_{max} was performed on the untransformed data using the non-parametric Wilcoxon test.

Tolerability/safety analysis

In order to prevent the occurrence of an adverse events during the study, the following measures have been taken: the drug administration was limited to a single oral dose of 100 or 400 mg/study period; only healthy adult volunteers with no history of hypersensitivity reactions to the drug or other related molecules were enrolled; the investigator has checked each volunteer's well being prior to his/her discharge from the clinic. Tolerability and safety were determined by monitoring vital signs (blood pressure, heart rate, body temperature) at baseline and at the end of each period. Laboratory results (hematology, urinalysis, blood biochemistry) collected before and after the study of all the subjects were also considered. The participants were interviewed by the physician as well as nonspecific questioning. All the subjects were advised to report any adverse event or undesirable sign or symptom at any time during the study period.

RESULTS

Study population

The study was conducted in 43 (Imatynib-Biofarm 100 mg, film coated tablet) and in 42 (Imatynib-Biofarm 400 mg, film coated- tablet) Caucasian non-smoking healthy male and female subjects. In each studies 37 subjects completed all phases of the study. The results of the physical examination for all subjects during the pre-study visit and post-study visit were found to be normal. No subject abandoned the study for any reason. The summary of the demographic data of the population is presented in Table 1. The clinical part of the study with 100 mg of Imatynib-Biofarm was completed without deaths, serious adverse events and suspected unexpected serious adverse reactions. During the study, a total number of adverse events was 35. During the whole study period, 35 non-serious adverse events were reported in 18 subjects. No deaths or serious adverse events were reported during the study. Twelve adverse events occurred in treatment period I, 1 adverse event in wash-out period, 15 in treatment period II and 7 adverse events occurred during the follow-up examination. Twenty adverse events were of mild intensity and 15 of moderate. There was 1 adverse event classified as significant adverse event – allergic reaction. Moreover, there were 6 adverse

events in post-study laboratory values estimated as clinically relevant abnormalities. One adverse event was related to the study drug, 20 adverse events were possible related and 14 were not related to the study drug. All adverse events were resolved.

In the study for Imatynib-Biofarm 400 mg dose, the 43 non-serious adverse events were reported in 21 subjects for both product (test and reference). No death or serious adverse events were reported during the study. Twenty three adverse events occurred in treatment period I, 2 adverse events in wash-out period, 8 in treatment period II and 10 adverse events occurred during the follow-up examination. Thirty one adverse events were of mild nature, 11 moderate and 1 severe. Twenty nine adverse events were assessed as possibly related to the study drug and 14 as not related to the study drug. All adverse events were resolved. The adverse events were: ALT increased (4 events), vascular access complication (4 events), blood glucose increased (3 events), blood triglycerides increased (3 events), constipation (3 events), AST increased (2 events), CRF increased (2 events), weakness (2 events), abnormal body temperature (1 event), back pain (1 event), bad feeling (1 event), blood cholesterol increased (1 event), blood pressure increased (1 event), cough (1 event), diarrhea (1 event), dry skin (1 event), faint (1 event), headache (1 event), loose stools (1 event), muscle pain (1 event), nausea (1 event), pain of orchis (1 event), proteinuria (1 event), rhinitis (1 event), sleep disturbances (1 event), WBC abnormal (1 event), wind (1 event) and vertigo (1 event).

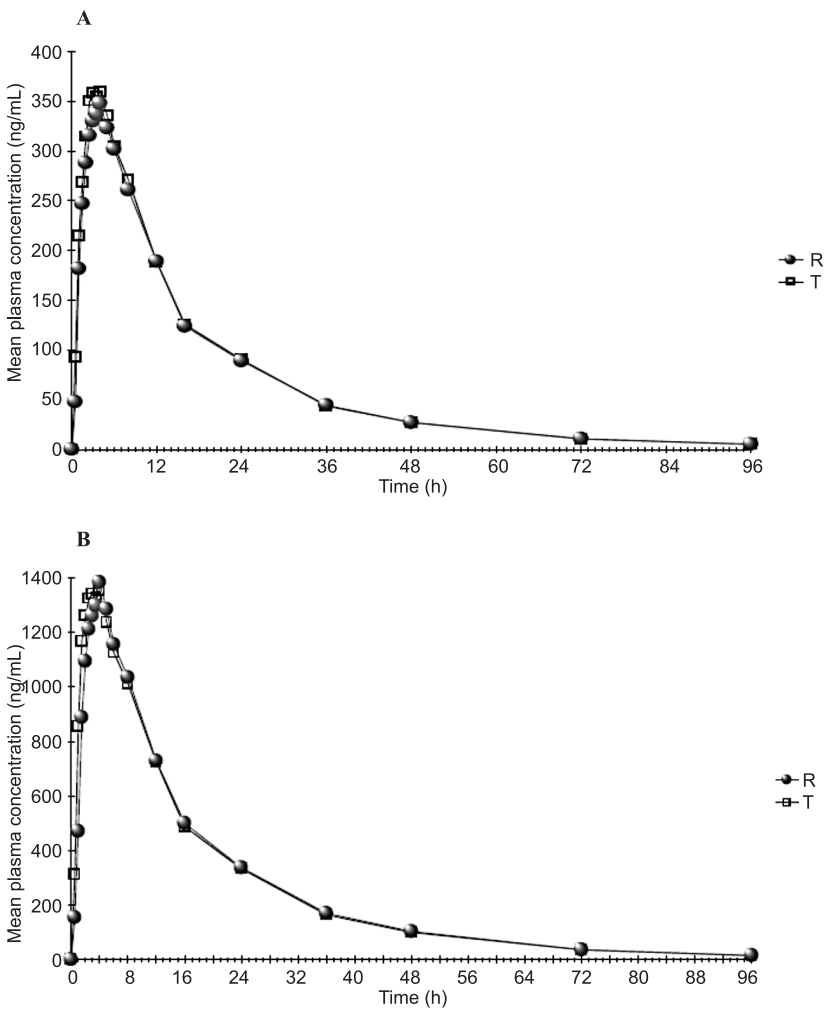

Figure 1. Linear plot of mean plasma concentration of imatinib *versus* time curves after administration of reference product- R (Glivec) and test product-T (Imatynib-Biofarm) under fed conditions in healthy volunteers. **A**: Glivec – 100 mg, Imatynib-Biofaam – 100 mg; **B**: Glivec – 400 mg, Imatynib-Biofarm – 400 mg

Pharmacokinetics and bioequivalence analysis

The mean plasma concentrations *vs*. time profiles after a single oral administration of both products with imatinib in two doses are shown in Figure 1, whereas its metabolite, N-desmethyl imatinib, in Figure 2. The descriptive statistics of pharmacokinetic parameters is shown in Tables 2, 4 for imatinib and in Tables 3, 5 for metabolite – N-desmethyl imatinib, respectively. It appeared that one cannot reject the hypothesis on the ln-normal distribution of the AUC_{0-t}, $AUC_{0-\infty}$, AUC_%Extrap _obs, C_{max}, $t_{1/2}$ and lz for both test and reference products at the significance level $\alpha = 0.05$. The $T_{max(s)}$ distribution values were significantly different from the normal distribution; therefore, in the subsequent analysis nonparametric tests were used for the evaluation. All primary pharmacokinetic parameters, i.e., AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, met the bioequivalence regulatory criteria and there were no statistical differences between proper values of the pharmacokinetic parameters (Tables 6, 7). The descriptive statistics for /λz and $t_{1/2}$ were similar for the test and reference products and there were no statistical differences between proper values of these pharmacokinetic parameters (Tables 2–5). The descriptive statistics of T_{max} of imatinib were similar for the test and reference products (Tables 2, 4), but lower variability in T_{max} was observed for the test product (CV = 47.8% (100 mg) and 39.6% (400 mg)) than for the reference product (CV = 51.7% (100 mg) and 58.6% (400 mg)), respectively. However, since p-value for Wilcoxon-Signed-Rank Test was $p > 0.05$, therefore

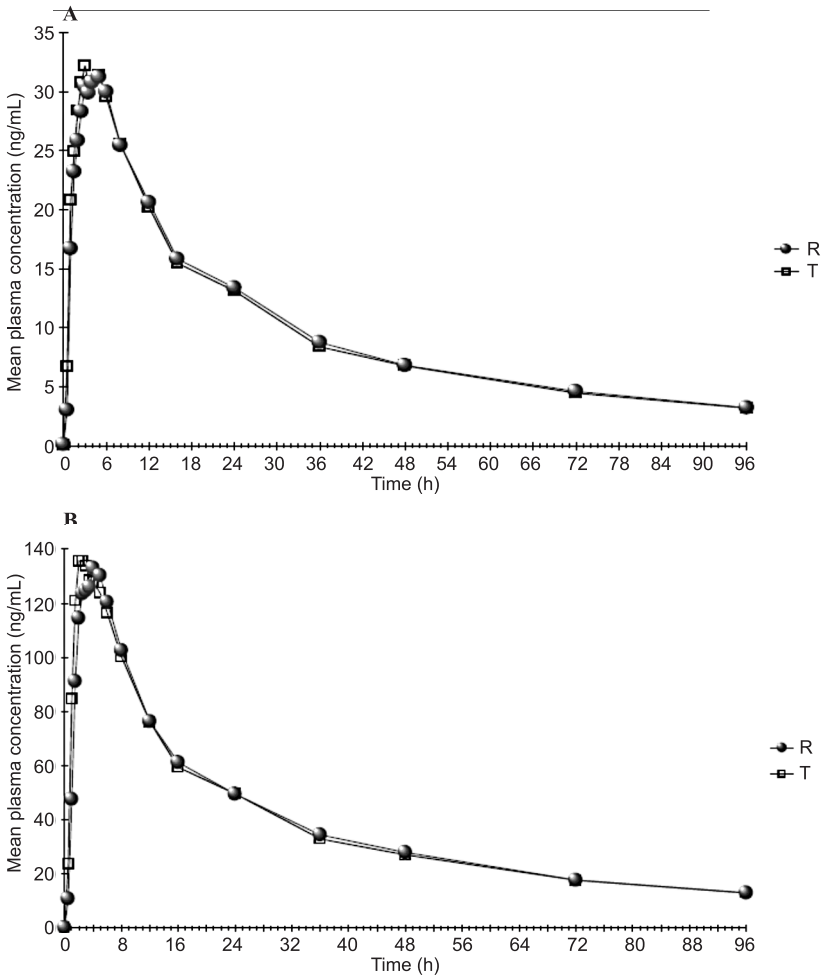

Figure 2. Linear plot of mean plasma concentration of N-desmethyl imatinib *versus* time curves after administration of reference product – R (Glivec) and test product –T (Imatynib-Biofarm) under fed conditions in healthy volunteers. **A**: Glivec – 100 mg, Imatynib-Biofarm – 100 mg; **B**: Glivec – 400 mg, Imatynib-Biofarm – 400 mg

the null hypothesis was not rejected and led to conclude that the difference between the two treatments R and T (both for 100 and 400 mg) with respect to pharmacokinetic parameter T_{max} was not significant. Similarly, there were not significant differences between T_{max} of N-desmethyl imatinib for test and reference products both in the dose of 100 and 400 mg (Tables 3, 5).

Tolerability/safety

Both products in two doses were well tolerated. Thirty seven subjects completed the study without any significant serious adverse events. No clinically significant abnormalities on physical examination including vital signs measurement, ECG recordings and laboratory results were observed.

DISCUSSION

The aim of the study was to evaluate the bioequivalence between the test (Imatynib-Biofarm manufactured by Biofarm Sp. z o.o.) and the reference (Glivec manufactured by Novartis Pharma GmbH) products. The clinical part of the study was designed in compliance with the respective EMA guidelines (28, 29). Based on the imatinib elimination half-life of 18 h (22, 26), and taking into account imatinib metabolite long half-life (about 40 h) (34) and that to avoid carryover effect in second study period washout period should be at least 5 times of the half-life long, wash-out period of 14 days was chosen in that study**.** For all profiles, the AUC_{0-t} was at least 80% of the AUC_{0-8}, which confirmed the proper duration of sampling. The current study demonstrated comparable bioavailability of Imatynib-Biofarm 100 and 400 mg film-coated tablets with Glivec 100 and 400 mg film-coated tablets, respectively. The pharmacokinetic values (C_{max}, T_{max}, and AUC) for N-desmethyl imatinib were also found to be nearly identical for both the test and reference dose forms of imatinib. If the 90% confidence interval of two one-sided tests for ratio of geometric means (test formulation Imatynib-Biofarm over reference formulation Glivec) for both C_{max} and AUC_{0-t} of imatinib were included entirely in the acceptance range of 80.00–125.00%, the test formulation (Imatynib-Biofarm) was assumed as bioequivalent to the reference formulation (Glivec). The similar pharmacokinetic property of the metabolite further supports the bioequivalence of the tablet product. This study indicated relatively rapid absorption of the 100 and 400 mg tablet (median T_{max} 2.5 and 2.5 h, respectively) after oral administration that was comparable with that of Glivec tablets (T_{max} 3.0 and 4.0, respectively). The coefficient for varia-

Table 4. Plasma pharmacokinetic parameters of imitanib after a single dose administration of the Imatynib-Biofarm 400 mg (test product) and the Glivec 400 mg (reference product).

Imatynib-Biofarm 400 mg (test product)					
Pharmacokinetic parameter	Arithmetic mean	Median	Geometric mean	SD	CV [%]
T_{max} (h)	2.9	2.5	2.5	1.7	58.6
C_{max} (ng/mL)	15477	1549	1452	579	37.4
AUC_{0-t} (ng × h/mL)	24705	22562	23295	8902	36.0
$AUC_{0-\infty}$ (ng × h/mL)	25065	22808	23640.	9005	35.9
λz (1/h)	0.043	0.043	0.043	0.0065	15.1
$t_{1/2}$ (h)	16.4	16.0	16.2	2.6	15.9
AUC_%Extrap_obs (%)	1.45	1.14	1.27	0.86	59.0
Glivec 400 mg (reference product)					
T_{max} (h)	3.5	4.0	3.2	1.38	39.6
C_{max} (ng / mL)	1506	1439	1443	459	30.5
AUC_{0-t} (ng × h / mL)	24545	24149	23437	778	31.7
$AUC_{0-\infty}$ (ng × h / mL)	24913	24304	23772	7957	31.9
λz (1 / h)	0.044	0.044	0.044	0.007	15.6
$t_{1/2}$ (h)	15.9	15.9	15.8	2.44	15.3
AUC_%Extrap_obs (%)	1.41	1.20	1.19	0.88	62.7

Table 5. Plasma pharmacokinetic parameters of N-desmethyl imatinib after a single dose ad-ministration of the Imatynib-Biofarm 400 mg (test product) and the Glivec 400 mg (reference product).

