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Design and Characterization of Carbopol-HPMC-Ethyl Cellulose based Buccal Compact containing Propranolol HCl

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Abstract

The purpose of this work was to evaluate the effect of formulation variables on release properties and bioadhesive strength in development of three layered buccal compact containing highly water-soluble drug propranolol hydrochloride (PRPH). Formulations were prepared based on rotatable central composite design with peripheral layer polymer ratio (carbopol 934P: HPMC 4KM) and core layer polymer ratio (HPMC 4KM: sodium alginate) as two independent formulation variables. The three layered buccal compact comprises a peripheral layer, core layer and backing layer. In order to provide the unidirectional drug release towards the mucosa and avoid backward diffusion, ethyl cellulose (EC) and magnesium stearate were included as backing layer. The dependent (response) variables were considered: bioadhesion force, and percentage PRPH release at 8 h. The decrease in PRPH release was observed with an increase in both the formulation variables and as the carbopol: HPMC ratio increases the bioadhesive strength also increases. The desirability function was used to optimize the response variables, each having a different target and the observed responses were highly agreed with experimental values. The results demonstrate the feasibility of the model in the development of three layered buccal compact containing highly water-soluble drug propranolol hydrochloride.

Key words: *Bioadhesive strength, Buccal compact, Backing layer, Peripheral layer.*

INTRODUCTION

Delivery of therapeutic agents through various transmucosal routes has received significant attention owing to the agents' presystemic metabolism or instability in the acidic environment associated with oral administration. The oral cavity is being increasingly used for the administration of drugs, which are mainly designed for the medicaments through the oral mucosa into the systemic circulation. Buccal mucosa consist of stratified squamous epithelium supported by a connective tissue lamina propia was investigated as a site for drug delivery several decades ago and the interest in this area for the transmucosal drug administration is still growing [1]. Buccal mucosa makes a more appropriate choice of site if prolonged drug delivery is desired because buccal site is less permeable than the sublingual site. Buccal compacts or buccal bioadhesive drug devices designed to remain in contact with buccal mucosa and release the drug over a long period of time in a controlled fashion. Such a delivery of drug through buccal mucosa overcomes premature drug degradation with in the GI tract, as well as active drug loss due to first pass metabolism, and another is inconvenience of parenteral administration. In addition, there is excellent acceptability and the drug can be applied, localized and may be removed easily at any time during the treatment period.

The strategy for designing buccoadhesive is based principally on the utilization of polymers with suitable physicochemical properties. Combined usage of HPMC and carbopol in delivering the clotrimazole for oral canididiasis has been reported [2]. Similar polymer combination was studied by Perez Marcos et al and concluded that the amount of water penetrated in HPMC K4M was higher[3]. The control release mucoadhesive tablet of eugenol for gingival application has been prepared by using carbopol 934P and HPMC as polymers [4]. Different ratio of carbopol 934P and optimize the controlled release mucoadhesive hydrophilic compressed matrices of diltiazem for buccal delivery [5].

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However, there has been no study to date designed to evaluate the release rate and mucoadhesive property of three layered buccal compacts by using combination of polymers (carbopol 934P, sodium alginate and HPMC K4M).

The typical three layered buccal compacts was prepared containing peripheral layer, core layer and backing layer as shown in Fig. 1. The peripheral layer contains lactose, different ratio of carbopol and HPMC K4M which acts has as a rate controlling layer. The core layer consists of drug PRPH, HPMC K4M and sodium alginate at different ratio. In order to provide the unidirectional drug release towards the mucosa and avoid backward diffusion, ethyl cellulose (EC) and magnesium stearate were included in backing layer.

Fig.1

Propranolol hydrochloride (PRPH), a nonselective β-adrenergic blocking agent, is widely used in the treatment of hypertension, angina pectoris, and many other cardiovascular disorders. Although it is well absorbed in the gastrointestinal tract, its bioavailability is low (15%-23%) as a result of extensive first-pass metabolism [6, 7]. Since the buccal route bypasses the hepatic first-pass effect, the dose of PRPH can be reduced. The physicochemical properties of PRPH, its suitable half-life (3-5 hours), and its low molecular weight 295.81make it a suitable candidate for administration by the buccal route.

MATERIALS AND METHODS

Materials

Propranolol hydrochloride was received as gift sample from Alkem laboratories, Mumbai, India. Hydroxylpropylmethylcellulose (Methosil®) K4M, sodium alginate (alginic acid sodium salt) and ethyl cellulose (EC) were also obtained from Alkem laboratories, Mumbai, India. Other materials were purchased from commercial source; Magnesium stearate, and directly compressible lactose. All other chemicals used in the study were of analytical grade.

Preparation of three layered buccal compacts

The composition of various batches is shown in Table 1. All the ingredients were screened through 120 µm sieve and then thoroughly blended in glass mortar with pestle. Before direct compression blending was carried out separately for peripheral, core and backing layer. The blended powder of backing layer was compressed on 13 mm diameter flat faced punch and die set in an IR hydraulic press at a force of 50 kg cm^{-2}. Above this, blended powder of core layer was added and compressed at a force of 50 kg cm^{-2}. Finally, the blended powder of peripheral layer was added to get three layered buccal compact by compressing at a force of 240 kg cm^{-2}.

Table 1

Evaluation of buccal compacts

Weight and thickness:

Weight of five compacts of every formulation were taken and weighed individually on a digital balance (Fisher Brand PS-200). The average weights were taken and the film thickness was measured using digital micrometer (Mitituo, New Delhi, India) at different places and the mean value was calculated.

Surface pH:

For determination of surface pH, three buccal compacts of each formulation were allowed to swell for 2 h on the surface of agar plate. The surface pH was measured by using a pH paper placed on the surface of swollen patch. A mean of three reading was recorded [8].

Percent swelling:

Buccal compact was weighed, placed in a 2% agar gel plate and incubated at 37 ± 1 °C. At regular interval of one-hour time intervals (for 3 h), the dosage form was removed from the Petri dish and excess surface water was removed carefully using the filter paper. The swollen patch was then reweighed and the swelling index was calculated [9]. The experiments were carried out in triplicate and average values were reported.

Folding endurance:

Folding endurance was determined by repeatedly folding a compact at the same place till it broke[10]. The number of times, the compact could be folded at the same place without breaking gave the value of folding endurance (Table 2).

Content Uniformity:

Drug content uniformity was determined by dissolving the compact by homogenization in 100mL of an isotonic phosphate buffer (pH 6.8) for 8h with occasional shaking. The 5 mL solution was taken and diluted with isotonic phosphate buffer pH 6.8 up to 20 mL, and the resulting solution was filtered through a 0.45 mm Whatman filter paper. The drug content was determined after proper

dilution at 290 nm using a UV spectrophotometer (Shimadzu, SPD-10 A VP, Japan) [11]. The experiments were carried out in triplicate.

Ex Vivo Residence Time:

The ex vivo mucoadhesion time was studied after application of buccal compact on freshly cut sheep buccal mucosa.[12] The fresh sheep buccal mucosa was fixed in the inner side of a beaker, about 2.5 cm from the bottom, with cyanoacrylate glue. One side of each compact was wetted with 1 drop of phosphate buffer (pH 6.8) and pasted to the sheep buccal mucosa by applying a light force with a fingertip for 30 seconds. The beaker was filled with 200 mL of phosphate buffer (pH 6.8) and was kept at $37^{o}C \pm 1^{o}C$. After 2 minutes, a 50-rpm stirring rate was applied to simulate the buccal cavity environment, and patch adhesion was monitored for 12 hours. The time required for the compact to detach from the sheep buccal mucosa was recorded as the mucoadhesion time.

In Vitro Drug Release:

The US Pharmacopoeia XXIII rotating paddle method was used to study drug release from the buccal compacts; 200 mL of phosphate buffer (pH 6.8) was used as the dissolution medium, at $37.0 \pm 0.5^{o}C$, and a rotation speed of 50 rpm was used. One side of the buccal compact was attached to the glass disk with instant adhesive (cyanoacrylate adhesive). The disk was put in the bottom of the dissolution vessel. 5 ml Samples from each formulation were withdrawn at 1 hour interval and replaced with fresh medium. The samples were filtered through 0.45- m Whatman filter paper and analyzed using a UV spectrophotometer at 290 nm.

RESULTS AND DISCUSSION

Formulation of three layered buccal compact

The amount of PRPH in the formulation was established according to its clinical use [13]. Three layered buccal compact was prepared at various concentration of polymers as presented in Table 1. The backing layer contains EC and Magnesium stearate. EC was selected because of its hydrophobic nature and low water permeability, moderate flexibility, thus preventing drug loss by backward diffusion. Magnesium stearate was included as anti-adherent [14, 15]. PRPH, sodium alginate and HPMC K4M comprises the core layer. HPMC K4M is a water swellable polymer which controls the release of drug from the core layer by forming a matrix or gel layer [16]. To increase the release of drug, sodium alginate was included as a water soluble polymer results in formation of porous channel [17]. In order to study the effect of concentration of HPMC K4M, ratio of HPMC K4M: sodium alginate was increased by keeping the total polymer content at 1:1 ratio with respect to drug. Peripheral layer which adheres to the mucosa should possess good bioadhesive strength and also control the release. Hence, carbopol 934P, a potential mucoadhesive polymer along with HPMC K4M was included in peripheral layer [18]. Directly compressible lactose was included as diluent for it's high aqueous solubility and increase in the rate and amount of water imbibitions to peripheral layer thereby increasing the rate of swelling of polymers in peripheral layer which in turn forms a gelled matrix to control the release [19]. As reported previously in formulating a water-soluble drug MT for sustained release, hydration of polymer is necessary in a short time, hence HPMC K4M and lactose were included [20].