Imatynib-Biofarm 400 mg (test product)					
Pharmacokinetic parameter	Arithmetic mean	Median	Geometric mean	SD	CV [%]
T_{max} (h)	2.6	2.0	2.3	1.7	61.7
C_{max} (ng/mL)	168	151	160	55	32.8
AUC_{0-t} (ng × h/mL)	3698	3477	3476	1333	36.0
$AUC_{0-\infty}$ (ng × h/mL)	4561	4318	4273	1691	37.1
λz (1/h)	0.017	0.017	0.016	0.0036	21.6
$t_{1/2}$ (h)	43.9	40.8	42.6	11.8220	26.9
AUC_%Extrap_obs (%)	18.4	16.7	17.4	6.5798	35.8
Glivec 400 mg (reference product)					
T_{max} (h)	3.2	2.5	2.9	1.7	53.8
C_{max} (ng / mL)	162	150	153	55	34.0
AUC_{0-t} (ng × h / mL)	3705	3796	3447	1446	39.0
$AUC_{0-\infty}$ (ng × h / mL)	4481	4311	4151	1852	41.3
λz (1 / h)	0.018	0.018	0.017	0.0030	16.7
$t_{1/2}$ (h)	40.3	39.4	39.7	7.1	17.7
AUC_%Extrap_obs (%)	16.8	16.1	16.2	4.8	28.8

Table 6. The 90% confidence intervals based on the Schuirmannis TOST test and using mean square error estimated from ANOVA analysis of pharmacokinetic parameters for imatinib.

100 mg			
Pharmacokinetic parameter	Point estimate [%]	90% confidence interval [%]	Estimated intrasubject CV [%]
AUC_{0-t}	104.2	99.3–109.2	12.1
$AUC_{0-\infty}$	104.1	99.2–109.1	12.2
C_{max}	104.6	98.4–111.2	15.7
400 mg			
AUC_{0-t}	99.6	94.8–104.7	12.7
$AUC_{0-\infty}$	99.7	94.8–104.8	12.7
C_{max}	100.7	94.8–107.0	15.5

tion for T_{max}, C_{max} and AUCs showed considerable intersubject variability (up to 15.7%). Nikolova et al. (34) reported that the intra subject variability for C_{max} was on the level of 20%. Although the cause of this was not clear, it is below observed in the above mentioned study and may be attributed to intersubject differences in plasma proteins binding to the parent compound or to variations in CYP3A4, the major CYP isoenzyme involved in the microsomal metabolism of imatinib. It is known that variability in CYP3A activity between individuals is large (35) and may in part have contributed to the large intersubject variability.

There were no serious adverse events reported during the conduct of the trials. Only 1 adverse event was assessed as significant. Based on the clinical results for the parent drug, the study clearly demonstrated that new formulations of imatinib (Imatynib-Biofarm 100 and 400 mg film-coated tablet) were tolerated in the same way as reference drug (Glivec 100 and 400 mg film-coated tablet).

Table 7. The 90% confidence intervals based on the Schuirmannis TOST test and using mean square error estimated from ANOVA analysis of pharmacokinetic parameters for N-desmethyl imatinib.

100 mg			
Pharmacokinetic parameter	Point estimate [%]	90% confidence interval [%]	Estimated intrasubject CV [%]
AUC_{0-t}	101.2	96.9-105.6	10.9
$AUC_{0-\infty}$	100.6	96.6-104.7	10.3
C_{max}	103.7	96.8-111.0	17.5
400 mg			
AUC_{0-t}	104.2	95.4-107.3	15.0
$AUC_{0-\infty}$	103.4	96.6-110.6	17.4
C_{max}	104.3	97.6-111.4	16.9

CONCLUSION

The results of this single-dose study in healthy white volunteers indicated that Imatynib-Biofarm 100 and 400 mg film-coated tablets manufactured by Biofarm Sp. z o.o. (test products) are bioequivalent to Glivec 100 and 400 mg film-coated tablets manufactured by (Novartis Pharma GmbH) (reference products), even though the imatinib mesylate as the active substance in these preparations differs in polymorphic forms, as it was mentioned earlier. Both products were well tolerated. It should be noted that the results for AUC (for imatinib and N-desmethyl imatinib in the study for 100 mg and 400 mg) are generally fitting in the limited acceptance interval for drug in narrow therapeutic index 90.00–111.11%. It is an important premise allowing to conclude the high quality of tested products in term of bioequivalence.

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REFERENCES

1. Druker B.J., Tamura S., Buchdunger E., Ohno S., Segal G.M., Fanning S., Zimmermann J., Lydon N.B.: Nat. Med. 2, 561 (1996).
2. Druker B.J., Talpaz M., Resta D.J., Peng B., Buchdunger E., Ford J.M., Lydon N.B. et al.: N. Engl. J. Med. 344, 1031 (2001).
3. Buchdunger E., O'Reilly T., Wood J.: Eur. J. Cancer. 38, Suppl. 5, S28 (2002).
4. Druker B.J, Lydon N.B.: J. Clin. Invest. 105, 3 (2000).
5. Buchdunger E., Cioffi C.L., Law N., Stover D., Ohno-Jones S., Druker B.J., Lydon N.B.: J. Pharmacol. Exp. Ther. 295, 139 (2000).
6. Mughal T.I., Goldman J.M.: Eur. J. Cancer 37, 561 (2000).
7. O'Brien S.G., Guilhot F., Larson R.A., Gathmann I., Baccarani M., Cervantes F., Cornelissen J.J. et al.: N. Engl. J. Med. 348, 994 (2003).
8. Hughes T.P., Kaeda J., Branford S., Rudzki Z., Hochhaus A., Hensley M.L., Gathmann I. et al.: N. Engl. J. Med. 349, 1423 (2003).
9. Druker B.J., Guilhot F., O'Brien S.G., Gathmann I., Kantarjian H., Gattermann N., Deininger M.W. et al.: N. Engl. J. Med. 355, 2408 (2006).
10. Lee H.J., Thompson J.E., Wang E.S., Wetzler M.: Cancer 117, 1583 (2011).
11. Mechtersheimer G., Egerer G., Hensel M., Rieker R.J., Libicher M., Lehnert T., Penzel R.: Virchows Arch. 444, 108 (2004).
12. Chabner B.A., Amrein P.C., Druker B.J., Michaelson M.D., Mitsiades C.S., Goss P.E., Ryan D.P. et al.: Chapter 62. Antineoplastic agents. in Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th edn., McGraw-Hill, New York 2011.
13. Mac K., Wójcik M.: Postepy Hig. Med. Dosw. 62, 272 (2008).
14. Prenen H., Dumez H., Stefan C., Hoeben A., Wouters C., Van Lierde M.A., Sciot R. et al.: Acta Gastroenterol. Belg. 69, 367 (2006).
15. Nishida T., Shirao K., Sawaki A., Koseki M., Okamura T., Ohtsu A., Sugiyama T. et al.: Int. J. Clin. Oncol. 13, 244 (2008).

16. Chen P., Zong L., Zhao W., Shi L.: World J. Gastroenterol. 16, 4227 (2010).
17. Gronchi A., Blay J.Y., Trent J.C.: Cancer 116(8), 1847 (2010).
18. Klion A.D., Robyn J., Maric I., Fu W., Schmid L., Lemery S., Noel P. et al.: Blood 110, 3552 (2007).
19. Wang L.N., Pan Q., Fu J.F., Shi J.Y., Jin J., Li J.M., Hu J. et al.: Chin. Med. J. (Engl) 121, 867 (2008).
20. Intermesoli T., Delaini F., Acerboni S., Salmoiraghi S., Spinelli O., Guerini V., Vannucchi A.M. et al.: Br. J. Haematol. 147, 681 (2009).
21. Zhang Y., Qin T.J., Zhou C.L., Liu L., Xiao Z.J.: Zhonghua Xue Ye Xue Za Zhi 30, 381 (2009).
22. Peng B., Lloyd P., Schran H.: Clin. Pharmacokinet. 44, 879 (2005).
23. Gschwind H.P., Pfaar U., Waldmeier F., Zollinger M., Sayer C., Zbinden P., Hayes M. et al.: Drug Metab. Dispos. 33, 1503 (2005).
24. Peng B., Dutreix C., Mehring G., Hayes M.J., Ben-Am M., Seiberling M., Pokorny R. et al.: J. Clin. Pharmacol. 44, 158 (2004).
25. Fitos I., Visy J., Zsila F., Mády G., Simonyi M.: Biochim. Biophys. Acta. 1760, 1704 (2006).
26. Cross S.A., Lyseng-Williamson K.A.: Drugs 67, 2645 (2007).
27. Gleevec – Rx List. The Internet Drug Index [database on the Internet]. WebMD, Inc. [last revised May 2011; cited: 2013 April 26]. Available from: http:// www. rxlist.com/gleevec -drug. htm
28. The European Agency for the Evaluation of Medicinal Products, Note for Guidance on the Investigation of Bioavailability and Bioequivalence CPMP/EWP/QWP/1401/98, London, 26 July 2001.
29. The European Medicines Agency, Guidance on the Investigation of Bioequivalence CPMP/EWP/QWP/1401/98 Rev.1, London, 20 January 2010.
30. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Adopted by the 18th WMA General Assembly, Helsinki June 1964 and amended by the 59th WMA General Assembly Seoul, Korea 2008.
31. European Agency for Evaluation of Medicinal Products, International Conference on Harmonization, in World Health Organization. Guideline for Good Clinical Practice, ICH topic E6. Geneva 2002.
32. FDA Guidance for Industry. Statistical approaches to establishing bioequivalence. U.S. Food and Drug Administration, Rockville, MD 2001.
33. Schuirmann D.J.: J. Pharmacokinet. Biopharm. 15, 657 (1987).
34. Nikolova Z., Peng B., Hubert M., Seiberling M., Keller U., Ho Y.-Y., Schran H., Capdeville R.: Cancer Chemother. Pharmacol. 53, 433 (2004).
35. Wilkinson G.R.: J. Pharmacokinet. Biopharm. 24, 475 (1996).
36. Grillo D., Polla G., Vega D.: J. Pharm. Sci. 101, 541 (2012).
37. Guidance for Industry. ANDAs: Pharmaceutical Solid Polymorphism. Chemistry, Manufacturing, and Controls Information. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), July 2007, OGD, http://www.fda.gov/cder/guidance/index.htm
38. Szczepek W., Samson-Lazinska D., Zagrodzki B., Glice M., Maruszak W., Korczak K., Modzelewski R. et al.: "Crystalline methanesulfonic acid addition salts of imatinib", WO 2005/095379 A2 (2005).
39. Zimmermann J. et al.: Polish patent 188348 B1 (1998).

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COMPARATVE URIC ACID LOWERING STUDIES OF ALLOPURINOL WITH AN INDIGENOUS MEDICINAL PLANT IN RABBITS

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Abstract: The aim of this research was to carry out a comparative study of lowering of uric acid by the use of dried powder of *Colchicum luteum* and allopathic drug (allopurinol) in rabbits, to determine whether herbal drugs can be used by patients instead of allopathic drugs. The herbal medicine, dried corm powder of *Colchicum luteum* 2.5 mg/kg/day and dried powder of allopurinol 2 mg/kg/day an allopathic medicine, was used in the study. The results of these medicines were observed in animal model, using 12 adult rabbits, which were divided into three groups A, B and C, respectively, where group C was taken as control. The SPSS version 17 was used for statistical analysis and analysis of variance (ANOVA) was used for comparing the data in different groups and the level of significance was 5%. It was resulted that dried corm of *Colchicum luteum* significantly reduced the uric acid in adult rabbits as reduced by allopathic medicine – allopurinol. In the light of present research we concluded that the herbal medicines can be used in lieu of allopathic drugs. Thus, the risk of side effects that are associated with the prolonged use of allopathic drugs can be minimized.

Keywords: herbal medicine, *Colchicum luteum,* allopurinol, reduction in uric acid

Hyperuricemia, a common biochemical abnormality can be termed as the increased concentration of serum uric acid beyond the limit of solubility (approximately 6.8 mg/dL) (1). Hyperuricemia is the abnormally raised uric acid concentration in the blood (2), usually defined as more than 6.8 mg/dL (3). Serum uric acid levels greater than 6 mg/dL in females and 7 mg/dL in males deal with hyperuricemia (4). Hyperuricemia can also be termed as uric acid concentration greater than 7.0 mg/dL in the blood (5), which results in diseases like gout and arthritis, i.e., rheumatoid arthritis. For a long time, hyperuricemia and gout were used interchangeably, but uric acid is now confirmed as a nuisance factor for many other abnormalities in the body metabolism. It is well established that hyperuricemia is the actual cause of gout (6). Deposits of crystals of monosodium urate in tissues lead to arthritis and gout i.e., gouty arthritis characterized by swelling of one or more joints in the lower extremities, erythema and recurring attacks of pain (7). Elevated serum uric acid level in combination with joint symptoms help in diagnosing gouty arthritis (8). Hyperuricemia may lead to an autosomal dominant renal disease i.e., familial juvenile hyperuricemic nephropathy (FJHN). The diseases caused by hyperuricemia include gout or arthritis, multiple complications and metabolic syndromes like insulin resistance and dyslipidemia and it may also be a cause of severe cardiovascular diseases and chronic kidney diseases and renal stones, if not managed properly (9).

Uric acid is the final product of protein metabolism in human beings (10). Allantoin is the end product in lower animals, which is much more soluble than uric acid found in humans. Deficiency of hepatic enzyme, uricase and lower fractional excretion of uric acid are the main causes of higher levels of uric acid in the blood in human beings (11). Hyperuricemia may result from decreased excretion (90%) of uric acid, overproduction (10%) of uric acid, or as a result of combined mechanism (12).

Food and Drug Administration approved allopurinol in 1966 for treatment of hyperuricemia and gout (13). Allopurinol helps in the management of hyperuricemia and thus helps in the treatment of

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gout and arthritis by inhibiting the biosynthesis of purines and pyrimidines in man (14).

Colchicum luteum is being used generally in the Unani system of medicines as a main ingredient in many herbal formulations for the treatment of several diseases since a long time. *Colchicum luteum* belongs to the family Liliaceae (15). The genus colchicum includs almost 42 species mostly endemic to the Middle East and South Africa to Western Europe and Asia (16). Corms of the *Colchicum luteum* are widely used in the treatment of gout, arthritis and several diseases of the spleen and liver. The corms are also used for the purification of blood (17). The seeds and corms of the plant are used by practitioners for the treatment of arthritis, gout, rheumatic fever and several complications of spleen and liver (18).

The active principle in colchicum is the alkaloid "colchicine", which provides dramatic relief from acute attacks of gouty arthritis. This antirheumatic effect is highly specific for gout and colchicum has little effect on non-gouty arthritis and no analgesic property.

MATERIALS AND METHODS

All the drugs used were of pharmaceutical/analytical grade with known assay. Fructose (BDH Laboratory Supplies, London), allopurinol USP30/BP2008, Batch No. 20111102, (Shanghai Chemicals & Pharmaceuticals Co. Limited, China) provided by Pharmedic Laboratories, Lahore, dried corms of *C. luteum* (R&D Department, Qarshi Laboratories, Lahore) and uric acid determination kit (Crescent Diagnostic, Lahore). Analytical balance (Mettler Toledo AB54-S), centrifuge (BHG), incubator/oven (Memmert Schwabach, Germany), spectrophotometer (UV-1700, Shimadzu), refrigerator/freezer (Dawlance), BD syringes, capsule shells and wooden restraining boxes were use in the study.

Animals

Twelve adult healthy albino rabbits with weights ranging from 1.3 kg to 1.6 kg were bought from the city local market and they were housed in clean metal hutches in the animal house of University College of Pharmacy, University of the Punjab, Lahore, Pakistan. The animals were exposed to their usual feeding pattern and green fodder and water were provided to all of the rabbits. The temperature of the animal house was maintained at 22.5 ± 2^{o}C. Before conducting the study, the rabbits were acclimatized in the animal house for a week.

Study protocols

All the twelve rabbits were administered with oral fructose syrup for ten days to induce hyperuricemia. A daily dose of fructose syrup, i.e., 1 gram per kg per day was given to all of the rabbits (19). The weighed amount of fructose was dissolved in distilled water at 37^{o}C. Syringes were used to dispense the appropriate preparation and amounts into the mouth of the animals. After attaining a specific

Table 1. Comparison of uric acid at different follow-ups in group "A" (allopurinol).

No.	Follow up duration	Mean (mg/dL)
0	Base line (day 0)	8.50 ± 1.41
1	1st Follow up (day 01)	6.96 ± 0.42
2	2nd Follow up (day 04)	6.95 ± 1.87
3	3rd Follow up (day 07)	3.99 ± 0.26
4	4th Follow up (day 10)	5.85 ± 0.72
5	5th Follow up (day 13)	4.75 ± 0.68
6	6th Follow up (day 16)	4.16 ± 0.57
7	7th Follow up (day 19)	3.78 ± 0.39
8	8th Follow up (day 22)	3.53 ± 0.40
9	9th Follow up (day 25)	3.16 ± 0.29
10	10th Follow up (day 28)	2.94 ± 0.29
11	11th Follow up (day 31)	2.85 ± 0.28
12	12th Follow up (day 34)	2.63 ± 0.24

level of animal's uric acid, they were divided into three groups with four rabbits in each group. Each group was labeled and received the treatment as follows:

GROUP A: In this group, all rabbits were administered with allopurinol 2 mg/kg/day for six weeks.

GROUP B: In this group, all rabbits were administered with fine powder of *C. luteum* filled in capsules with dose of 2.5 mg/kg/day and fructose syrup (1 g/kg/day) for six weeks.

GROUP C: This group was kept as control group and all rabbits of this group received fructose syrup 1 g/kg/day for six weeks.

Serum uric acid was measured as an indicator of hyperuricemia.

Collection of blood samples

Blood samples of all the rabbits were collected at the start of the experiment to measure the normal values of serum uric acid. Then, blood samples were collected after an intervals of two days till the end of study.

Before the start of sampling, the rabbit was held in the wooden restraining box in such a way that its head protrudes outwards. The rabbit's ear was shaved with the help of the sterilized blade, to make vein more prominent. Blood of each rabbit was collected into the blood collecting tubes after puncturing the vein of the right ear with 21-gauge syringes after an interval of two days. After collecting the blood samples, tubes were marked for identification with specific codes and kept in container containing ice cubes in order to coagulate the blood samples for almost an hour. The coagulated blood samples were then centrifuged at 4000 rpm for about 15 min at room temperature to separate the serum. The clear supernatant serum was then collected in previously marked Eppendroff tubes and stored at –23°C to –18°C in a freezer before analysis.

In order to make multi blood sample collection possible from the one rabbit, the blood sampling started from the apex to base of the ear, so that vein would not be blocked.

Analysis of serum uric acid

Analysis of uric acid was performed by using the enzymatic colorimetric test, URICASE/PAP method.

Quantitative analysis of uric acid helps in diagnosing hyperuricemia, gout, renal dysfunction, diabetes and other several conditions. Uricase catalyzes the reaction and uric acid is oxidized to allantoin and H_2O_2 reacts with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonate in the presence of peroxidase, which resulted in the formation of a quinoneimine dye, the concentration of which is in direct proportion to the concentration of uric acid at 546 nm (20).

Statistical analysis

Statistical analysis was performed using SPSS version 17. The quantitative data were given as the mean ± SD along with their SEM and range (lowest thorough highest value). The appropriate line charts

Table 2. Comparison of uric acid at different follow-ups in group "B" (*Colchicum luteum*).

No.	Follow up duration	Mean (mg/dL)
0	Base line (day 0)	7.87 ± 1.79
1	1st Follow up (day 01)	7.77 ± 0.41
2	2nd Follow up (day 04)	7.37 ± 0.85
3	3rd Follow up (day 07)	4.20 ± 0.75
4	4th Follow up (day 10)	6.34 ± 0.50
5	5th Follow up (day 13)	5.27 ± 0.29
6	6th Follow up (day 16)	4.40 ± 0.14
7	7th Follow up (day 19)	4.05 ± 0.13
8	8th Follow up (day 22)	3.81 ± 0.10
9	9th Follow up (day 25)	3.57 ± 0.24
10	10th Follow up (day 28)	3.36 ± 0.17
11	11th Follow up (day 31)	3.28 ± 0.18
12	12th Follow up (day 34)	3.11 ± 0.13

along their description were incorporated for visual presentation and visual comparison of data. For statistical analysis, analysis of variance (ANOVA) was used for comparing the data in different groups. Friedman test (alternative to repeated measurement ANOVA), was used for the comparison of different follow ups in three treatment groups. The level of significance was established at (less or equal) the 5% probability level.

RESULTS AND DISCUSSION

In this study, hyperuricemia was induced in twelve rabbits for the comparative study of indigenous medicinal plant (*Colchicum luteum*) and allopathic drug (allopurinol) for lowering uric acid level. At each follow up study, it was observed that there was a prominent fall in uric acid level, which gave significant results till the end of studies. Allopurinol is an isomer of hypoxanthine and inhibits the activity of xanthene oxidase, the main enzyme responsible for oxidation of hypoxanthine and xanthene, which give rise to uric acid synthesis (21). Increased concentration of xanthene and hypoxanthine are converted to closely related ribotides i.e., adenosine and guanosine monophosphates. The raised level of ribotides can cause inhibition of amidophosphoribosyl transferase i.e., the rate limiting and the first step in the synthesis of purines in the body, by feedback mechanism (22).

In the present study, allopurinol dramatically reduced uric acid in Group A of rabbits from 8.50 mg/dL to 2.63 mg/dL in twelve follow ups. Allopurinol helps in lowering serum concentration of uric acid by decreasing the purine biosynthesis. This reduction in the level of uric acid was very significant as shown in Table 1.

In group A (treated with allopurinol), the mean uric acid at base line was 8.50 mg/dL, which decreased at 12th follow up to 2.63 mg/dL, respectively. The mean uric acid was statistically significantly decreased over a period of 12 follows up with a p-value < 0.05 as compared to Group C (control group).

On the other hand, in Group B of rabbits the herbal medicine *Colchicum luteum* showed similar significant effects as it gradually reduced the uric acid from 7.87 mg/dL to 3.11 mg/dL in 12th follow ups as shown in Table 2.

The mean uric acid was statistically significantly decreased over a period of 12 follows up with p-value < 0.05 as compared to Group C (control group).

The main phytochemical principles in *Colchicum luteum* possess alkaloids i.e., colchicine, lumicolchicine, N-desacetyl-N-formylcolchicine, 2-desmethylcolchicine and luteidine (23). Among these alkaloids, colchicine is believed to be one of the most important anti-inflammatory compounds procured from *Colchicum luteum* and its anti-inflammatory activity is characterized by inhibiting microtubules in the proinflamatory cells comprising of macrophages (24). The mean uric acid in group C (control group i.e., hyperuricemic) at base line 7.74 mg/dL was raised to 10.58 mg/dL at the end of study (p-value > 0.05).

Table 3. Comparison of uric acid at different follow-ups in group "C" (control).

No.	Follow up duration	Mean (mg/dL)
0	Base line (day 0)	7.74 ± 0.66
1	1st Follow up (day 01)	8.18 ± 0.2
2	2nd Follow up (day 04)	8.23 ± 0.25
3	3rd Follow up (day 07)	8.48 ± 0.91
4	4th Follow up (day 10)	8.81 ± 5.08
5	5th Follow up (day 13)	9.86 ± 0.85
6	6th Follow up (day 16)	10.78 ± 0.54
7	7th Follow up (day 19)	10.53 ± 1.34
8	8th Follow up (day 22)	10.38 ± 0.71
9	9th Follow up (day 25)	9.99 ± 0.38
10	10th Follow up (day 28)	10.14 ± 0.84
11	11th Follow up (day 31)	10.0 ± 0.59
12	12th Follow up (day 34)	10.58 ± 0.00

Group A (treated with allopurinol) gave the best results and lowered uric acid level to the lowest level as compared to the Group B (treated with *Colchicum luteum*). However, *Colchicum luteum* can be used as a good alternative in case of patients suffering from severe liver damage or renal failure or those patients who are found hypersensitive to allopurinol.

CONCLUSION

The *Colchicum luteum* reduced the uric acid at significant level as does allopurinol, which is a synthetic allopathic drug. *Colchicum luteum* is not only used in gout but it can also be used as analgesic and in rheumatoid arthritis. One of the major problems with this herbal formulation is that the active ingredients are not well defined. It is important to know the active component and their molecular interaction, which will help to analyze therapeutic efficacy of the product and also to standardize the product. Efforts are now being made to investigate the mechanism of action of some of these plants using model systems. Care must be taken while using *Colchicum luteum*. It must be free from toxic adulterations. Due to the poisonous nature of the herb, it must be used only under strict professional supervision. Dose of the herbal drug must not exceed beyond limits, as it may cause severe gastric disturbances. If this herb is used according to the prescribed instructions, then it can be a better and competent alternative of allopathic medication in the management of hyperuricemia and for the treatment of diseases associated with it. Newer approaches utilizing collaborative research and modern technology in combination with established traditional health principles will yield rich dividends in the near future in improving health, especially among people who do not have access to the use of more costly western systems of medicine.

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REFERENCES

1. Becker M.A., Schumacher H.R., Wortmann R.L., MacDonald P.A., Eustace D., Palo W.A., Streit J., Joseph-Ridge N.: New Eng. J. Med. 353, 2450 (2005).
2. Kutzing M.K., Firestein B.L.: J. Pharm. Exp. Ther. 324, 1 (2008).
3. Kim S.Y., Guevara J.P., Kim K.M., Choi H.K., Heitjan D.F., Albert D.A.: Arthritis Rheum. 61, 885 (2009).
4. Feig D.I., Kang D.H., Johnson R.J.: New Eng. J. Med. 359, 1811 (2008).
5. Krishnan E., Kwoh C.K., Schumacher H.R., Kuller L.: Hypertension 49, 298 (2007).
6. Gaffo A.L., Edwards N.L., Saag K.G.: Arthritis Res. Ther. 11, 240 (2009).
7. Eggebeen A.T. : Am. Fam. Physician 76, 801 (2007).
8. Grayzel A.I., Liddle L., Seegmiller J.E.: New Eng. J. Med. 265, 763 (1961).
9. Eraly S.A., Vallon V., Rieg T., Gangoiti J.A., Wikoff W.R., Siuzdak G., Barshop B.A., Nigam S.K.: Physiol. Genomics 33, 180 (2008).
10. Álvares-Lario B., Macarrón-Vicente J.: Rheumatology 49, 2010 (2010).
11. Hayashi S., Fujiwara S., Noguchi T.: Cell. Biochem. Biophys. 32, 123 (2000).
12. Choi H.K., Mountand D.B., Reginato A.M.: Ann. Intern. Med. 143, 499 (2005).
13. Wortmann R.L.: Curr. Opin. Rheumatol. 17, 319 (2005).
14. Rawlins M.D., Smith S.E.: Br. J. Pharmacol. 48, 693 (1973).
15. Nair V., Singh S., Gupta Y.K.: J. Ethnopharmacol. 133, 303 (2011).
16. Adnan S.M., Khan A.A., Latif A., Shiwari Z.K.: Lyonia 11, 91 (2006).
17. Khan H., Tariq S.A., Khan M.A.: J. Med. Plant. Res. 5, 7031 (2011).
18. Singh P.B., Aswal B.S.: J. Econ. Taxon. Bot. 18, 715 (1994).
19. Perheentupa J., Raivio K.: Lancet 2, 528 (1967).
20. Trivedi R. Berta E., Rebar L.: Clin. Chem. 22, 1223 (1976).
21. Machado-Vieira R., Soares J.C., Lara D.R., Luckenbaugh D.A., Busnello J.V., Marca G., Cunha A. et al.: J. Clin. Psychiatry 69, 1237 (2008).
22. Pea F.: Contrib. Nephrol. 147, 35 (2005).
23. Chommadov B., Yusupov M.K., Sadykov A.S.: Chem. Nat. Comp. 6, 77 (1970).
24. Rao P., Falk L.A., Dougherty S.F., Sawada T., Pluznik D.H.: J. Immunol. 159, 3531 (1997).