Thickness, weight variation and assay

The average thickness of all prepared buccal compacts ranged from 0.89 ± 0.02 to 1.16 ± 0.03 mm. The average weight of 05 buccal compacts of each formula was range from 78- 84 mg, which provided good weight uniformity. In all the formulations, the assay for drug content was found to be uniform among different batches of the buccal compacts and ranged from 64.83 to 91.63% of the theoretical value (Table 2).

Surface pH and Swelling studies:

Compacts surface pH ranges from 5.81 – 6.42 were found around neutral pH and hence no mucosal irritation was expected. The swelling of the compacts were observed in agar plate and shown in table 2. Swelling was more pronounced in (patches) all formulation except (comparatively less) in 6 which contain HPMC and carbopol in a ratio of (1:2) which may due to approximate quantity of ethyl cellulose as that of HPMC.

Table 2

In vitro release:

The release data of PRPH from all the patches are given in Fig. 2. A perusal to fig. 2 indicated that the drug release was higher in compact with higher concentration of HPMC (compact 1, 2, and 3). At pH 6.6, carbopol is present in the ionized state and as a result the polymeric network gets loosened comparatively, attributing for the

higher drug release [21]. An increase in the polymer content was associated with a corresponding decrease in the drug-release rate [22].

Fig. 2

The compact No. 6 was considered to be the optimal compact on the basis of its moderate swelling, convenient ex vivo residence time, *ex-vivo* mucoadhesive strength, and adequate *in-vitro* drug release. Thus compact from this batch was thus optimized for investigation of in vitro drug permeation through sheep buccal mucosa and a stability study in natural human saliva.

Measurement of bioadhesion:

Bioadhesion studies were carried out ex-vivo using freshly obtained mucosa without any further treatment. The peak force of detachment was determined by measuring the tensile strength required for complete breakdown of bioadhesive bond between the dosage form and the surface of mucosa. The apparatus and procedure adapted was previously described [23]. The backing layer was glued to the Teflon® cylinder while the peripheral layer was exposed to the mucosal surface (Fig. 3). Each measurement was carried out in triplicate and the results averaged.

Residence time

The **ex-vivo** residence time with sheep buccal mucosa in phosphate buffer (pH 6.8) varied from 2.85 to 4.30 hours (Table 2). The three layer patch containing carbopol in higher concentration indicates good residence time as it is bioadhesive polymer.

CONCLUSION:

The present study indicates that, the peripheral layer, core layer and backing layer of buccal dosage form have their own characteristic which gives novel ideas. Different ratios of carbopol and HPMC have rate controlling effect over the time. At higher polymer concentration in peripheral layer, the PRPH release from the system can be controlled with good bioadhesion. The peripheral polymer ratio is a major factor affecting the release and bioadhesive strength of the three layered buccal patches. Ethyl cellulose and magnesium stearate plays important role to avoid backward diffusion through backing layer. So lastly we conclude that, three layered can meet the ideal requirement for buccal devices, which can be good way to bypass the extensive hepatic first pass metabolism, avoid the loss of drug into the saliva and increase bioavailability.

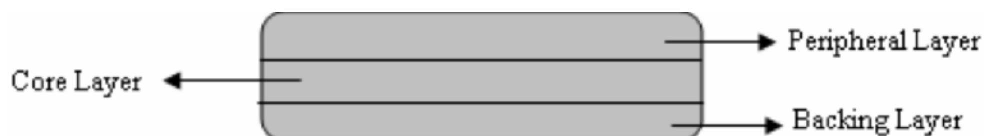

Fig. 1: A typical three layered buccal compact

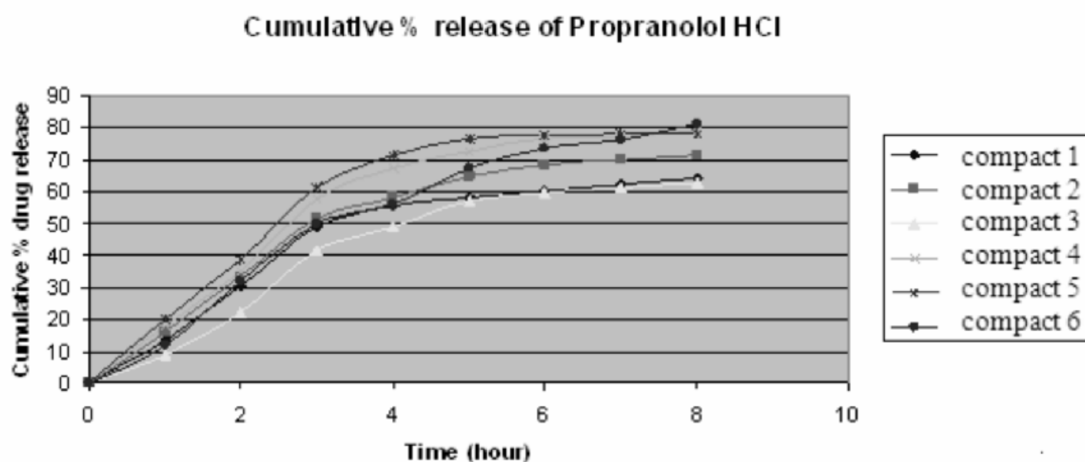

Fig. 2: Release data of Propranolol HCl upto 8 Hour for P 1 to P 6

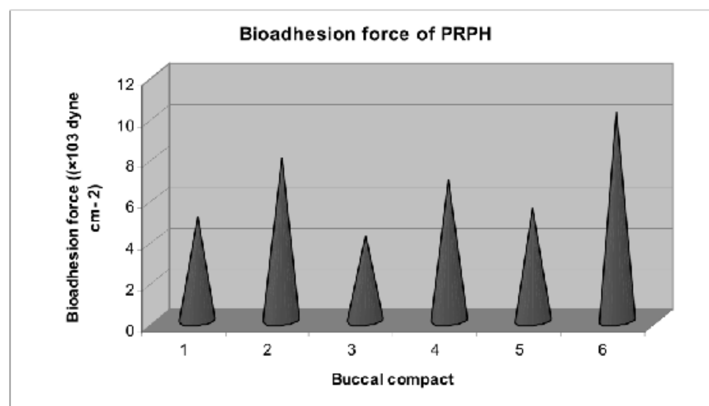

Fig. 3: Bioadhesion study for the Compact 1 to 6

Table 1: Composition of three layered buccal compacts (in mg)

Compact Code	Peripheral layer			Core layer			Backing layer		Total weight
	Carbopol	HPMC	Lactose	PRPH	HPMC	Sodium alginate	Mg stearate	Ethyl cellulose	
1	45	35	20	60	20	20	25	25	250
2	50	30	20	60	25	15	25	25	250
3	55	25	20	60	30	10	25	25	250
4	60	20	20	60	20	20	25	25	250
5	65	15	20	60	25	15	25	25	250
6	70	10	20	60	30	10	25	25	250

Table 2: Primary evaluation parameter for buccal compact

Compact Code	Thickness (mm)	eight (mg)	Swelling index(%) (3h)	Folding endurance	Content uniformity	In vitro residemce time(h)
1	1.13 ± 0.02	84 ± 0.36	48.4 ± 1.21	>200	77.21	2.85
2	0.89 ± 0.02	78 ± 0.41	43.0 ± 1.08	>200	91.63	3.30
3	1.09 ± 0.01	86 ±1.09	45.7 ± 0.87	>200	86.28	4.10
4	0.92 ± 0.04	87 ± 1.11	43.1 ± 0.36	>200	82.81	3.75
5	1.16 ± 0.03	82 ± 0.78	46.8 ± 1.03	>200	64.83	3.15
6	1.12 ± 0.03	79 ± 0.41	38.3 ± .021	>200	71.26	4.30

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Formulation and In-vitro Evaluation of Bilayered Buccal Tablets of Carvedilol

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Abstract

Carvedilol was formulated as a bilayered buccal tablet in order to avoid the first-pass effect and decrease the drug loss using different polymers and excipients. Eight formulations were made using different ratio of carbopol 934P and HPMC K4M.The formulations were tested for in vitro drug release, in vitro bioadhesion, moisture absorption and in vitro drug permeation through porcine buccal mucosa. The dissolution of Carvedilol from all the prepared tablets into phosphate buffer (pH 6.8) was controlled and followed by non-fickian release mechanism. Dissolution studies of the tablets of optimized batch containing 5% Carbopol 934P/65% HPMC K4M/30% lactose showed 82.7 % release of drug in 6 h. The mucoadhesive strength and residence time of the optimized batch are 17.93 g and 9.45 h respectively. The swelling index and microenvironment pH of the optimized batch after 6 h are 77.54 and 6.76 respectively. Procured sample of carvedilol was tested for its identification by taking FTIR of pure drug. Drug excipient compatibility was done at 30°C ,65% ± 5%RH, and 40°C 75% ± 5%RH using open and closed vial for four weeks and observed for physical changes. Result does not show any physical changes to mixture after 4 weeks. The results indicate that suitable bioadhesive bilayered buccal tablets with desired permeability using 5-6% Carbopol 934P,65-68%HPMC K4M and 30% Lactose.

Key words: *Buccoadhesive tablets; Carvedilol; Mucoadhesion; Carbopol 934P; Polymer*

INTRODUCTION

The unique environment of the oral cavity offers its potential as a site for drug delivery. Because of rich blood supply and direct access to systemic circulation, the oral mucosal route is suitable for drugs, which are susceptible to acid hydrolysis in the stomach or which are extensively metabolized in the liver (first pass effect). particularly in overcoming deficiencies associated with the latter mode of administration Mucoadhesive drug delivery system utilize the property of bioadhesion of certain water soluble polymer which become adhesive on hydration and hence can be used for targeting a drug to a particular region of the body for extended period of time[1-4].

The term mucoadhesion is implied when there is an adhesion between a polymer and a mucous tissue[5]. Carvedilol is a non selective β-adrenergic blocking agent with α_1-blocking activity [6]. It is used in the management of hypertension and angina pectoris, and as an adjunct to standard therapy in sympatomatic heart failure[7]. After oral administration, only 25 – 30% of the total dose reaches to systemic circulation because of its high first pass metabolism. Additionally plasma half life is also less i.e. 2-8 hr. The drug is basic and lipophilic in nature with low molecular weight. The drug is low aqueous solubility[8]. Because of its low bioavailability and short half life, attempt has been made to develop sustained release mucoadhesion formulation with extended clinical effect and reduce dosing frequency.

MATERIALS AND METHODS

Carvedilol was obtained as a gift sample from Zydus Cadila, Mumbai. Carbopol 934P, sodium CMC and HPMC K4M were obtained from Pioma chemicals Ltd., Mumbai. Lactose and mannitol were obtained from Famy care Ltd., Gujarat. All other reagents and chemical used were of analytical grade. Procured sample of drug confirmed by FTIR spetra.

Preparation of mucoadhesive tablets:

The tablets were prepared by direct compression method. Medicated tablets (120 mg) containing 5 mg drug

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(Carvedilol) were compressed using flat face punch, 6.5 mm in diameter (Erweka ltd.). Tablet compositions are listed in Table 1. Before direct compression, the powder were screened through a 60µm sieve and then thoroughly blended. To prepare bilayer tablets, the non medicated layer first compressed, then the medicated layer was filled into the die cavity and both are compressed together.

In-vitro buccal permeation studies:

In-vitro diffusion study of pure drug was carried out using fresh porcine oral mucosa tissue, which was procured from local slaughter house and placed in Krebs buffer pH 7.4. Isolation of the epithelium was done mechanically by using scissors and forceps. These studied were carried out using Franz's diffusion cell. It consists of upper cylindrical compartment open from above and containing the porcine buccal mucosa at its base. Lower compartment was in form of a closed cylinder having the sampling port and had Teflon coated magnetic needle at the base. The junction between two compartments was designed in such a manner that the buccal membrane did not shift from its place. The donor compartment was filled with 10 ml of phosphate isotonic buffer pH 6.8 containing 20% of methanol. 5.00 mg drug was dissolved in the buffer and methanol mixture. The receptor compartment contains 20 ml isotonic phosphate buffer having 20% methanol to maintain sink conditions. The whole assembly was maintained at 37 ± 1^0C. Five ml of samples was withdrawn from receptor compartment and replaced with the same amount of fresh medium. The withdrawn samples were then diluted suitably, then assayed spectrophotometrically at 244 nm, and calculated the % cumulative drug diffused[9,10].

In-vitro release study:

The USP paddle (USP Apparatus type II) method was employed for the in-vitro dissolution studies. To study the drug release from only one side of the tablet, the tablets were attached on the surface of the plastic dishes with the help of adhesive so that only one surface exposed to the dissolution medium. In dissolution study, 900 ml of isotonic phosphate buffer solution pH 6.8 were taken. The rate of stirring was 50 rpm. The temperature was maintained 37ºC ± 0.5ºC for a period of 6 hrs. At appropriate time interval (0.5, 1.5, 2, 3, 4, 5 and 6 hr), 10 ml of sample was taken and filtered. In the dissolution media, 10 ml of fresh dissolution fluid was added after each withdrawal in order to maintain constant volume. The samples were assayed at 244 nm by UV Spectrophotometer. The mean of six determinations were used to calculate the drug release from each formulation[11,12,13]

Statistical analysis:

Release data were analyzed using the following equation:

$$M_t / M \;= Kt^n \qquad (1)$$

Where, M_t / M : is the fractional release of drug,

t : Denotes the release time,

K : Constant incorporating structural and geometrical characteristics of the device and

n : Diffusional exponent that characterized the type of release mechanism during the dissolution process.

For non-fickian release, the value of n falls between 0.5 and 1.0; while in case of fickian diffusion, n = 0.5; for Zero order release (case II transport), n = 1; and for super case II transport, n is greater than 1. The values of 'n' were estimated by linear regression of log (M_t / M)ersus log (t) of different formulations are shown in Table 2. This model is used, when the release mechanism is not known or when more then one type of release phenomenon could be involved[10,14,15]

Determination of residence time:

The in-vitro residence time was determined using a locally modified USP disintegration apparatus. The disintegration medium was 800 ml isotonic phosphate buffer solution pH 6.8, maintains at 37°C ± 1°C. A segment of goat buccal mucosa was glued to glass slide, attached to glass slab which is vertically attached to apparatus. The tablet was hydrated from one surface using little amount of isotonic phosphate buffer solution, and then the hydrated surface was brought into the contact with the mucosal membrane. The glass slab was vertically fixed to the apparatus and allows moving up and down so that the tablet was completely immersed into the solution at lowest point and was out at highest point. The time necessary for complete erosion or detachment of the tablet from the mucosa surface were recorded[16].

Measurement of mucoadhesion strength and surface pH:

An apparatus designed earlier in laboratory was used for this study. The assembly of the apparatus and the method was same as described by Soliman Mohammad-Samani et al. [68]. This instrument consists of two jaws from flat glasses. The upper glass was fixed, but the lower glass was mounted on a screw elevating surface. The upper fixed glass was attached to a sensitive digital balance. Tablets from each formulation were suspended in water (pH 7) for 15 min. Then this adhesive tablets were located on the surface of lower glass and were elevated until they contact the surface of the upper glass. The lower glass was then lowered until the tablet clearly pulled free from the upper glass. The maximum tensile force needed to detach the jaws was recorded in g/cm^2. The method use to determine the surface pH is similar to that used by Fergany et al., where the tablet is placed in small beaker with 4 ml of isotonic phosphate buffer solution (pH 6.8) and the pH was measured at time interval of 2, 4, 6 hr by placing the electrode in contact with the microenvironment of the swollen tablets. The average pH of three determinations were recorded[17].

Swelling index:

3 tablets from each batch were weighed individually (W_1) placed separately in Petri dishes containing 4 ml of isotonic phosphate buffer solution (pH 6.8). At regular interval (1, 2, 4, and 6 hr), the tablet were removed from the Petri dishes and excess water surface water was removed carefully using filter paper. The swollen tablet were reweighed (W_2) and swelling were calculated using the formula,

$$S.I = (W_2 - W_1) / W_1 \qquad (2)$$

Where, S.I = swelling index, W_1= initial weight of tablet, W_2= weight at time 't' [10]

RESULTS

Evaluation of mucoadhesive tablets:

The results of in-vitro buccal permeation studies shows in Fig. 1. The in-vitro release curves from all the formulation are shown in Fig. 2. Out of all the formulation, developed optimized batch exhibit 82.7 % release (n = 6, SD = ± 1.36). The kinetic studies showed the non-fickian diffusion. The results are listed in Table 2. The mucoadhesive strength of all the formulations is shown in Table 3 and Fig. 3. The mucoadhesive strength of the optimized batch(D6) was found to be 17.93 g ± 2.59. The surface pH and residence time of the optimized batch(D6) was found to be 6.72 hr and 9.45 hr and values of all the formulation are shown in Table 4.The swelling index of the optimized batch was found to be 77.54. The results are shown in Table 5, Fig. 4, 5 .