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CARDIOPROTECTIVE EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED CARDIOTOXICITY IN RATS

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Abstract: The *in vivo* antioxidant properties of green tea extract (GTE) were investigated against doxorubicin (DOX) induced cardiotoxicity in rats. In this experiment, 48 Wistar albino rats (200–250 g) were divided into eight groups (n = 6). Control group received normal saline for 30 days. Cardiotoxicity was induced by DOX (20 mg/kg *i.p.*), once on 29th day of study and were treated with GTE (100, 200 and 400 mg/kg, *p.o.*) for 30 days. Aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LDH), lipid peroxidation (LPO), cytochrome P_{450} (CYP), blood glutathione, tissue glutathione, enzymatic and non-enzymatic antioxidants were evaluated along with histopathological studies. DOX treated rats showed a significant increased levels of AST, CK, LDH, LPO and CYP, which were restored by oral administration of GTE at doses 100, 200 and 400 mg/kg for 30 days. Moreover, GTE administration significantly increased the activities of glutathione peroxidase (GPX), glutathione reductase (GR), glutathione s-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), in heart, which were reduced by DOX treatment. In this study, we have found that oral administration of GTE prevented DOX-induced cardiotoxicity by accelerating heart antioxidant defense mechanisms and down regulating the LPO levels to the normal levels.

Keywords: green tea extract, doxorubicin, cardiotoxicity, antioxidants, lipid peroxides

Doxorubicin is a potent broad-spectrum chemotherapeutic agent that is highly effective in treating patients with acute lymphoblastic leukemia, Hodgkin's lymphoma, aggressive non-Hodgkin's lymphomas, breast carcinoma, ovarian carcinoma and many solid tumors (1). However, the clinical use of this drug has been seriously limited by undesirable side effects especially dose-dependent myocardial injury, leading to potentially lethal congestive heart failure (2).

Due to the great importance of DOX in chemotherapy for the treatment of many types of cancer, researchers have exerted great efforts to attenuate the side effects of DOX. In view of this several strategies have been followed for dosage optimization and use of analogues or combined therapy but no promising results have been found (3, 4). The use of several DOX analogues available clinically did not show stronger antitumor efficacy as compared to DOX (5). Antitumor action of DOX is mediated by a wide number of mechanisms but one of the activities, i.e., generation of the free radicals, is among the main causes of cardiotoxicity. This fact allows the researchers to develop strategies to reduce the toxic effects of DOX without interfering with its antitumor properties.

Herbal extracts have many properties like antioxidant, anti-allergic, anti-inflammatory, antiviral, anti-proliferative and anti-carcinogenicity (6). Natural antioxidants, which are capable of protecting the cells from oxidative injury, should be included in the potential antioxidant therapy. Therefore, there is a need for identifying alternative, natural and safer sources of antioxidants (7).

Green tea (*Camellia sinensis*) is one of the most popular beverages, approximately three billion

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kilograms of tea is produced and consumed yearly throughout the world. Green tea is favored in Asian countries and initial research on the benefits of green tea showed that daily consumption of green tea is safe and has no adverse effects on human health (8). The major catechin present in green tea extract is epigallocatechin-3-gallate (EGCG), which attributes to its beneficial effects and also reduces the risk of a variety of diseases (9, 10). Thus, the present study was designed to investigate whether pre-treatment of GTE has any protective effect on lipid peroxidation, activities of enzymatic and non-enzymatic and histopathological examination of myocardium in DOX treated rats.

MATERIALS AND METHODS

Experimental animals

This study was conducted in Wistar albino rats (200–250 g), which were kept in the animal house of Faculty of Pharmacy, Jamia Hamdard, New Delhi, India for one week prior to starting the experimental protocol for proper acclimatization under controlled condition of illumination (12 h light/12 h darkness) and temperature 20–25°C. They were housed under ideal laboratory conditions, maintained on standard pellet diet (Lipton rat feed, Ltd., Pune, India) and water *ad libitum* throughout the experimental period. The study was approved by the Institutional Animal Ethics Committee (IAEC), Jamia Hamdard, New Delhi, India.

Drugs and chemicals

Standardized powdered, aquous extract of green tea was a gift sample by Sanat Products Ltd., India. DOX was procured from Dabur Pharmaceuticals Ltd., New Delhi, India. Vitamin E was procured from Merck Ltd., India. LDH, CK and AST assay kit were purchased from Span Diagnostics Ltd., Surat, India. All other chemicals used during the study were of analytical grade.

Experimental design

In this experiment, a total of 48 Wistar albino rats were used. The rats were randomly divided into eight groups comprising of six animals in each group as follows:

Group I: Normal control, received normal saline (1 mL/kg *p.o.*) for 30 days.

Group II: Toxic control, received DOX (20 mg/kg *i.p.*) once on 29th day.

Group III: GTE control, received GTE (400 mg/kg *p.o.*) for 30 days.

Group IV: Vitamin E control, received vitamin E (100 mg/kg *p.o.*) for 30 days.

Group V: GTE treated-1, received GTE (100 mg/kg *p.o.*) for 30 days and DOX on 29th day.

Group VI: GTE treated-2, received GTE (200 mg/kg *p.o.*) for 30 days and DOX on 29th day.

Group VII: GTE treated-3, received GTE (400 mg/kg *p.o.*) for 30 days and DOX on 29th day.

Group VIII: Vitamin E treated, received vitamin E (100 mg/kg *p.o.*) for 30 days and DOX on 29th day.

On 31st day, blood samples were collected from rat tail vein for biochemical determinations. Later, the rats were sacrificed under the influence of anesthesia. The hearts were excised out immediately, rinsed in ice-cold normal saline and used for the following assays.

Post mitochondrial supernatant (PMS)

The heart were quickly removed and perfused immediately with ice-cold saline (0.85 %, w/v NaCl) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) that contained KCl (1.17%, w/v). The homogenate was centrifuged at 800 × *g* for 5 min at 4°C in a refrigerated centrifuge to separate the nuclear debris. The supernatant was centrifuged at 10,500 × *g* for 20 min at 4°C to obtain PMS, which was used as a source of enzymes. A portion of the PMS was further centrifuged in an ultracentrifuge (Beckman, L7-55) at 34,000 × *g* for 60 min at 4°C to isolate microsomal fraction, which was finally suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17 %, w/v).

Biochemical determinations

Aspartate aminotransferase (AST) was determined by the method of Reitman and Frankel (11). The activity of creatine kinase (CK) was determined by the method of Tsung (12), whereas the lactate dehydrogenase (LDH) activity was assayed by the method of Lum and Gambino (13). The microsomal cytochrome P_{450} content was determined according to the method of Omura and Sato (14). Lipid peroxidation was determined by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids (15). Protein content in various samples was estimated by the method of Lowry et al. (16). Blood glutathione was assayed by the method of Beutler et al. (17). Tissue GSH content was determined by method of Sedlak et al. (18). GPX and GR activities were measured by the oxidation of NADPH (19), GST activity was assayed by the method of Haque et al. (20) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, SOD activity was measured according to the method of Marklund et al. (21), in which the

enzyme activity was expressed as units/mg protein and 1 unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50% and CAT activity was determined by the method of Claiborne (22).

Histopathological examination of heart

The heart was isolated immediately after sacrificing the animal and washed with ice-cold normal saline, and fixed in 10% buffered neutral formalin solution. After fixation, the heart tissue was processed by embedding in paraffin. Then, the heart tissue was sectioned and stained with hematoxylin and eosin (H.E.) for histopathological examination.

Statistical analysis

Data were expressed as the mean ± standard error (SE). For a statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) with *post-hoc* analysis. The Tukey-Kramer *post-hoc* test was applied to identify significance among groups; $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of GTE on serum levels of AST, CK and LDH level

The effect of GTE on serum levels AST, CK and LDH levels are summarized in Figures

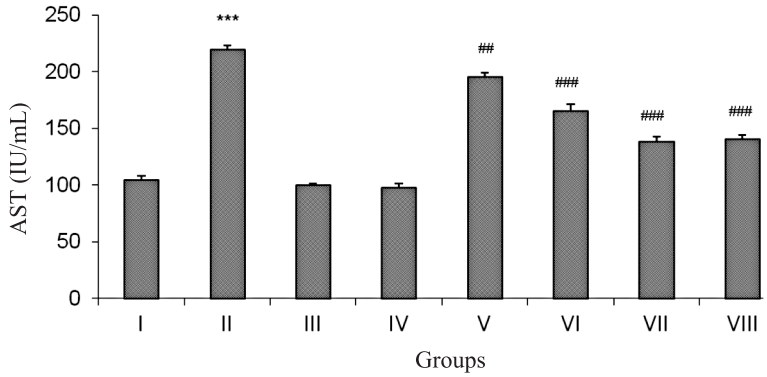

Figure 1. Effect of GTE on serum AST level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I); $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the corresponding value for DOX treated rats (group II)

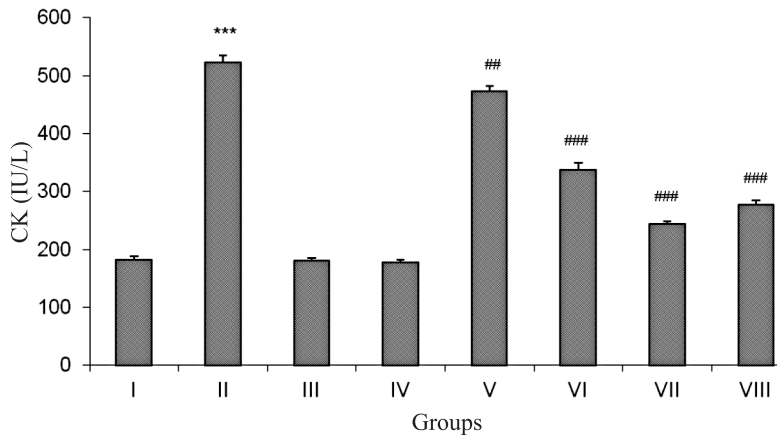

Figure 2. Effect of GTE on serum CK level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I); $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the corresponding value for DOX treated rats (group II)

1–3, respectively. GTE treated groups V, VI and VII and vitamin E treated group VIII showed a significant ($p < 0.001$) decrease in the level of serum marker enzymes when compared with DOX alone treated rats (group II). No significant difference was observed in control groups (group III and IV) when compared to normal control rats (group I).

Effect of GTE on CYP contents

Figure 4 illustrates the effect of GTE on CYP contents in various groups. DOX alone treated rats showed a significant ($p < 0.001$) decrease in CYP contents when compared with normal control rats. GTE treated groups V, VI, VII and vitamin E treated group VIII significantly ($p < 0.001$) increased the CYP contents when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on LPO levels

Table 1 illustrates the effect on malondialdehyde (MDA), a secondary product of LPO, in various groups. DOX treatment resulted in a significant ($p < 0.001$) increase in MDA level in DOX alone treated rats when compared with normal control rats (group I). The level of MDA were significantly ($p < 0.001$) decreased in GTE treated groups V, VI and VII and vitamin E treat-

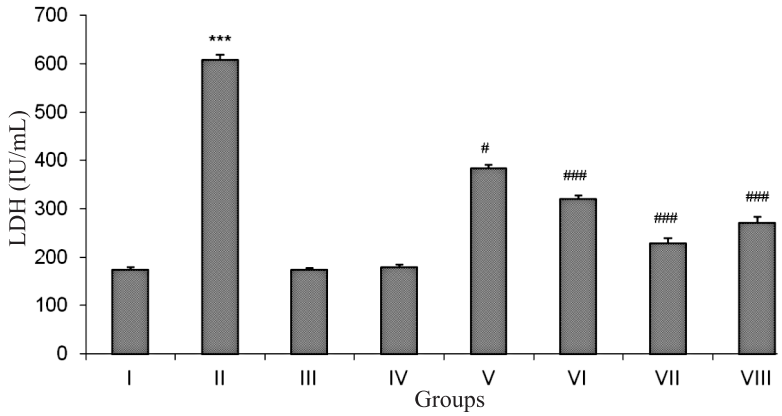

Figure 3. Effect of GTE on serum LDH level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I); $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the corresponding value for DOX treated rats (group II)

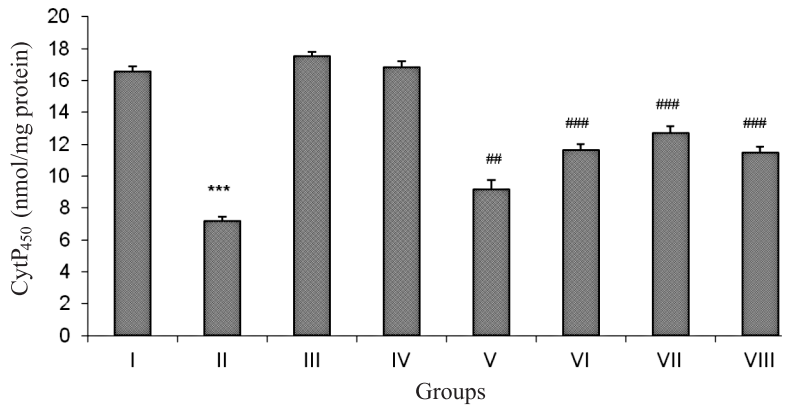

Figure 4. Effect of GTE on cytochrome P_{450} level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I); $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the corresponding value for DOX treated rats (group II)

Table 1. Effect of GTE on LPO, blood GSH and tissue GSH levels in DOX treated rats.