Spectroscopic Characterization

FTIR spectra of carvedilol, and optimized buccal tablets (D6) were obtained. Carvedilol alone showed principal peaks of 2923.68, 1449.96, 1340.13, and 1097.31 cm^{-1}.However, some additional peaks were observed with the physical mixture, which could be due to the presence of the polymer. These results suggest that no interaction took place between the drug and the polymer.

DISCUSSION

Swelling index was calculated with respect to time. Swelling index increased with time as the weight gain by the tablet was increased proportionally with the rate of hydration. Maximum swelling was obtained in 4 hr, after which polymer started eroding slowly in the medium. The tablets of batch D8 showed cracking on drug load layer after 2 hr. This batch contain only sodium CMC with drug in drug loaded layer, and this batch may show this property due to presence of Sodium CMC. As Sodium CMC has viscosity less as compared to HPMC K4M, and Carbopol 934P, resulting in low binding forces between the molecules. Maximum swelling was seen with the batches D6, D7 (containing Carbopol 934P in combination with HPMC K4M, lactose and mannitol respectively) and D9 and D10 (Containing HPMC K4M and Sodium CMC in different ratio). The linearity in the swelling index shows the sustained release of the drug. The tablets of all the batches had shown a surface pH in the range of 5-7; that indicates no risk of mucosal damage or irritation because of similar pH to the oral cavity. As the concentration of carbopol increases, the pH of surface decreases. The tablets of all batches except D2, D3 and D5 showed moderate residence time (around 8 hr). Tablets of batch D2, D3, D5 showed residence more than 10 hr; due to possible presence of carbopol 934P in higher concentration. The mucoadhesive property of mucoadhesive buccal tablets of Carvedilol containing varying proportion of polymers was determined with a view to develop a contact with good adhesiveness

without any irritation and other problems. The bioadhesion characteristics were affected by the type and ratio of the polymers. The highest strength of mucoadhesive bonds (22.23 gm) was proposed for batch D3 i.e. the formulation containing Carbopol 934P (10%) and HPMC K4M (90%) and the least force of adhesion was proposed for batch D8 i.e. the formulation containing only sodium CMC. By observing the results from tablets of batches D1 – D7, it was concluded that as the concentration of Carbopol 934P increases the mucoadhesive strength increases in combinations with HPMC K4M. The combination of HPMC K4M and SCMC shows less mucoadhesion strength; this may be due to absence of proton donating carboxyl group in HPMC which reduces the its ability for the formation of hydrogen bond. The in-vitro release study showed satisfactory sustained release of Carvedilol from all medicated formulae. CP and HPMC are hydrophilic swellable polymer matrices. They are able to form a viscous gel layer; which controls the drug release via diffusion through the gel and erosion of the gel layer. The release of Carvedilol was slow in tablets of batches D1 –D3 due to the presence of carbopol 934P and the higher concentration of HPMC K4M. The release of drug from D4 and D5 was greater than D1 – D3; most probably due to the presence of lactose and mannitol which enhances the erosion of the tablets. The bilayer tablets (batch D6 and D7) show greater release rate at all intervals compared to the single layer tablet. Reducing the amount of the polymer to 40% showed faster release. This can be attributed to the reduction of the strength of the gel layer, which enhances drug diffusion and water uptake through the matrix. The tablets of batch D8, D9 and D10 showed maximum cumulative drug release as it contains SCMC alone or in combination with other polymers, mainly due to the higher swelling property or erodible nature of SCMC. But the formulation containing higher percentage of SCMC showed slow drug release in first 1.5 hr; may be due to the higher swelling of the SCMC, which cause the formation of the strong gel layer on the surface of the tablet which resists the penetration of the medium inside the tablets. From the above characterization, D6 shows the promising release profile. Thus, formulation D-6 was selected for further study depending on its swelling study, bioadhesion, and drug release properties. The data obtained from the release kinetic study shows all the 'n' values between 0.5 to 1; indicating non-fickian release kinetics. The in-vitro diffusion study of Carvedilol using porcine mucosa shows that more than 90 % drug permeation through the mucosa in 2 hr. These results indicate that the diffusion or absorption was not the rate-limiting step in the absorption of Carvedilol from the sustained released system. The rate-limiting step was the release of drug from the tablets.

CONCLUSION

From the study it can be concluded that mucoadhesive tablet formulations containing 5-6% carbopol 934P,65-68%HPMC K4M and 30% Lactose give promising results with water insoluble drug carvedilol for susutained release action of carvedilol with adequate swelling, mucoadhesive strength, suitable residence time, and no irritation effect on buccal mucosa. It also reduces polymer loss, and allows sustained release of drug from the tablets.

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The authors are grateful to Department of Research and Development, Famy Care Ltd. and Department of Pharmaceutics, Padmavathi College of Pharmacy, for providing facilities.

Table 1: The ingredients of each formulation (all formulation contain 5 mg Carvedilol)

Batch code	CP934p	HPMCK4M	SCMC	Lactose	Mannitol
D1	2.3	112.4	-	-	-
D2	5.73	108.7	-	-	-
D3	11.47	103.23	-	-	-
D4	5.73	74.55	-	34.41	-
D5	5.73	74.55	-	34.41	
A	3.34	43.43	-	20.04	-
D6 B	2.4	31.1	-	14.4	-
A	3.34	43.43	-	-	20.04
D7 B	2.4	31.1	-	14.4	-
A	-	-	67	-	-
D8 B	2.4	31.1	-	14.4	-
A	-	53.45	13.4	-	-
D9 B	2.4	31.1	-	14.4	-
A	-	13.4	53.45	-	-
D10 B	2.4	31.1	-	14.4	-

*A and B indicates the drug loaded layer and backing layer in bilayer tablets.
**Each formulation contains 0.25 % magnesium stearate as lubricating agent and 0.1 % sunset yellow colour in backing layer, in bilayer tablets.

DRUG : Carvedilol
CP 934P : Carbopol 934
HPMC : Hydroxypropylmethylcellulose
SCMC : Sodiumcarboxymethylcellulose

Table 2: Estimated values of n (Diffusional Exponent) and r^2 (correlation coefficient) of log (M_t/M_∞) Vs Log (T)

Batch code	Kinetic Parameters for Peppas model	
	n (Diffusional Exponent)	r^2 (Correlation Coefficient)
D1	0.7399	0.9726
D2	0.7053	0.9858
D3	0.6698	0.9978
D4	0.5886	0.9940
D5	0.5945	0.9853
D6	0.5258	0.9935
D7	0.5433	0.9821
D8	0.9597	0.9530
D9	0.9546	0.9817
D10	0.9653	0.9950

Table 3: In-Vitro Mucoadhesive Strength Study of the Prepared Carvedilol Tablets

Batch Code	Mucoadhesive strength (gm)* (mean ± S.D)
D1	9.80 ± 0.09
D2	10.81 ± 0.52
D3	22.82 ± 1.26
D4	9.22 ± 0.57
D5	14.99 ± 1.19
D6	17.93 ± 2.59
D7	16.39 ± 1.80
D8	6.15 ± 0.95
D9	10.36 ± 0.43
D10	9.34 ± 0.48

Table 4: Observations of surface pH and residence time of prepared mucoadhesive buccal tablets of Carvedilol

Batch Code	Surface pH*			Mucoadhesion time* hr
	2 hr	4 hr	6 hr	
D1	5.98 ± 0.11	6.14 ± 0.09	6.07 ± 0.06	7.67 ± 0.42
D2	5.71 ± 0.09	5.73 ± 0.03	5.64 ± 0.15	> 10
D3	5.46 ± 0.04	5.43 ± 0.06	5.29 ± 0.04	> 10
D4	5.94 ± 0.03	5.86 ± 0.04	5.84 ± 0.02	9.32 ± 0.19
D5	5.64 ± 0.03	5.54 ± 0.03	5.55 ± 0.04	> 10
D6	6.82 ± 0.09	6.81 ± 0.04	6.76 ± 0.05	9.45 ± 0.10
D7	6.89 ± 0.13	6.88 ± 0.04	6.85 ± 0.03	8.88 ± 0.39
D8	6.47 ± 0.04	6.45 ± 0.04	6.40 ± 0.02	7.93 ± 0.54
D9	6.73 ± 0.02	6.72 ± 0.05	6.71 ± 0.05	8.08 ± 0.83
D10	6.55 ± 0.03	6.52 ± 0.04	6.48 ± 0.04	7.88 ± 1.50

* n = 3

Table 5 In-Vitro Swelling Study Of Prepared Mucoadhesive Buccal Tablets of Carvedilol

Batch code	Swelling index (mean ± S.D)* Time in hr			
	1	2	4	6
D1	17.58 ± 0.56	30.46 ± 1.06	36.32 ± 0.53	42.82 ± 1.68
D2	18.64 ± 0.87	27.73 ± 0.78	36.86 ± 1.03	43.77 ± 0.83
D3	23.21 ± 1.56	30.41 ± 1.16	36.09 ± 1.39	48.11 ± 0.90
D4	25.72 ± 0.11	30.45 ± 1.25	37.73 ± 1.11	49.31 ± 1.41
D5	25.79 ± 0.77	37.04 ± 1.61	42.43 ± 1.75	55.59 ± 0.65
D6	28.33 ± 0.34	40.34 ± 0.81	61.80 ± 0.96	77.54 ± 0.90
D7	30.80 ± 0.74	39.60 ± 0.99	65.31 ± 0.97	79.45 ± 1.01
D8	46.53 ± 0.52	60.27 ± 1.99	Tablet	breaks
D9	40.95 ± 0.30	49.05 ± 0.62	80.81 ± 1.34	92.53 ± 0.65
D10	43.47 ± 0.87	53.39 ± 0.35	85.63 ± 0.62	91.26 ± 1.05

* n = 3

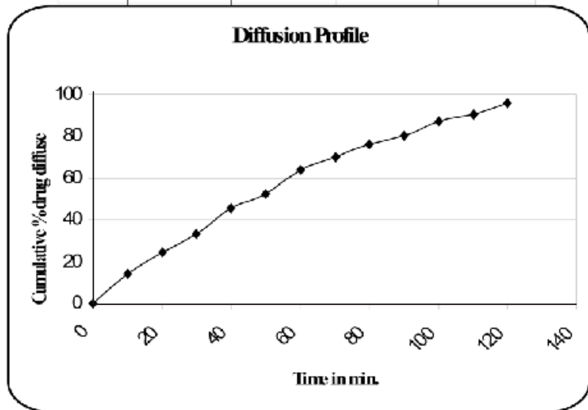


Fig. 1 : Drug Diffusion profile of Carvedilol through Porcine Buccal Mucosa.

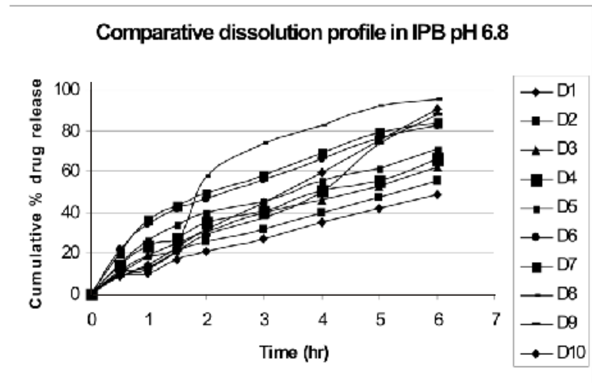


Fig. 2 : Comparative drug release profile prepared Buccoadhesive Carvedilol tablets of all batches (D1 to D10)

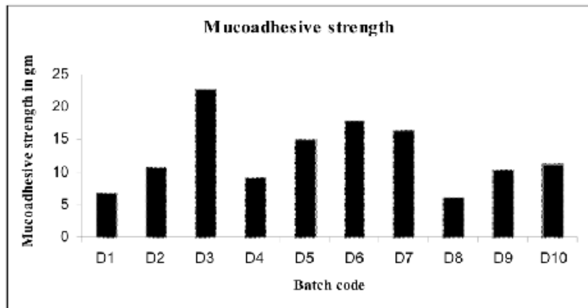


Fig. 3: Graphical representation of mucoadhesive strength of buccoadhesive Carvedilol tablets

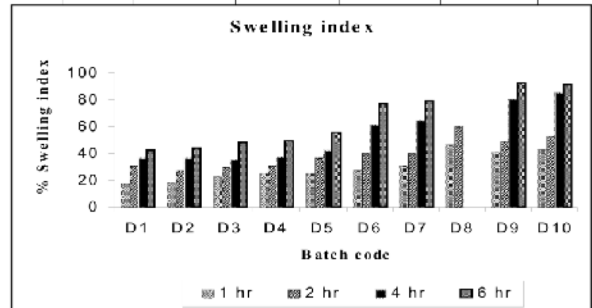


Fig. 4: Graphical representation of swelling index of prepared buccoadhesive Carvedilol tablets

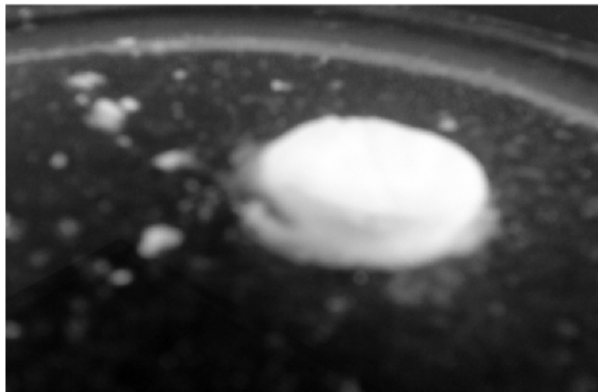
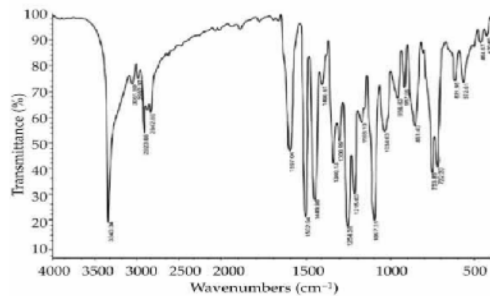


Fig 5:Photograph of optimized tablet (D6) before swelling

Fig 6:Photograph of optimized tablet(D6) after swelling.



FTIR SPECTRA OF CARVEDILOL (PURE DRUG)

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Effect of Polymer Concentration and Viscosity Grade on Atenolol Release from Gastric Floating Drug Delivery Systems

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Abstract

Gastroretentive floating drug delivery systems of atenolol, an antihypertensive drug with an oral bioavailability of only 50% (because of its poor absorption from lower gastrointestinal tract) have been designed. Hydroxypropyl methylcelluloses of different viscosity grades (K4M and 50 cps) were used as polymers and sodium bicarbonate as gas generating agent to reduce floating lag time. Tablets were prepared by direct compression method. The prepared formulations were further evaluated for hardness, friability, weight variation, drug content, swelling index, in vitro drug release pattern, short-term stability and drug-excipient interactions. Majority of the designed formulations displayed nearly first order release kinetics releasing more than 75% drug in 10 hours and remained buoyant for more than 24 hours. Drug release data shows that as the proportion and viscosity of polymer increases, drug release decreases. The formulation containing atenolol 50 mg, hydroxypropyl methylcellulose (50 cps) 100 mg and 37 mg sodium bicarbonate (20% w/w of tablet) as gas generating agent, appears to be a promising gastroretentive floating drug delivery system of the drug atenolol, releasing more than 90% of the drug in 10 hours.

Keywords: *Atenolol, gastroretentive floating drug delivery system, hydroxypropyl methylcellulose, hydrodynamically balanced system*

INTRODUCTION

Gastroretentive drug delivery systems can remain in the gastric region for several hours and hence significantly prolong the gastric residence time of drugs. Prolonged gastric retention improves bioavailability, reduces drug waste, and improves solubility for drugs that are less soluble in a high pH environment. It has applications also for local drug delivery to the stomach and proximal small intestines. Gastroretention helps to provide better availability of new molecular entities with new therapeutic possibilities and substantial benefits for patients.

The gastric emptying time has been reported to be from 2 to 6 hours in humans in the fed state[1]. Drugs that are required to be formulated into gastroretentive dosage forms include: (a) drugs acting locally and primarily absorbed in the stomach; (b) drugs that are poorly soluble at an alkaline pH; (c) those with narrow window of absorption; (d) drugs absorbed rapidly from GI tract and (e) drugs that degrade in colon. Various approaches have been worked out to improve the retention of oral dosage forms in the stomach. Depending on the mechanism of buoyancy, two distinctly different methods, viz., effervescent and non-effervescent systems have been used in the development of floating drug delivery systems[2]. Effervescent drug delivery systems utilize matrices prepared with swellable polymers such as methocel[3] or polysaccharides and effervescent components e.g., sodium bicarbonate and citric acid or tartaric acid[4].

Atenolol, a beta-blocker used in the treatment of hypertension and angina pectoris. It is incompletely absorbed from the gastrointestinal tract[5] and has an oral bioavailability of only 50%, while the remaining is excreted unchanged in faeces. This is because of its poor absorption in lower gastrointestinal tract[9]. It undergoes little or no hepatic first pass metabolism and its elimination half-life is 6 to 7 hours[6]. Therefore, it is selected as a suitable drug for the design of a gastroretentive floating drug delivery system (GFDDS)

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with a view to improve its oral bioavailability.

The objective of this work is to develop GFDDS of atenolol, employing swellable polymer hydroxypropyl methylcellulose (HPMC) of different viscosity grades (K4M and 50 cps) and sodium bicarbonate as gas generating agent, and to evaluate the effect of polymer concentration and viscosity on atenolol release from the prepared GFDDS.

MATERIALS AND METHODS

Atenolol IP and HPMC K4M were gift samples from M/s.Vapi Care Pharma Ltd., Vapi and M/s.Colorcon Asia Ltd., Goa respectively. HPMC 50 cps, sodium bicarbonate, talc and magnesium stearate were purchased from SD Fine Chem, Boisar, Maharashtra. All other chemicals used were of analytical reagent grade.