Group	Treatment	TBARS (nmol of MDA/mg protein)	Blood GSH (mg %)	Tissue GSH (mmole/ mg protein)
I	Normal control	0.76 ± 0.013	1.71 ± 0.045	1.38 ± 0.049
II	DOX (20 mg/kg *i.p.*)	1.81 ± 0.050 ***	0.91 ± 0.031 ***	0.54 ± 0.044 ***
III	GTE (400 mg/kg *p.o.*)	0.73 ± 0.022	1.79 ± 0.035	1.49 ± 0.040
IV	Vit. E (100 mg/kg *p.o.*)	0.75 ± 0.017	1.77 ± 0.034	1.39 ± 0.029
V	GTE (100 mg/kg *p.o.*) + DOX	1.61 ± 0.021$^{\#\#}$	1.13 ± 0.055$^{\#\#}$	0.76 ± 0.044$^{\#}$
VI	GTE (200 mg/kg *p.o.*) + DOX	1.29 ± 0.033$^{\#\#\#}$	1.34 ± 0.048$^{\#\#\#}$	0.89 ± 0.049$^{\#\#\#}$
VII	GTE (400 mg/kg *p.o.*) + DOX	1.07 ± 0.046$^{\#\#\#}$	1.49 ± 0.024$^{\#\#\#}$	1.11 ± 0.038$^{\#\#\#}$
VIII	Vit. E (100 mg/kg *p.o.*) + DOX	1.23 ± 0.037$^{\#\#\#}$	1.41 ± 0.022$^{\#\#\#}$	0.96 ± 0.030$^{\#\#\#}$

The data are expressed as the mean ± S.E.; n = 6 in each group. $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I). $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.1$ compared with the corresponding value for DOX treated rats (group II).

ed group when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on blood glutathione and tissue glutathione level

Table 1 also illustrates the activities of blood glutathione and tissue glutathione in various groups. DOX alone treated rats showed a significant ($p < 0.001$) decrease in blood and tissue glutathione levels. GTE treated groups V, VI, VII and vitamin E treated group VIII significantly ($p < 0.001$) increased the blood and tissue glutathione levels when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on antioxidant enzymes (GPX, GR, GST, SOD & CAT) levels

Table 2 represents the effect on these antioxidant enzymes in various groups. Rats treated with DOX alone showed a significant ($p < 0.001$) decrease in the activity of these enzymatic antioxidants in heart as compared to the normal control rats. GTE treated groups V, VI, VII and vitamin E treated group VIII showed a significant ($p < 0.001$) increase in the activity of these enzymatic antioxidant when compared with DOX alone treated rats (group II). No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on histopathological changes in the heart

As shown in Figure 5, section of rat heart from normal control group showed normal myocardial fibres. There was no vacuolation, necrosis or inflammation found in the group I rats (Fig. 5A), but DOX alone treated rats (group II) showed a large and irregularly shaped hypertrophic myocardial fibre with other fibres in the vicinity with small and large vacuoles (Fig. 5B). The histopathology of the heart was improved in GTE treated groups V, VI, VII and vitamin E treated group VIII and showed a normal shape, size and configuration of cardiac muscle fibres (Figs. 5C, 5D, 5E and 5F, respectively).

DISCUSSION

DOX-induced generation of reactive oxygen species (ROS) seems to be a leading cause of cardiomyopathy (9, 23). The diagnostic serum marker enzymes of cardiotoxicity are AST, CK, and LDH (24). It has been reported that the enzymes (AST, CK and LDH), that leak from the tissue damage, are the best marker of cardiotoxicity due to their tissue specificity and serum catalytic activity.

Our study also reveals an increase in the activities of these marker enzymes in DOX alone treated rats. Administration of DOX may leads to the damage of the myocardial cell membrane or it become permeable, that resulted in the leakage of AST, CK and LDH in the blood. This probably accounts for the increase in the level of these marker enzymes in the serum. Pretreatment with GTE (100, 200 and 400 mg/kg *p.o.*) restored the activities by reducing these marker enzymes level toward normal in serum. This may be due to the protective role of GTE on the myocardium, reducing the myocardial damage, thereby restricting the leakage of these enzymes in serum.

The target organelle of DOX induced cardiotoxicity is the mitochondria within which DOX accumulates (25, 26). Mitochondrial enzymes (e.g., NADH dehydrogenase) act on DOX in such a way that the quinone ring undergoes redox cycling between quinone and semiquinone states. During this

process, electrons are generated and captured by oxidizing agents, including oxygen, which then initiate a chain reaction leading to the generation of ROS (27). Cytochrome P_{450} reductase and xanthine oxidase also have been found to catalyze the reduction of anthraquinone to a semiquinone free radical (28). DOX has the ability to suppress cytochrome P_{450}, stimulating its own metabolism and thus could accelerate its elimination and increase the production of reactive toxic metabolites. Cytochrome P_{450} is not rapidly organized in the microsomal membrane and possesses lateral mobility, which largely depends on fluidity of the membrane (29). Exogenous supplementation of different doses of GTE to the DOX treated rats resulted in an increase in the cytochrome P_{450} contents. This may be attributed to the decreased activity of hemoxygenase, which in turn increased the cytochrome content. Moreover, reduction in the formation of lipid peroxides may likely to contribute to increment in detoxification process (30).

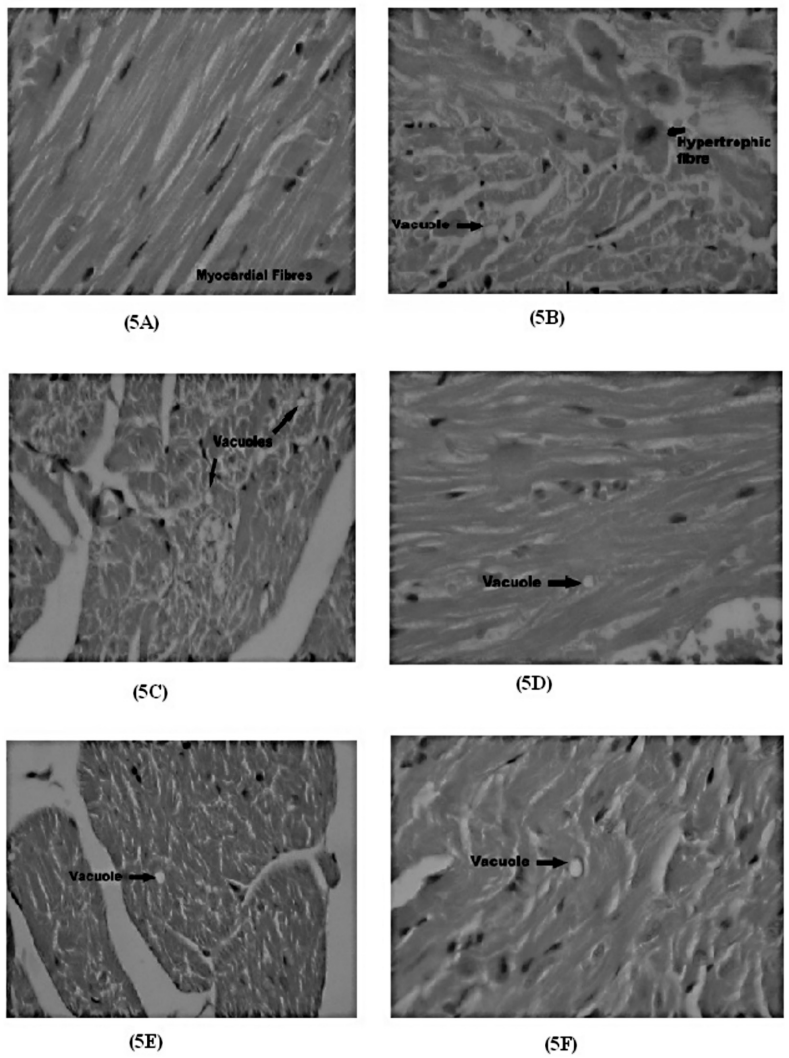

Figure 5. Hematoxylin and eosin-stained sections of rat heart, which were examined under high power (400×) of light microscope: (5A) Represents normal control rat showing normal myocardial fibers, no vacuolation, necrosis or inflammation. (5B) represents DOX alone treated rats, showing a large and irregularly shaped hypertrophic myocardial fiber with other fibers in the vicinity showing small and large vacuoles. (5C) represents GTE (100 mg/kg *p.o.*) + DOX treated rats, showing scattered vacuoles in the myocardial fibres. (5D) represents GTE (200 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single myocardial fiber with an intracytoplasmic vacuole is seen in the photograph. (5E) represents GTE (400 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single myocardial fiber with small intracytoplasmic vacuole is seen. (5F) represents Vit. E (100 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single vacuole is seen in one of the myocardial fibers

Table 2. Effect of GTE on GPX, GR, GST, SOD and CAT in DOX treated rats.

Group	Treatment	GPX (nmol of NADPH oxidized/ minute/mg protein)	GR (nmol of NADPH oxidized/ minute/mg protein)	GST (nmol of CDNB conjugate/ minute/mg protein)	SOD (U/mg protein)	CAT (nmol of H_2O_2 consumed/ min/mg protein)
I	Normal control	143.56 ± 4.66	385.80 ± 5.75	140.10 + 4.07	9.61 + 0.26	28.40 + 0.47
II	DOX (20 mg/kg *i.p.*)	71.01 ± 3.54***	194.02 ± 5.0***	69.57 ± 2.71***	3.82 ± 0.14***	10.88 ± 0.43***
III	GTE (400 mg/kg *p.o.*)	152.40 ± 2.52	394.32 ± 6.92	148.38 ± 4.32	9.88 ± 0.10	30.95 ± 0.40
IV	Vit. E (100 mg/kg *p.o.*)	150.13 ± 4.55	393.52 ± 6.47	148.21 ± 4.25	9.56 ± 0.10	30.30 ± 0.69
V	GTE (100 mg/kg p.o) + DOX	89.33 ± 4.37$^{\#}$	238.11 ± 5.4$^{\#\#}$	92.61 ± 4.10$^{\#\#}$	4.76 ± 0.10$^{\#\#}$	13.55 ± 0.45$^{\#}$
VI	GTE (200 mg/kg *p.o.*) + DOX	101.26 ± 4.09$^{\#\#\#}$	301.05 ± 7.35$^{\#\#\#}$	108.19 ± 2.56$^{\#\#\#}$	6.46 ± 0.12$^{\#\#\#}$	17.27 ± 0.62$^{\#\#\#}$
VII	GTE (400 mg/kg *p.o.*) + DOX	117.35 ± 3.12$^{\#\#\#}$	340.33 ± 6.76$^{\#\#\#}$	121.02 ± 2.98$^{\#\#\#}$	7.92 ± 0.15$^{\#\#\#}$	23.34 ± 0.39$^{\#\#\#}$
VIII	Vit. E (100 mg/kg *p.o.*) + DOX	102.11 ± 3.26$^{\#\#\#}$	309.36 ± 7.98$^{\#\#\#}$	112.42 ± 2.84$^{\#\#\#}$	6.81 ± 0.084$^{\#\#\#}$	18.80 ± 0.47$^{\#\#\#}$

The data are expressed as the mean ± S.E.; n = 6 in each group. $^{***}p < 0.001$ compared with the corresponding value for normal control rats (group I). $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.1$ compared with the corresponding value for DOX treated rats (group II).

Oxidative stress is characterized by increased lipid peroxidation (LPO) and altered enzymatic and non-enzymatic antioxidant systems (31). In the present study, a significant increase in the level of LPO, in cardiac tissue of DOX alone treated rats was observed. Free radicals initiate LPO of biological membranes. GTE have been shown to neutralize reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite, thereby reducing the damage to lipid membranes, proteins and nucleic acids in cell-free systems. The assessment of the relative contribution of different pathways of free radical production elicited by DOX, is an important tool for toxicological mechanisms of these agents and for the implementation of adequate therapeutic approaches towards limiting their toxicological effects.

The glutathione antioxidant system plays a fundamental role in cellular defence against free radicals and other oxidant species (32). GSH plays a crucial role in both scavenging reactive oxygen species and the detoxification of the drugs. GSH with its –SH group functions as a catalyst for disulfide exchange reactions, and plays a major role in H_2O_2 detoxification. GSH depletion results in impaired cell defence and tissue injury. Following DOX administration, glutathione status get greatly impaired as indicated by a decrease in the GSH level in both blood and the heart. In this study, reduction in levels of MDA and elevation in GSH in GTE treated rats suggest that it scavenges free radicals, generated during oxidative stress (35).

In our study, the activity of all the antioxidant enzymes like: glutathione peroxidase, glutathione reductase, glutathione s-transferase, superoxide dismutase and catalase were significantly decreased in DOX alone treated rats. This result showed that DOX generates free radicals in heart and decreases its ability to detoxify ROS. However, GTE significantly increased the level of these antioxidant enzymes in DOX treated groups. The protection thus offered could be attributed to its antioxidant and ROS scavenging properties.

The histopathological changes of DOX induced cardiotoxicity, consist in order of increasing severity, swelling of sarcoplasmic reticulum, cytoplasmic vacuolization, myofibrillar degeneration, myocyte disruption and fibrosis (34–36). In our study, we have observed hypertropic fibre, disruption of myocyte structure, including damage to microtubules, vacuolization, dilation of sarcoplasmic reticulum, loss of myofibrils and alteration of mitochondrial functions such as decreased mito-

chondrial enzyme activities, which goes fine with the above finding. Rats pretreated with GTE showed cardiac muscle fibers of normal shape, size and configuration. Only a single vacuole is seen in one of the myocardial fibres. The protection exhibited by GTE as evident by the histopathological changes may be due to its antioxidant potential to counteract free radicals.

The overall protective effect of GTE is probably due to counter action of free radicals by its antioxidant nature hence its ability to restore normalcy in tissue under oxidative stress. However, the precise molecular mechanism by which GTE exerts its protective action against oxidative damage remains to be established. If this protective function is confirmed in cancer patients, GTE may have an important clinical significance as an adjuvant therapy with DOX.