1. Preparation of Atenolol GFDDS:

In this work, direct compression method has been employed to prepare gastric floating drug delivery systems (GFDDS) of atenolol. HPMC of two different viscosity grades viz., K4M and 50 cps, with different concentration and fixed concentration of sodium bicarbonate (20% w/w) have been used for preparation of GDDS. Tablets were compressed on a single punch tablet machine (Cadmach, Ahmedabad, India) using 8 mm flat round punches.

The formulation codes for the prepared batches of GFDDS are given in Table 1. A batch of 50 tablets was prepared for each of the designed formulations.

2. *In Vitro* Characterization of GFDDS:

a) Drug Release Study: *In vitro* dissolution studies of GFDDS of atenolol were carried out in USP XXIII tablet dissolution test apparatus-II (Electrolab, Model:TDT-06N), employing paddle stirrer at 50 rpm and 900 ml of 0.1N HCl at 37±0.5ºC as dissolution medium. At predetermined time intervals, 5ml of the samples were withdrawn by means of a syringe fitted with a prefilter. The volume withdrawn at each interval was replaced immediately with same quantity of fresh dissolution medium maintained at 37±0.5ºC. The samples were analyzed for drug release by measuring the absorbance at 224.6nm using UV-visible spectrophotometer (Shimadzu UV-1700) after suitable dilution. All the studies were conducted in triplicate.

b) *In Vitro* Floating Studies: Floating time was determined by the same apparatus of dissolution study. The duration of floating is the time the tablet floats in the dissolution medium (including floating lag time, which is the time required for the tablet to rise to the surface), is measured by visual observation.

c) Swelling Index: The individual tablets were weighed accurately and kept in 50 ml of water. Tablets were taken out carefully after 60 minutes, blotted with filter paper to remove the water present on the surface and weighed accurately. Percentage swelling (swelling index) was calculated using the formula[7]:

$$\text{Swelling index} = \frac{\text{Wet weight - Dry weight}}{\text{Wet weight}} \times 100$$

a) Stability Studies: According to ICH guidelines for Accelerated stability testing of new drug substance, stability studies were performed at a temperature of 40±2ºC and 75%±5% RH, in Humidity chamber (Tempo, Mumbai), over a period of 6 months on the promising formulation (T_5) . The samples were analyzed at monthly intervals for any physical changes and drug content (by measuring the absorbance at 225.3 nm on methanolic extracts of the drug). At the end of storage period, dissolution test and *in vitro* floating studies were performed.

3b) Drug-Polymer Interaction Studies: IR spectroscopy is one of the most powerful analytical techniques, which offers the possibility of detecting chemical interaction. The IR spectra of atenolol, HPMC (50 cps) and promising formulation (T5) were obtained by KBr pellet method (Perkin-Elmer FTIR 1516 series spectrometer).

RESULTS AND DISCUSSION

In the present study, an attempt was made to design GFDDS of atenolol using hydroxylpropyl methylcellulose of different viscosity grades (K4M and 50 cps) as the polymers and sodium bicarbonate as a gas generating agent, to reduce floating lag time. The tablets were prepared by direct compression method. Six batches of formulations were designed and evaluated for various physical and floating characteristics, drug content uniformity and drug release profiles (Tables 2 and 3). Short term stability and drug- polymer interaction studies were also performed on the promising formulation.

The hardness of prepared GFDDS of atenolol was found to be in the range of 3.92 to 4.65 Kg/cm². The friability of all tablets was less than 1% and the percentage deviation from the mean weight of all the batches of prepared HBS were found to be within the prescribed limits as per IP. The low values of standard deviation indicate uniform drug content in all the batches prepared.

Swelling Index studies

Tablets composed of polymeric matrices build a gel layer around the tablet core when they come in contact with water. This gel layer governs the drug release. Kinetics of swelling is important because the gel barrier is formed with water penetration. Swelling is also a vital factor to ensure floating and drug dissolution. To obtain floating, the balance between swelling and water acceptance must be restored.[7] The swelling index of the tablets increases with an increase in the polymer content and the content of gas generating agent ($NaHCO_3$) and was found to be in the range of 5.26 to 65.33.

***In Vitro* Floating Studies**

For all formulations, floating lag time was found to be in the range of 0.4 to 3.5 min. With increase in concentration of polymer of same viscosity grade, lag time increased and for same concentration of the polymers of different grades, lag time increases with increase in viscosity of polymer. All the designed formulations have displayed a floating time of more than 24 hours.

Floating mechanism

Effervescent Systems utilize effervescent reactions between carbonate/bicarbonate salts and gastric fluid to liberate CO_2, which gets entrapped in the gellified hydrocolloid layer of the systems thus decreasing its specific gravity and making it to float over gastric fluid. How the dosage form float is shown in the following figure (Fig.1)

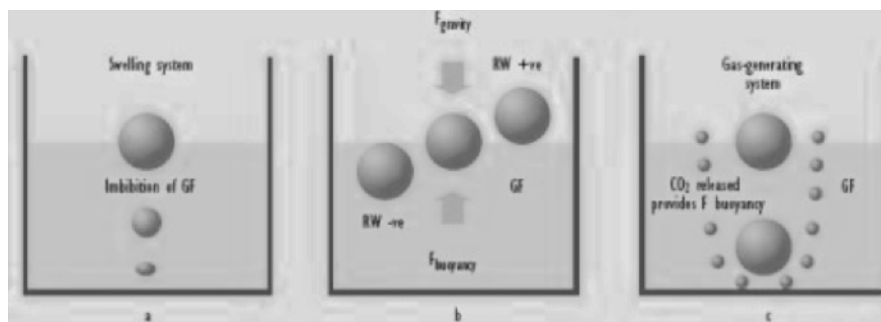

Fig.1:The Mechanism of Floating Systems

Drug Release Study:

The formulations T_1 to T_3 (prepared from HPMC K4M) have released only 67% to 76% drug in 10 h, whereas, the formulations T_4 to T_6 (prepared from HPMC 50 cps), have released 81 to 95% during the same period (Fig. 2). This increased drug release from the latter formulations can be attributed to the lower viscosity grade of HPMC. *In vitro* drug release data of all the formulations was subjected to goodness of fit test by linear regression analysis according to zero-order and first-order kinetic equations, Higuchi's and Korsmeyer–Peppas models (PCP DISSO 2000 V3 software) to ascertain the mechanism of drug release. The results of linear regression analysis including regression coefficients are summarized in Table 4 and cumulative percent drug released vs time plots shown in Fig. 2 and 3.

From the above data, it is evident that except formulation T_3, all the formulations have displayed first order release kinetics ('r' values in the range of 0.9714 to 0.9933). Higuchi and Peppas data reveals that the drug is released by non-Fickian diffusion mechanism (n=0.48 to 0.79) except formulation T_3 (n=1.05), which displays zero-order release by erosion-dominated mechanism.

Formulation T_5 has displayed $t_{50\%}$, $t_{70\%}$ and $t_{90\%}$ values of 4.8, 6.4 and 8.2 h respectively (Table 3) and released nearly 91% drug in 10 h. Hence, this formulation was found to be promising (compared to T_4 which releases 95% drug in 10 h, in order to preclude any chances of dose-dumping from this formulation as it contains only 50 mg of the matrix polymer, i.e., HPMC 50 cps) and therefore, selected for accelerated stability study according to ICH guidlines.

Stability Studies

The results of accelerated stability study on the promising formulation T_5 indicated that there were no significant changes in physical appearance, drug content and dissolution profile ($p<0.05$).

Drug-Polymer Interaction Studies

IR spectrum of pure drug (atenolol) exhibits characteristic peaks at 3357.02 cm^{-1} and 1637.4 cm^{-1} due to N–H stretching and C=O stretching of amide group respectively. The peaks at 1417.27 and 1243.06 cm^{-1} are due to alcoholic –OH group. IR spectrum of formulation T_5 shows peaks at 3359.06 cm^{-1} and 1637 cm^{-1} due to N–H stretching and C=O stretching of amide group respectively. The peaks at 1416.53 cm^{-1} and 1243.04 cm^{-1} are due to alcoholic –OH group. The presence of above peaks confirm undisturbed drug in the formulation; hence there are no drug-carrier interactions. IR spectra of atenolol, HPMC (50 cps) and T_5 shown in figure-4.

CONCLUSION

The results of the study indicated that as the proportion and viscosity of the matrix polymer increases, cumulative percent drug release in 10 h decreases. Hence, these two factors have a great influence on the overall performance of the designed GFDDS formulations. The formulation (T_5) containing atenolol 50 mg, HPMC (50 cps) 100 mg and 37 mg sodium bicarbonate (20% w/w of tablet) as gas generating agent, appears to be a promising GFDDS of atenolol, releasing nearly 91% of the drug in 10 h.