REFERENCES

1. Clement Y.: Prev. Med. 49, 83 (2009)
2. Choi E.H., Chang H.J., Cho J.Y., Chun H.S.: Food Chem. Toxicol. 45, 1873 (2007).
3. Steinherz L.J., Steinherz P.G., Tan C.T.: JAMA 266**,** 1672 (1991).
4. Ewer M.S., Jaffe N., Reid H., Zietz H.A., Benjamin R.S.: Med. Pediatr. Oncol. 31, 512 (1998).
5. Weiss R.B.: Semin. Oncol. 19, 670 (1992).
6. Bast A., Haenen G.R., Doelman C.J.: Am. J. Med. 30, 2S (1991).
7. Youdim K.A., Joseph J.A.: Free Radic. Biol. Med. 30, 583 (2001).
8. Frank J., George T.W., Lodge J.K., Rodriguez-Mateos A.M., Spencer J. P. E., Minihane A. M., Rimbach G.: J. Nutr. 139, 58 (2009).
9. Zheng J., Lee H.C.M., Mukmin bin Sattar M., Huang Y., Bian J.-S.: Eur. J. Pharmacol. 652, 82 (2011).
10. Nagle D.G., Ferreira D., Zhou Y.D.: Phytochemistry 67, 1849 (2006).
11. Reitman S., Frankel A.S.: Am. J. Clin. Pathol. 28**,** 56 (1957).
12. Tsung S.H.: Creatine Clin. Chem. 29, 2040 (1983).
13. Lum G., Gambino S.R.: Am. J. Clin. Pathol. 61, 108 (1974).
14. Omura T., Sato R.: J. Biol. Chem. 239, 2370 (1964).
15. Ohkawa H., Ohishi N., Yagi K.: Anal. Biochem. 95, 351 (1979).
16. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J.: J. Biol. Chem. 193, 265 (1951).
17. Beutler E., Duron O., Kelly B.M.: J. Lab. Clin. Med. 61, 882 (1963).
18. Sedlak J., Lindsay R.H.: Anal. Biochem. 25, 192 (1968).
19. Mohadas J., Marshall J.J., Duggin G.G., Horvath J.S., Tiller D.J.: Cancer Res. 44, 5086 (1984).
20. Haque R., Bin-Hafeez B., Parvez S., Pandey S., Sayeed I., Ali M., Raisuddin S.: Hum. Exp. Toxicol. 22, 473 (2003).
21. Marklund S., Marklund G.: Eur. J. Biochem. 47, 469 (1974).
22. Claiborne A.: Catalase activity, in Handbook of Methods for Oxygen Radical Research. Greenwald R.A. Ed., pp. 283–284, CRC Press, Boca Raton 1985.
23. Doroshow J.H.: Cancer Res. 43, 460 (1983).
24. Singal P.K., Deally C.M., Weinberg L.E.: J. Mol. Cell Cardiol. 19, 817 (1987).
25. Wittevean S.A.G., Hemker H.C., Hollar L., Hermens W.: Br. Heart J. 37, 795 (1975).
26. Sarvazyan N.: Am. J. Physiol. 271, H2079 (1996).
27. Konorev E.A., Kennedy M.C., Kalyanaraman B.: Arch. Biochem. Biophys. 368, 421 (1999).
28. Davies K.J., Doroshow J.H.: J. Biol. Chem. 261, 3060 (1986).
29. Berlin V., Haseltine W.A.: J. Biol. Chem. 256, 4747 (1981).
30. Yamamoto K., Masubuchi Y., Narimatsu S., Kobayashi S., Horie T.: Pharmacol. Toxicol. 88, 76 (2001).
31. Padmanbhan M., Stanley M., Prince P.: J. Sci. Food Agric. 86, 772 (2006).
32. Parvez S., Tabassum H., Rehman H., Banerjee B.D., Athar M., Raisuddin S.: Tox. 225, 109 (2006).
33. Meister A., Anderson M.E.: Ann. Rev. Biochem. 52, 711(1983).
34. Bristow M.R., Mason J.W., Billingham M.E., Daniels J.R.: Am. Heart J. 102, 709 (1981).
35. Torti F.M., Bristow M.M., Lum B.L.: Cancer Res. 46, 3722 (1986).
36. Billingham M.E., Mason J.W., Bristow M.R., Daniels J.R.: Cancer Treat. Rep. 62, 865 (1978).

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GENERAL

QUANTITATIVE STUDY EVALUATING PERCEPTION OF GENERAL PUBLIC TOWARDS ROLE OF PHARMACIST IN HEALTH CARE SYSTEM OF PAKISTAN

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Abstract: To investigate general public perception towards the role of pharmacist in developing countries' healthcare system was the main aim of this study, which would be the basic foundation for researching the treatment pattern of cognitive disorder after stroke in communities. The study population (sample size = 385) consisted of general public from Islamabad, Faisalabad and Lahore, Pakistan. Main sections of the questionnaire comprised of series of statements pertaining to consumer's perception and experience with the pharmacists. The response rate of study was 77.1%. A majority (80.1%) of the consumers knows who is pharmacist; 49.8% (n = 148) of the respondents found the pharmacist working in the pharmacies; 74.1% (n = 220) believed that pharmacist can guide them regarding their medicine. With respect to government efforts to improve services provided by community pharmacies, less percentage (31.0%) of the consumers were satisfied. Half of the respondents (59.9%) were expecting from the pharmacists to be knowledgeable drug therapy experts, whereas 61.3% (n = 182) expect from the pharmacists to educate them regarding safe and appropriate use of medication. The findings of this study conclude that the quality of pharmaceutical services provided is very low in Pakistan. There is a gap between the public and the pharmacist, which can only be filled by creating awareness among public regarding the pharmacist's role in healthcare system and by focusing on how services provided by the pharmacists can add improvement to general public health.

Keywords: general public, perception, pharmacist role, pharmaceutical services, cognitive disorder, stroke

In recent years, pharmacy profession has extended its role of product-oriented to consumer-oriented with an emphasis on the provision of consumer care services (1, 2). Although there are barriers to pharmacist-consumers communication due to personal and social factors that influence pharmacy practice (3), but the involvement of the patients in their own healthcare is the present need of today (4). The consumers' perception of the benefits of pharmaceutical care is based on the ability of the pharmacist to help them. More frequent interaction with the consumers increases the opportunities to improve outcomes of therapy (5). In developed countries, studies showed that pharmacists were willing to provide evidence based advice to people. It also showed that patients believed that they were at lower risk if they were able to meet with a pharmacist regularly, and the pharmacist was identified as the predominant source of information for medicines (4–6). This scenario in developing countries is totally different. The pharmacist population ratio is very low, and a majority of the population, especially in poor and rural areas rarely come in contact with the trained pharmacist (7, 8). Pharmacy services throughout the world play a broad range of activities in provision of health services provided to the general public. Comparatively small number of studies focused towards the role of pharmacist in this context (9). However, there are couple of studies, which do focused with the use of medicines and pattern of practice in low and middle income countries (10–15). There are very few studies, which focused

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on the consumer's perception regarding community pharmacist. At present, this study is first of its kind, which focuses directly on the perception of consumer towards community pharmacists' role in healthcare delivery in developing countries, and currently many subsequent symptoms such as cognitive disorder after stroke have a long course for treatments, so Community Clinic rather than Integrated Hospitals has been the main choice for many patients (16–18), and in this condition, community physicians and pharmacists' role are more and more important. So the research will be a basic foundation for the further design of treatment pattern of cognitive disorder after stroke in communities.

EXPERIMENTAL

The questionnaire was developed after the extensive literature review and from the qualitative findings of the study. The questionnaire had five parts, which included the demographic information, general awareness about pharmacists and places of work, perception, expectation and experience regarding pharmacists' role. Each section of the questionnaire included a set of statements for which the respondents were asked. There are questions which required a "yes" or "no" response. To indicate the level of agreement, 4-point Likert scale, where 1 = strongly disagree, 2 = disagree, 3 = agree, 4 = strongly agree, was used in order to avoid confusion with the 'neutral' responses.

The primary version of the questionnaire consisting of 30 items was viewed by the professionals at the School of Pharmacy, Universiti Sains Malaysia (USM). These professionals were asked to assess the questionnaire by providing their overall opinion and by listing the questions in the order of relevance and importance. The more relevant and important questions were highlighted. To assess face validity of the questionnaire, thirty participants were solicited. These participants were asked for their views on the significance, worth, and simplici-

Table 1. General public demographic characteristics.

Variable	Frequency	Percent (%)
Age (mean ± SD, 31.53 ± 10.8)		
15–25	126	42.4
26–35	74	24.9
36–45	57	19.2
46–55	34	11.4
> 56	6	2.0
Gender		
Male	149	50.2
Female	148	49.8
Education		
No education	10	3.4
Religious studies only	13	4.4
Primary	20	6.7
Matric/SSC	20	6.7
FA /FSc	46	15.5
Graduation	88	29.6
Post graduation	105	35.4
Employment		
Government employee	69	23.2
Private employee	67	22.6
Businessman	58	19.5
Unemployed	103	34.7

ty of each question and to identify, which questions they would point out to be removed so to make the questionnaire brief and comprehensive. In addition to this, the participants were also welcomed to suggest further comments on questions whether they are understandable or not. Most of them suggested simplifying the questions. The reliability test was applied to all variables comprising all domains. The reliability of tool was estimated on the basis of Cronbach's Alpha ($\alpha = 0.74$).

The study population consisted of general public from three cities of Punjab province named Islamabad, Faisalabad and Lahore.

Sample size was calculated by Roasoft soft sample size calculator (19) with confidence interval of 95 and 5% margin of error. Due to a lack of sampling frame and up-to-date electronic population database, a convenient sampling technique was used. A sample of 385 was calculated and by adding 20% drop-out rate, sampling size was 462 consumers who were selected from the pharmacies (chain and independent) of three cities of Pakistan.

The questionnaire was distributed to those people visiting 25 pharmacies (chain and independent) (14), which were selected from three cities. Data were obtained through a 4 hour visit made to each pharmacy by a data collecting team over a period of 3 months. During these visits, questionnaires were filled by consumers of all pharmacies who requested advice from the pharmacists.

Survey administration and time frame

Survey was conducted for a period of three months from January through March 2011. Community pharmacists were informed regarding survey of general public perceptions regarding the role of pharmacists, the aim, objective and nature of the study and verbal consent was given.

Secondly, general public who visited the pharmacies were informed regarding survey. After obtaining their consent for participation in this study, the execution of the study took place.

Data collection

Questionnaires were collected. Responses were exported to Statistical Package for Social Sciences (SPSS®) for Windows, version 15, to perform statistical analysis (20).

Data analysis

Non-parametric statistical test and appropriate descriptive statistics for demographic characteristics (mean and standard deviation for age) were performed using SPSS® for Windows, version 15. The demographic information that was collected included age, gender, education and employment, frequencies and descriptive statistic of each variable was reported. The χ^2-test was used to test significance of association between independent variables (age, gender, education and employment) and dependent variables (general awareness about pharmacists, perception regarding pharmacist's role, expectation from pharmacist's role and experience with pharmacists). Statistical significance was accepted at p value of < 0.05.

RESULTS AND DISCUSSION

Response rate

During the period of three months of data collection from January to March 2011, a total of 385 questionnaires were distributed, 297 questionnaires were returned giving a response rate of 77.1%.

Demographic characteristics

Demographic characteristics of general public who took part in this study are shown in Table 1. Mean age was 31.53 years with standard deviation (SD) = 10.8 years. Among the respondents, 50.2% (n = 149) were male, while 49.8% (n = 148) were female. A majority (34.4%) of the consumers were post graduates, 29.6% (n = 88) had graduation degree, the education of 15.5% (n = 46) were FA/FSc. There is equal (6.7%, n = 20) number of primary and matric level consumer, whereas 4.4% (n = 13) were having religious education only, while 3.4% (n = 10) of the consumers have no education. With respect to employment of the consumers, a majority (34.7%) of the consumers were unemployed. Government employee were 23.2% (n = 69), while 22.6% (n = 67) belonged to private organizations and 19.5% (n = 58) were businessmen.

General awareness about pharmacists

A majority (80.1%) of the consumers knows who is pharmacist; 49.8% (n = 148) of the respondents found the pharmacists working in the pharmacies. Only half (56.9%) of the respondents replied that they can got medicine without prescription; 74.1% (n = 220) believed that pharmacist can guide them regarding their medicine; 67.7% (n = 201) of consumers felt comfortable while taking advice from the pharmacists, while 65.3% (n = 194) trust on pharmacists advice. Beside medicines, 53.5% (n = 159) of the consumers trust on pharmacist's advice on health related issues. Only half (50.2%) of the respondents showed their satisfactions with services provided by the community pharmacies. With

respect to the government efforts to improve the services provided by the community pharmacies, less percentage (31.0%, n = 92) of the consumers were satisfied.

Perception regarding pharmacist's role

Table 3 indicates perception regarding the pharmacist's role in healthcare. Approximately half (45.8%, n = 136) of the consumers agreed on pharmacists providing patient education, and 48.8% (n = 145) pharmacists suggesting use of non-prescription medications. Respondents were asked about their opinion suggesting the use of certain prescription medications to patients and only 61.6% (n = 183) of the respondents agreed. With respect to treating minor illness only half (50.5%) of the respondents agreed and 43.1% (n = 128) of the respondents agreed with the pharmacist role as identifying and preventing prescription errors. Respondents were asked regarding designing and monitoring pharma-

Table 2. General awareness about pharmacist.

Items in questionnaire	Frequency of "Yes"	Percent of "Yes"
Do you know who is pharmacist in healthcare setting?	238	80.1
When you go to pharmacy / medical store you find pharmacist working?	148	49.8
Can you get medicine without prescription?	169	56.9
Did you know pharmacist can guide you regarding your medicine?	220	74.1
Would you feel comfortable talking to pharmacist regarding your minor illness?	201	67.7
Do you already talk to pharmacist for advice on medicines?	160	53.9
Do you trust your pharmacist's advice about your medicine?	194	65.3
Would you trust your pharmacist's advice on other health issues besides medicines?	159	53.5
Are you satisfied with the services provided by the community pharmacies?	149	50.2
Are you satisfied by the steps taken by government to improve the services provided by the community pharmacies?	92	31.0

Table 3. Perception regarding pharmacist's role.

Items in questionnaire	Responses *				p value **		
	n (%)				Age	Gender	Employment
	SD	D	A	SA			
1	9 (3.0)	136 (45.8)	131 (44.1)	21 (7.1)	0.512	0.738	0.482
2	39 (13.1)	98 (32.3)	145 (48.8)	17 (5.7)	0.125	0.958	0.696
3	16 (5.4)	80 (26.9)	183 (61.6)	18 (6.1)	0.740	0.724	0.326
4	18 (16.1)	111 (37.4)	150 (50.5)	18 (16.1)	0.197	0.128	0.115
5	22 (7.4)	126 (42.4)	128 (43.1)	21 (7.1)	0.277	0.891	0.591
6	47 (15.8)	137 (46.1)	95 (32.0)	18 (6.1)	0.471	0.513	**0.044**
7	50 (16.8)	143 (48.1)	87 (29.3)	17 (5.7)	0.505	0.866	**0.025**

* Responses were for all respondents ** χ^2; SD = strongly disagree, D = disagree, A = agree, SA = strongly agree. 1 = Providing patient education. 2 = Suggesting use of non-prescription medications. 3 = Suggesting use of certain prescription medications to patients. 4 = Treating the minor illnesses. 5 = Identifying and preventing prescription errors. 6 = Designing and monitoring pharmacotherapeutic regimens. 7 = Monitoring outcomes of pharmacotherapeutic regimens and plans.

Table 4. Expectation from pharmacist's role.