ACKNOWLEDGEMENTS

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Table 1: Composition of atenolol GFDDS formulations

Ingredients(mg/tablet)	T_1	T_2	T_3	T_4	T_5	T_6
Atenolol	50	50	50	50	50	50
HPMC K4M	50	100	150	--	--	--
HPMC 50 cps	--	--	--	50	100	150
NaHCO	3 25	37	50	25	37	50
Magnesium stearate	2.5	3.7	5.0	2.5	3.7	5.0
Purified Talc	1.25	1.85	2.50	1.25	1.85	2.50

Table 2: Evaluation of atenolol GFDDS formulations

Formulation code	Mean Hardness	Friability (%) kg/ cm²	Average weight mg	Mean% content*	Swelling Index* ±SD	Floating lag (min)	Floating time (hr)
T_1	4.18	0.51	126.45	96.30±1.17	5.26±0.152	3.5	24
T_2	4.59	0.54	185.75	94.92±3.10	14.33±0.25	2.71	24
T_3	4.77	0.57	250.25	97.71±1.69	40.50±1.47	0.70	24
T_4	3.92	0.64	125.95	96.60±1.02	8.60±0.257	3.1	24
T_5	4.46	0.61	185.60	94.49±0.54	22.36±1.15	2.20	24
T_6	4.65	0.56	249.00	93.65±1.72	65.33±0.32	0.41	24

* Average of three determinations

Table 3: Dissolution parameters of atenolol GFDDS formulations

Sl. No.	Formulation Code	$t_{50\%}$(h)	$t_{70\%}$(h)	$t_{90\%}$(h)	Cumulative percent drug release in 10 h
1.	T1	4.9	7.6	>10	76.44
2.	T2	5.2	8.9	>10	72.67
3.	T3	5.5	>10	>10	67.19
4.	T4	4.5	6.2	8.0	95.21
5.	T5	4.8	6.4	8.2	90.65
6.	T6	5.9	8.2	>10	81.34

Table 4: Kinetic data of atenolol GFDDS formulations

Batch		Zero order	First order	Higuchi 's equation	Peppas equation
	r	0.9798	-0.9919	0.9823	0.9945
T 1	a	8.6400	2.0060	-7.9368	1.1924
	b	7.7062	-0.0669	27.0450	0.7220
	r	0.9185	-0.9714	0.9751	0.9292
T 2	a	17.2527	1.9016	1.2920	1.3343
	b	6.3074	-0.4735	23.2690	0.5559
	r	0.9742	-0.9858	0.9657	0.9836
T 3	a	3.2768	2.0200	-11.6286	0.8621
	b	7.3175	-0.5430	25.2148	1.0547
	r	0.8891	-0.7715	0.9689	0.9125
T 4	a	25.9400	1.8920	5.2363	1.5022
	b	7.5259	-0.0840	28.4053	0.4813
	r	0.9336	-0.9871	0.9710	0.9605
T 5	a	14.9350	1.9933	-6.2388	1.2654
	b	8.8811	-0.1029	32.1101	0.7584
	r	0.9861	-0.9762	0.9786	0.9851
T 6	a	4.7137	2.0313	-6.7894	1.1144
	b	7.8644	-0.6660	25.7071	0.7890

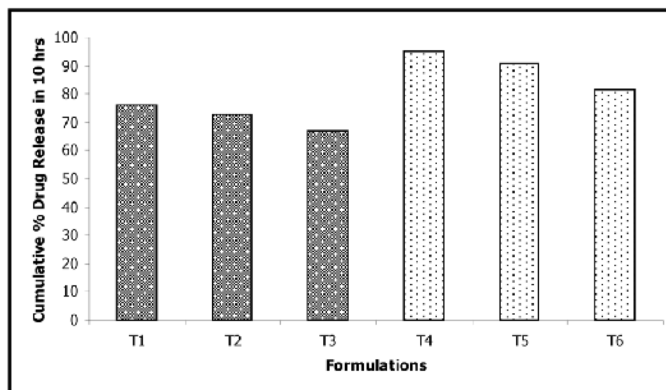


Fig. 2: Effect of polymer concentration and viscosity grade on atenolol release from GFDDS (T_1 to T_3 – HPMC K4M; T_4 to T_6 – HPMC 50 cps)

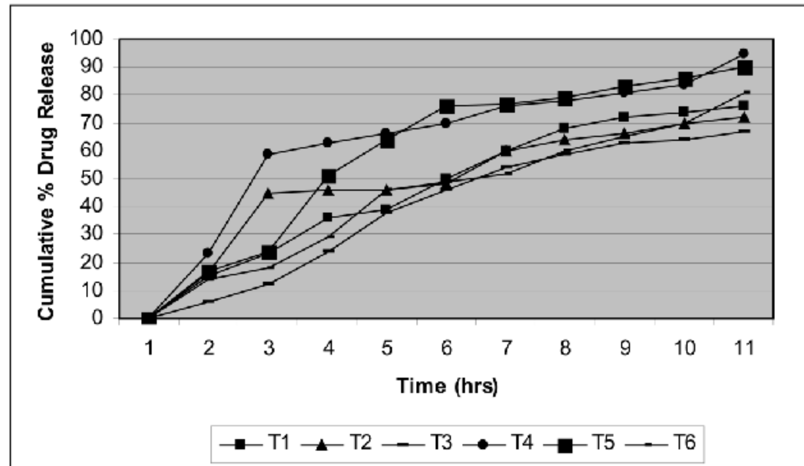


Fig. 3: Cumulative Percent Drug Released Vs Time Plots (Zero Order) of formulations T1, T2, T3,T4,T5 and T6

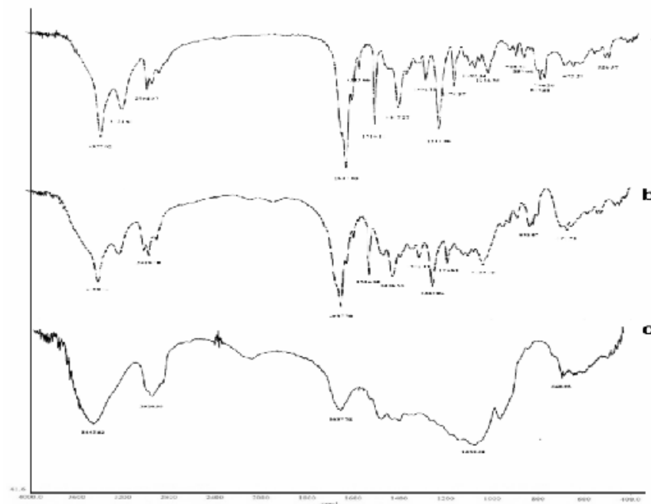
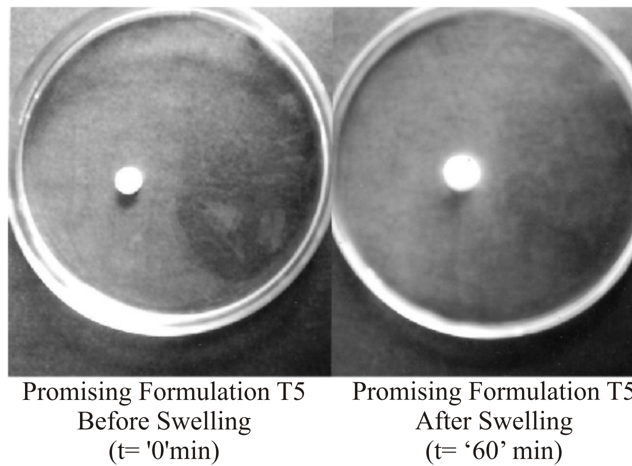


Fig. 4: IR Spectra of (a) Atenolol IP; (b) HPMC (50 cps); (c) Formulation T5



Promising Formulation T5 Before Swelling (t= '0'min)	Promising Formulation T5 After Swelling (t= ‘60’ min)

Fig.5: Determination of Swelling Index

Fig.6: Determination of Floating time and Floating lag time

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In-vitro Characterization and Cytotoxicity Analysis of 5-Fluorouracil loaded Chitosan Microspheres for Targeting Colon Cancer

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Abstract

The objectives of the present investigation were to prepare the 5-Fluorouracil (5-FU) loaded chitosan microspheres for colon targeting and its in vitro cytotoxicity analysis on HT-29 human colon cancer cell lines. Chitosan microspheres prepared by the emulsion polymerization method were analyzed for morphology, mean particle size, drug polymer interaction, entrapment efficiency, in vitro drug release and cytotoxicity on HT-29 colon cancer cell lines. The mean particle size of unloaded microspheres underwent significant change with increase in concentration of chitosan solution. The stirring speed had a significant effect only at the lower level (i.e. 1000 to 4000 rpm). Entrapment efficiency increased with increase in drug concentration. The formation of chitosan microspheres was heeled by the use of differential stirring. With an increase in the concentration of water-soluble drug, there was an increase in entrapment efficiency and drug load over a large concentration range. Cytotoxicity study indicated that 5-FU loaded chitosan microspheres prolonged the cytotoxic effect on HT-29 colon cancer cell lines in comparison to free 5-FU