Items in questionnaire	Responses *				p value **		
	n (%)				Age	Gender	Employment
	SD	D	A	SA			
1	18 (6.1)	66 (22.2)	173 (58.2)	40 (13.5)	0.000	0.051	0.007
2	17 (5.7)	54 (18.2)	178 (59.9)	48 (16.2)	0.059	0.655	0.082
3	13 (4.4)	53 (17.8)	182 (61.3)	49 (16.5)	0.295	0.506	0.167
4	14 (4.7)	87 (29.3)	148 (49.8)	48 (16.2)	0.130	0.430	**0.007**

* Responses were for all respondents ** χ^2; SD = strongly disagree, D = disagree, A = agree, SA = strongly agree. 1 = I expect pharmacists to take personal responsibility for resolving any drug-related problems. 2 = I expect pharmacists to be knowledgeable drug therapy experts. 3 = I expect pharmacists to educate me about the safe and appropriate use of medication. 4 = I expect pharmacists to monitor response to drug therapy and let me know if encounters any drug-related problem.

Table 5. Experience regarding pharmacist's role.

Items in questionnaire	Responses *				p value **		
	n (%)				Age	Gender	Employment
	SD	D	A	SA			
1	12 (4.0)	45 (15.2)	205 (69.0)	35 (11.8)	0.221	0.199	0.206
2	13 (4.4)	59 (19.9)	179 (60.3)	46 (16.5)	0.183	0.653	0.388
3	28 (9.4)	110 (37.0)	123 (41.4)	36 (12.1)	0.081	0.424	0.189
4	59 (19.9)	117 (39.4)	102 (34.3)	19 (6.4)	0.110	**0.014**	**0.001**

* Responses were for all respondents ** χ^2; SD = strongly disagree, D = disagree, A = agree, SA = strongly agree. 1 = In my experience, pharmacists are a reliable source of general drug information. 2 = In my experience, pharmacists are important health processional in the healthcare system. 3 = Pharmacists routinely counsel patients regarding the safe and appropriate use of their medications. 4 = In my experience, pharmacists appear willing to take personal responsibility for resolving any drug-related problems they discover.

cotherapeutic regimens as one of the roles of pharmacists, 46.1% (n = 137) of the respondents disagreed. The value was found to be statistically significant (p = 0.044) with respect to employment; 48.1% (n = 143) of the respondents disagreed with the pharmacist's role as monitoring outcomes of pharmacotherapeutic regimens and plans, again the value was found to be statistically significantly (p = 0.025) with respect to employment.

Expectation from pharmacist role

Part 4 of the questionnaire describes the expectation from pharmacist's role. Half of the respondents (58.2%) expect from the pharmacists to take personal responsibility for resolving any drug-related problems. The value was found to be statistically significant (p = 0.007) with respect to the employment. More than half (59.9%) of the respondents were expecting from the pharmacists to be knowledgeable drug therapy experts, 61.3% (n = 182) expect from the pharmacists to educate them regarding the safe and appropriate use of medication. Again only half of the respondents (49.8%, n = 148) agreed with the expectation from the pharmacists to monitor response to drug therapy and let them know if they encounter any drug-related problem.

Experiences with pharmacist

Consumer's experiences with the pharmacists are elaborated in Table 5; 69.0% (n = 205) of the respondents agreed on the statement that pharmacists are a reliable source of general drug information. 60.3% (n = 179) were agreed that pharmacists are important health processionals in the healthcare system. Less than half of the respondents (41.4%, n = 123) agreed on the statement that pharmacists routinely counsel patients regarding the safe and appropriate use of their medications. Some respondents

were disagreed (39.4%, n = 117) with the pharmacists willingness to take personal responsibility for resolving any drug-related problems they discover, whereas there was small number (34.3%) of respondents who agreed on the statements. The value was found to be statistically significant ($p = 0.014$) and ($p = 0.001$) with respect to the gender and employment, respectively.

In general, 385 questionnaires were hand delivered to the general public visiting community pharmacies and 297 were received by the principal researcher, thus giving response rate of 77.1%, which is adequate and found to be within the range (5–70%) quoted by researchers for self administered questionnaire (21).

The majority of the respondents knew about pharmacist in the healthcare system. On the other hand, only half of the population did not know whether they were present in community pharmacies, the possible reason could be the trend in Pakistan (22). They believed in the advice on common health problem from the person standing at the pharmacies even from the unqualified drug sellers. This problem has already been discussed in number of studies (23–25). There are also studies, which gave the possible reason of least awareness of pharmacist in the system, where the pharmacists are not recognized by the general public. The possible reason could be the small ratio of pharmacists available for population than they are required (12, 26). Respondents in this study believed that pharmacist can guide them in proper use of medicine and they also showed trust towards pharmacists. The finding is consistent with the studies in Qatar and Jordan where a majority of the public believed on pharmacists' role in provision of advice with respect to the rational use of medicines (13, 27).

Pharmacists play a very important role in solving all drug related problems for achieving optimal patient outcomes and ultimately improve the quality of life of the patient (28). The perception regarding pharmacists' role is different in different parts of the world. The finding in the study shows less difference between agreed and disagreed among the general public. Results from the studies showed that neither doctors nor consumers believed that pharmacists had momentous role in pharmaceutical care (29). Moreover, pharmacists and consumers did not understand each other's discernment regarding pharmaceutical care services (30). However, half of the respondents agreed on pharmacist's role in treating minor illness, it could be the reason the general public is saving the cost of doctors and will take direct advice from pharmacists. This can be beneficial as in case of limited resources available in developing countries and in this context, the role of pharmacists at the pharmacies has been diversified, integrating not only dispensing, but also health education and sometimes even diagnosis (22).

The expectations of general public in Pakistan are favorable towards pharmacists. They expect the pharmacists to provide broad range of pharmaceutical care services including safe use of medication along with monitoring of response from drug therapy. The finding from another study which focused on 'patient's expectation" revealed that the pharmacists also believed themselves to provide information related to appropriate use of medication (31). Studies from the developing countries also revealed that the people had great trust on pharmacists when they visit the pharmacies (11–13).

General public in Pakistan conceived the pharmacist as an authentic source of drug information, and believed in them as an important member of healthcare team. The finding supports the pharmacist's role in the country. As far as their experience with the pharmacist's willingness to take personal responsibility is concerned, they disagreed with the small difference with the agreed respondents. The possible reason could be the focus of pharmacists towards the managerial job rather than providing pharmaceutical services.

Study limitations

This study is subjected to certain limitations due to the general public involved from three cities only, namely Lahore, Faisalabad and Rawalpindi/Islamabad of Punjab province only, which cannot be generalized to the other provinces of the country. Moreover, only the general public involved who visited the chain or independent pharmacies within four hour visit of the data collecting team, were considered as the respondents for the study. There is no electronic data base available neither for the public living in these three cities, nor for the number where pharmacists are working.

CONCLUSION

The general public in Pakistan is aware of the pharmacists in the healthcare system. There are shortcomings in professional practice terms of providing pharmaceutical care services to the consumers. The trends of chain pharmacies in developing countries, which appoint pharmacists for 24 h at pharmacies, is greatly responsible for creating positive image of pharmacies in the society. The only need of time is to increase the number of pharma-

cists so that the pharmacists along with the management can equally focus on the provision of healthcare services to the community. Based on the role of pharmacists in community, the new treatment pattern of subsequent symptoms such as cognitive disorder after stroke could be considered in communities.

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REFERENCES

1. Kotecki J.E.: J. Commun. Health 27, 291 (2002).
2. Worley M.M.: Res. Soc. Admin. Pharm. 3, 47 (2007).
3. Paluck E.C.: Evaluation Health Prof. 26, 380 (2003).
4. Tio J., LaCaze A., Cottrell N.: Pharm. World Sci. 29, 73 (2007).
5. Nau D.P.: J. Am. Pharm. Assoc. 40, 36 (2000).
6. Silcock J.: BMC Musculoskel. Disord. 8, 10 (2007).
7. Viberg N:. Pharm. World Sci. 29, 25 (2007).
8. Goel P.: Soc. Sci. Med. 42, 1155 (1996).
9. Smith F.: Pharm. World Sci. 31, 351 (2009).
10. Smith F.: Health Pol. Plan. 19, 234 (2004).
11. Oparah A.C.: Res. Soc. Admin. Pharm. 2, 499 (2006).
12. Geetha Jayaprakash G.., Rajan M.L.., Shivam P.: Pharm. Pract. 7, 157 (2009).
13. Wilbur K., Salam S.E., Mohammadi E.: Patient Pref. Adher. 4, 87 (2010).
14. Al-Hassan M.: J. Med. Sci. 9, 36 (2009).
15. Abdel Moneim A., Al-Rabiy S., Aba Hussein E.: Med. Princ. Pract. 17, 315 (2008).
16. Makin S.D.J., Turpin S., Dennis M.S., Wardlaw J.M.: J. Neurol. Neurosurg. Psychiatry 10, 1136 (2013).
17. Douiri A., Rudd A.G., Wolfe, C.D.: Stroke 44, 1 (2013)
18. Scobbie L., McLean D., Dixon D., Duncan E., Wyke, S.: BMC Health Serv. Res. 13, 1 (2013).
19. Raosoft, An online sample size calculator. 2008.
20. SPSS, SPSS® base 15.0 user's guide. SPSS Inc., Chicago, IL 2003.
21. Bourque L., Fielder E.: How to conduct self-administered and mail surveys. 2nd edn. Thousand Oaks, Sage Publications, Inc., London 2003.
22. Butt Z.A.: Int. J. Quality Health Care 17, 307 (2005).
23. Syhakhang L.: Eur. J. Clin. Pharmacol. 57, 221 (2001).
24. Lansag M.A., Lucas A.R., Tupasi T.E.: J. Clin. Epidemiol. 43, 61 (1990).
25. Stenson B.: Soc. Sci. Med. 52, 393 (2001).
26. Owusu-Daaku F., Smith F., Shah R.: Pharm. World Sci. 30, 577 (2008).
27. Wazaify M.: Pharm. World Sci. 30, 884 (2008).
28. Hepler C.D., Strand L.M.: Am. J. Hospital Pharm. 47, 533 (1990).
29. Law A.V.: J. Am. Pharm. Assoc. 43, 394 (2003).
30. Assey-Eley M., Kimberlin C.: Health Commun. 17, 41 (2005).
31. Schommer J.C.: Am. J. Pharm. Educ. 16, 402 (1997).

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SHORT COMMUNICATION

QUANTITATIVE ANALYSIS OF FLAVONOIDS AND PHENOLIC ACIDS FROM INFLORESCENCES AND AERIAL PARTS OF SELECTED *CIRSIUM* SPECIES USING ASE METHOD

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Keywords: Extraction method, SPE HPLC, *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss., phenolic acids, flavonoids

According to the literature, plants from the *Cirsium* genus are rich in phenolic compounds (1–6). Phenolic acids are known to have various biological activities, especially fungistatic, bacteriostatic, antioxidant, anticancer, choleretic, potential sedative – hypnotic, antianxiety and anticonvulsant activity (1, 8, 9). Flavonoids display vasoprotective, hepatoprotective, anti-inflammatory, anticarcinogenic, and free radical-scavenging properties. Recent studies have shown that apigenin exhibits anti-proliferation effects on several forms of cancer cells such as prostate cancer cells, breast cancer cells, leukemia cells, colon cancer cells. Compared with other flavonoid substances, apigenin is characterized by low toxicity and non-mutagenesis (9).

So far, flavonoids and phenolic acids have not been studied in such plants as *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. Therefore, it is the first time when qualitative and quantitative analysis of flavonoids and phenolic acids in the flower and herb of *Cirsium* spp. has been carried out. The aim of this study was to carry out the separation of the active components of inflorescences and leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. using accelerated solvent extraction method. The obtained extracts were combined and purified by SPE. The SPE-eluates were analyzed by RP-HPLC. Quantitative analyses of flavonoids and phenolic acids were also carried out. Standard deviation was calculated for all the results, which allows to claim that the results are statistically significant. The isolation and separation of natural compounds (including phenolic acids) from plants is a very important problem in phytochemical analysis (10–14). Flavonoids were quantitatively analyzed for the first time. The obtained methanolic extracts proved to be very rich in flavonoids, some of which have not been identified.

EXPERIMENTAL

Plant material

Inflorescences and leaves were collected in 2009 in the Medicinal Plant Garden, Department of Pharmacognosy, Lublin, Poland. They were dried in air at room temperature and immediately powdered according to accepted normal procedures.

Plant material (1 g) was placed in the stainless-steel cell of Dionex (Sunnyvale, CA, USA) ASE 100 accelerated solvent extractor using methanol as solvent.

The extraction conditions were optimalized, giving the best parameters of extraction for: methanol concentration 70%, temperature: 85°C, number of cycles: 3. Extraction was performed at 100 bar.

Purification procedures

All the methanolic extracts were concentrated under reduced pressure, dissolved in small portion of methanol, and transferred to a 10 mL graduated flask. Solid phase extraction (SPE) is a popular procedure used for the isolation, purification and pre-

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concentration of organic compounds present in biological material. It is often considered an alternative to other methods. In this study, SPE was used for the isolation of flavonoids and phenolic acids from the *Cirsium* genus**.** Samples containing phenolic acids were purified from fatty components and chlorophylls with SPE. Samples were evaporated to dryness, dissolved in 30% aqueous methanol and applied to octadecyl BakerBond SPE microcolums (500 mg, 3 mL, J.T. Baker Phillipsburg, NJ, USA) previously activated with 10 mL of methanol and then 10 mL of water. The isolated compounds were obtained by the elution of columns with 7 mL methanol : water, 80 : 20 (v/v), under reduced pressure (SPE-12G chamber, J.T. Baker USA, Groß-Gerau, Germany). The eluates obtained were free from ballast compounds and contained aglycones and phenolic acids (15).

RP-LC analysis

LC was performed with an Agilent 1100 system coupled with an autosampler, a column thermostat; and diode array detector (DAD). Compounds were separated on 250 × 4.6 mm stainless-steel column packed with 5 μm Hypersil XDB- C18 (ZORBAX Eclipse), using a stepwise mobile phase gradient prepared from 1% aqueous acetic acid (component A) and methanol (component B) (v/v). The gradient was: 0 min, 2% B in A; 8 min 5% B in A; 12 min 10% B in A; 20 min 25% B in A; 35 min 45% B in A; 40 min 60% B in A, 45 min 75% B in A. The mobile phase flow rate was 1 mL/min, the sample injection volume was 10 μL, and elution was performed at 25°C. The LC pumps, autosampler, column oven, and DAD were monitored and controlled by use of HP Chem. Station rev.10.0 software (Agilent).

The identity of compounds examined was performed by the comparison of retention times (t_R) and UV spectra with standard substances analyzed under the same conditions. The qualitative and quantitative analysis was performed. Retention times were compared with those of standards, using UV spectra (λ = 254, 280 and 320 nm) as a comparative parameter. Quantitative determination was performed at the wavelength of maximum absorption of flavonoids and chlorogenic acid – 320 nm.

Recovery, repeatability and precision

Each extract was injected in triplicate on the same day. The RSD (relative standard deviation, %) of retention times and peak arrays were used as the measure of precision. The method of precision was evaluated by use of intra-day and inter-day tests. Intra-day experiments were performed by replicate analysis of three aliquots of the same sample on the same day, inter-day tests were performed on three consecutive working days in the same way as intraassay experiments (16). Peak area of each of the extract components was measured three times.

Calibration procedure

Each calibration plot was prepared three times after chromatography of five different concentrations (1, 0.75, 0.5, 0.25, 0.1 mg per 10 mL for all the flavonoids and chlorogenic acids except kaempferol-3-rhamnoglucoside, for which the additional concentration of 0.05 mg per 10 mL. was also

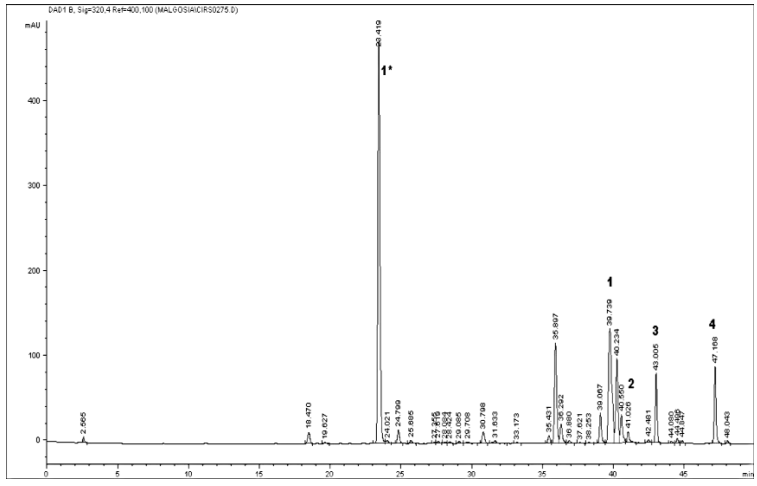

Fig. 1. HPLC chromatogram obtained from analysis of the flower of *C. ligulare* Boiss.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 3. kaempferol 3-glucoside, 4. apigenin

used). Quantification was performed by comparing the chromatographic peak areas for the external standard. The calibration plots were characterized by their regression coefficients, the slopes of plots *(b)* and the intercepts of the plots with the *y*-axis *(a)*. Calibration equations for flavonoids were: apigenin 7-glucoside y = 487.26x – 355.12 R^2 = 0.992; apigenin y = 966.45x – 739.91 R^2 = 0.9923; kaempferol 3-rhamnoglucoside y = 157.51x – 144.64 R^2 = 0.9917; and for phenolic acid: chlorogenic acid y = 646.75x – 629.77 R^2 = 0.9919.

RESULTS AND DISCUSSION

Calibration plots for the phenolic acids were highly linear ($R^2 > 0.991$) in the concentration range 0.05–1.00 mg per 10 mL (n = 3). The obtained methanol extracts proved to be very rich in flavonoids, some of which have not been identified. Therefore, more research is required. In the investigated inflorescences and aerial parts of leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. qualitative HPLC

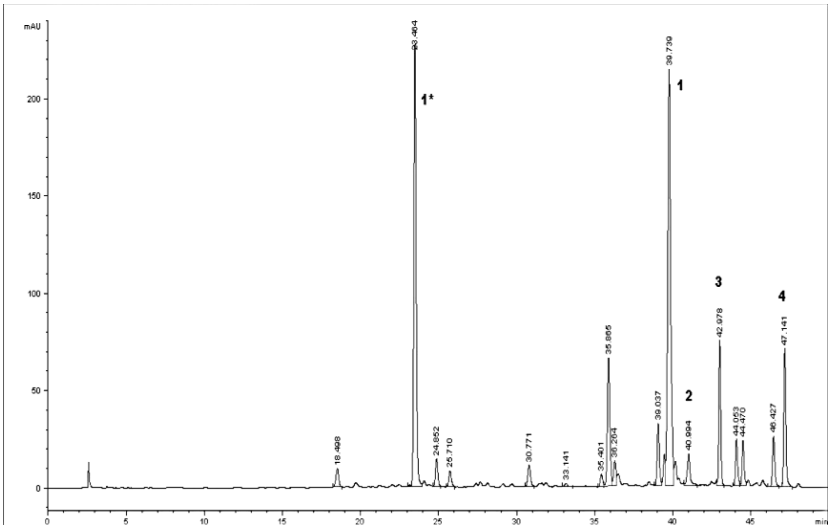

Fig 2. HPLC chromatogram obtained from analysis of the flower of *C. eriophorum* (L.) Scop.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 3. kaempferol 3-glucoside, 4. apigenin

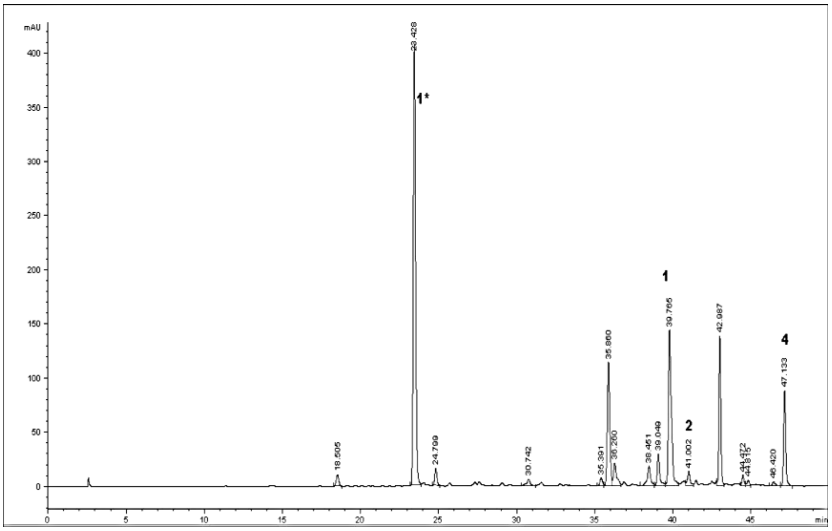

Fig 3. HPLC chromatogram obtained from analysis of the flower of *C. decussatum* Janka.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 4. apigenin

analysis was performed for selected identified compounds. Flavonoids, such as apigenin 7-glucoside, kaempferol 3-glucoside, kaempferol 3-rhamnoglucoside, apigenin, and chlorogenic acid were identified. Because we have not standard of kaempferol 3-glucoside we do not determinate this compound quantitatively.

Standard deviation was calculated for all the results, which allows to claim that the results are statistically significant. HPLC analysis of extracts enabled the identification of four flavonoids and one phenolic acid. Typical chromatograms obtained from the extracts from inflorescences are shown in Figures 1–3. The analysis of methanol extracts of three inflorescences and leaves from the *Cirsium* genus, as well as their quantitative analysis were carried out for the first time. The concentration of flavonoids was varied in selected investigated inflorescences and leaves. The amounts of all flavonoids and phenolic acid were estimated by HPLC. Chlorogenic acid was found in every fraction studied in different proportions, depending on species and organ. The acid was predominant in the inflorescences, mainly in the flowers of *C. ligulare* where amount was twice higher than in inflorescences of *C. eriophorum* (50.28 [mg/g] and 24.92 [mg/g], respectively). The leaves of *C. decussatum* were richer source of this acid (47.01 mg/g comparing to *C. ligulare* 26.34 mg/g and *C. eriophorum* – only 9.73 mg/g) (Table 1.) The results of the study suggest that chlorogenic acid may by responsible for the activity and applications of plants of the *Cirsium* genus. This is important because this acid is known for its antioxidant activity (16–18). Nazaruk et al. (19) performed studies concerning simultaneous identification of eight phenolic acids and three flavonods aglycones in Et_2O-fractions of inflorescences and leaves of five species of *Cirsium* genus – *C. arvense, C. oleraceum, C. palustre, C. rivulare and C. vulgare*. The contents of phenolic acids, expressed as caffeic acid, determined by the spectroscopic method with Arnov's reagent, were higher in leaves than in flowers heads.

Flavonoids are also predominant compounds in inflorescences. The main compound is apigenin 7-glucoside present in *C. ligulare* (27.75 mg/g and 23.55 mg/g), respectively, in *C. decussatum* and in very small amount in *C. eriophorum* (3.06 mg/g).

Table 1. Flavonoids' and phenolic acids content (*C*) of extracts obtained from the investigated plants.

		Apigenin	Apigenin 7-glucoside	Kaempferol 3- rhamnoglucoside	Chlorogenic acid
C. ligulare Boiss. inflorescences	*C*	5.29	27.75	3.50	50.28
	± SD	6.96	18.24	3.71	11.70
	RSD	0.13	0.065	0.10	0.02
C. ligulare Boiss. leaves	*C*	0.39	1.91	ND	26.34
	± SD	1.72	6.23		20.41
	RSD	0.43	0.33		0.08
C. decussatum Janka inflorescences	*C*	5.24	23.55	3.63	44.87
	± SD	2.53	3.26	25.32	11.14
	RSD	0.05	0.01	0.70	0.02
C. decussatum Janka leaves	*C*	0.82	3.97	ND	47.01
	± SD	1.26	25.12		4.43
	RSD	0.15	0.63		0.01
C. eriophorum (L.) Scop. inflorescences	*C*	4.24	3.06	4.73	24.92
	± SD	3.72	8.62	5.55	29.59
	RSD	0.09	0.28	0.12	0.12
C. eriophorum (L.) Scop. leaves	*C*	0.34	1.23	ND	9.73
	± SD	5.99	8.12		27.38
	RSD	1.78	0.66		0.28

Each value is the mean (mg per 1 g dry sample) from three replicate analyses. SD = standard deviation, RSD = relative standard deviation, ND = not determined.

The amounts of kaempherol 3-rhamnoglucoside were similar in the investigated inflorescences but was not identified in leaves. Flowers of *C. ligulare* and *C. decussatum* contain the similar amounts of apigenin. In leaves of *C. decussatum* concentration of this flavonoids was higher than in another investigated species. (0.82 mg/g) (Table1). These identified compounds: apigenin-7-glucoside, kaempferol 3-glucoside, kaempferol 3-rhamnoglucoside, apigenin, may by responsible for the activity and applications of plants of the *Cirsium* genus. Flavonoids are of particular interest because of their various pharmacological activities (including antianginal, antihepatotoxic, antimicrobial, antiulcer, spasmolytic, antiallergic, antiinflammatory, antiviral, anticarcinogenic and antioxidant) (1, 8, 15–17).

This is the first report of simultaneous quantification of three flavonoids and one phenolic acid in inflorescences and aerial parts (leaves) in different species of *Cirsium*. In the investigated aerial parts of flowering leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. have been identified: kaempferol 3-rhamnoglucoside, kaempferol 3-glucoside, apigenin, apigenin 7-glucoside and chlorogenic acid. (Figs. 1–3) The methanol extract is very rich in flavonoids but some of the flavonoids were not identified. Therefore, more research is required. The analysis of methanol extracts as well as their quantitative analysis have been carried out for the first time.

Calibration plots for the phenolic acids were highly linear ($R^2 > 0.991$) in the concentration range 0.05–1.00 mg per 10 mL (n = 3).

Standard deviation was calculated for all of the results leading to a conclusion that the results are statistically significant. The result of our investigation enabled us to establish a simple RP-HPLC method. ASE and SPE proved to be an inexpensive but very efficient methods for rapid isolation, separation and identification of the flavonoids and phenolic acids present in the extracts examined.

REFERENCES

1. Nazaruk J., Brzóska T.: Postępy Fitoterapii 3, 170 (2008).
2. Nazaruk J., Gudej J.: Acta Pol. Pharm. Drug Res. 60, 87 (2003).
3. Krzaczek T., Sokołowska-Woźniak A., Smolarz H.: Annales UMCS, Lublin, Sectio DDD 3, 51 (1990).
4. Harborne J.B.: The Biology and Chemistry of the Compositae. pp. 359–384, Academic Press, London 1998.
5. Iwashina T., Kadota Y., Ueno T., Ootani S.: J. Jpn. Botany 70, 280(1995).
6. Jordon-Thaden I.E., Louda S.M.: Biochem. Syst. Ecol. 31, 1353 (2003).
7. Mabry T.J., Markham K.R., Thomas M.B.: The ultraviolet spectra of flavones and flavonols. in: The systematic identification of flavonoids. pp. 41–164, Springer Verlag, New York 1970.
8. Nazaruk J.: Fitoterapia 79, 194 (2008).
9. Kun W., Lin-Hong Y., Wei X.: World J. Gastroenterol. 11, 4461 (2005).
10. Zhang Q.L., Li J., Wang C., Sun W., Zhang Z., Cheng W.: J. Pharm. Biomed. Anal. 43, 753 (2007).
11. Waksmundzka-Hajnos M., Oniszczuk A., Szewczyk K.: Acta Chromatogr. 19, 227 (2007).
12. Matysik G., Wójciak-Kosior M.: Chromatographia 61, 89 (2005).
13. Zgórka G., Hajnos A.: Chromatographia 57, 77 (2003).
14. Chao-Yang M., Jinan T., Hong-Xin W., Xiao-Hong G., Guan-Jun T.: Chromatographia 67, 383 (2008).
15. Zgórka G., Głowniak K.: Phytochem. Anal. 10, 268 (1999)
16. Xia D., Shi J., Gong J., Wu X., Yang Q., Zhang Y.: J. Med. Plants Res. 4, 1156 (2010).
17. Nakatani N., Kayano S., Kikuzaki H., Sumino K., Katagiri K., Mitani T.: J. Agric. Food Chem. 48, 5512 (2000).
18. Niggeweg R., Michael A.J., Cathie M.: Nat. Biotechnol. 22, 746 (2004).
19. Nazaruk J., Szoka Ł.: Herba Polonica 4, 32 (2009).

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