Keywords: *Chitosan microspheres, 5- Fluorouracil, Cytotoxicity.*

INTRODUCTION

Chitosan microspheres have been widely accepted for drug delivery, fabrication of biosensors as well as delivery of both hydrophilic and lipophillic drugs [1-3]. Chitosan, a polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine, being biodegradable and biocompatiable, is widely used in the formulation of particulate drug delivery systems to achieve controlled drug delivery [4]. Chitosan microspheres are prepared by chemical denaturation, of chitosan present in the inner phase of water/oil (w/o) emulsion. Denaturation is usually carried out using glutaraldehyde with continuous stirring. Various process parameters affecting characteristics of chitosan microspheres have been identified, along with their significance[5]. It has been reported that irrespective of molecular weight, chitosan microspheres are formed only at the minimum concentration of the chitosan solution at 1% w/v. However, no reason was offered for this observation. This finding has led the workers in this filed to restrict the minimum level of chitosan concentration to 1% w/v. In addition to concentration of chitosan solution, several other process parameters have been identified and optimized [6]. However, no attempt has been made to study the effect of the physical properties of the drug on the attributes of microspheres. The degree of stirring (ie, time and speed of stirring during emulsification) determines the size of droplets, which can be changed to obtain the product (i.e. chitosan microspheres) in the desired size range. However, no further division of quasi-solid or solid particles, formed during the process of cross-linking, are desired in order to protect the structural integrity of the microspheres [7]. Based on this hypothesis, it was planned to carry out stirring at a higher rate of agitation during initial emulsification followed by lower rate during the cross – linking stage. The present study was carried out with two objectives. The first objective was to change the method of preparation based on the above hypothesis to see whether microspheres could be obtained by the modified method using a chitosan solution of lower concentration.

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The second objective was planned to compare the basic characteristics of the prepared micro spheres with the microspheres obtained with a higher concentration of chitosan solution. The study was conducted in two different stages. In the first stage, optimization of the concentration of chitosan solution was carried out. In the second phase, in vitro cytotoxicity analysis of 5-FU solution and 5-FU loaded chitosan microspheres was carried out on HT-29 human colon cancer cell lines.

MATERIALS AND METHODS

Chitosan (85% deacetylated) was purchased from Sigma-Aldrich, USA. 5-FU was obtained as a gift sample from Shalaks Pharmaceutical Private Limited, New Delhi, India. Light paraffin oil and hard paraffin oil were purchased from Merck Chemicals, Mumbai, India. Glutaraldehyde, 25% in water, was purchased from S.D Fine chemicals, Mumbai, India.

Analytical estimation of 5-Fluorouracil

The estimation of 5-Fluorouracil was done by UV-Visible Spectrophotometric method. Aqueous solution of 5-Fluorouracil was prepared in distilled water and the absorbance was measured at 266 nm spectrophotometrically from 2.5 to 20 g mL^{-1} concentration (R^2=0.994).

Preparation of 5-Fluorouracil loaded chitosan microsphers

5-FU loaded chitosan microspheres were prepared by chemical crosslinking method.[8] 75 ml of light liquid paraffin and 75 ml of hard liquid paraffin oil were placed in a 250-ml plastic beaker. 1% w/w of span 80 was mixed with the oil with stirring and heated up to 80 ^{0}C. To this, 10 mL of chitosan solution of different concentration (prepared by dissolving chitosan in 2% v/v glacial acetic acid) was added drop wise using a 22-gauge hypodermic syringe. This addition was accompanied by stirring of paraffin oil at different speeds (1000 to 4000 rpm) with the help of a high – speed stirrer with propellers (Remi Motors, India). Stirring was continued for 1 h after the complete addition of chitosan solution into oil. After 1 h stirring, 10 mL of glutaraldehyde solution saturated with 30 ml of toluene was added dropwise to the mixture with continuous stirring at 500 rpm for next 1 h at the temperature 50-55 ^{0}C. Stirring was stopped after 1 hour of the final addition of glutaraldehyde. Suspension of chitosan microspheres in paraffin oil thus obtained was allowed to stand for 1 hour to settledown the microspheres under gravity. Clear supernatant was decanted and microspheres were washed three times with hexane. After the final wash, microspheres were allowed to dry in air and stored in desiccators at room temperature.

Determination of Mean Particle Size and its Particle Size Distribution

Particle size analysis of unloaded and drug-loaded chitosan microspheres was performed by optical microscopy using a compound microscope. A small amount of dry microspheres was suspended in purified waster. The suspension was sonicated for 5 seconds. A small drop of suspension, thus obtained, was placed on a clean glass slide. The slide containing chitosan microspheres was mounted on the stage of the microscope and diameter of at least 500 particles was measured using a calibrated ocular micrometer.

Morphological Study of Microspheres

The shape and surface morphology of the microspheres was investigated using scanning electron microscopy (SEM; Jeol, JSM – 6100). The microspheres were fixed on supports with carbon-glue, and coated with gold using a gold sputter module (JFC-1100) in a high vacuum evaporator. Samples were observed by SEM at 15kV.

Determination of Percent Drug Entrapment

5-Fluorouracil loaded chitosan microspheres (200mg) were digested in 50 ml of distilled water. The suspension was then warmed for few min, filtered with 0.2m membrane filter (MDI, India) and an aliquot of the filtrate was diluted appropriately with respective solvent system. Absorbance was measured at 266 nm and the concentration was calculated according to the standard regression.

Fourier transform infrared spectroscopy (FTIR)

Drug polymer interactions were studied by FTIR spectroscopy. The spectrum was recorded for 5-fluorouracil, blank chitosan microspheres, physical mixture of blank chitosan microspheres and 5-fluorouracil and 5-fluorouracil loaded chitosan microspheres using Spectrum BX (Perkin Elmer) infrared spectrophotometer. Samples were prepared in KBr disk (2 mg sample in 200 mg KBr) with a hydrostatic press at a force of 40psi for 4 min. The scanning range was 400-4400cm^{-1} and the resolution was 4 cm^{-1}.

Differential scanning calorimetry (DSC)

The thermal behavior of 5-fluorouracil, blank chitosan microspheres, physical mixture and 5-fluorouracil loaded chitosan microspheres was examined with a DSC 7 (Perkin-Elmer) Thermal analyzer. Argon was used as carrier gas and the DSC analysis was carried out at a heating rate of 10 ^{0}C/min and an argon flow rate of 35cc/min. The sample size was 5 mg and curves were recorded at a temperature range of 60-300 ^{0}C.

Powder X-ray diffraction analysis (PXRD)

PXRD was carried out to investigate the effect of microencapsulation process on crystallinity of the drug. PXRD patterns were recorded on a RIGAKU, Rotaflex , RV 200 (Rigaku Corporation, Japan) powder XRD using Ni-filtered, CuKradiation, a voltage of 60 kV, and a current of 50 mA. The scanning rate employed was 1^{0} /min over the 10^{0} to 40^{0} diffraction angle (2) range. The XRD patterns of 5-fluorouracil crystals, blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres were recorded. Microspheres were triturated to get fine powder before taking the scan.

In vitro release of 5-FU from chitosan microspheres

The *in vitro* drug release studies were performed using USP dissolution rate rest apparatus (paddle apparatus, 100 rpm, 37 0.1C).[9] Chitosan microspheres bearing 5-fluorouracil was suspended in simulated gastric fluid (SGF, pH 1.2, 900 ml) for 2 h. The dissolution media was then replaced with simulated intestinal fluid (SIF) pH 7.5 and the releases study was carried out for a further 3 h, which corresponds to the average small intestinal transit time. Aliquots of the dissolution medium were withdrawn at the pre-determined time interval and the amount of drug was quantified at 266 nm.

In vitro release of 5-FU from chitosan microspheres in the presence of rat cecal contents

In vitro drug release studies were also investigated in the presence of rat cecal contents. The animal experimentation protocols were conducted as per the guidelines of CPCSEA approved by Institutional Animal Ethics Committee. Male Albino rats weighing 15030g, maintained on standard normal diet and water *ad* libitum were selected for the present investigation. Rats were sacrificed and cecal were removed and transferred into simulated colonic fluid (SCF), pH 7.0 previously bubbled with CO_2. The contents of the cecum were weighed and transferred into SCF, pH 7.0, to produce 2% w/v cecal dilution. The release rate studies were carried out using USP type II dissolution rate test apparatus. The study was carried out with 100ml of dissolution medium at on 37 0.1C and rotated at a speed of 100 rpm. A 250 ml beaker containing 100 ml dissolution medium was immersed in the water contained in a 900 ml vessel, which was kept in the water bath of the dissolution rate test apparatus. The formulations, which were previously subjected to *in vitro drug* release studies in 0.1N Hydrochloric acid and SIF, pH 7.4, were kept in an empty gelatin capsule and immersed in the dissolution medium. At pre-specified time intervals, 5 ml of the dissolution media was withdrawn and compensated with the same amount of fresh SCF, pH 7.0, bubbled with CO_2. Samples were filtered through a 0.22m membrane filter and the amount of drug was quantified at 266 nm by UV Visible spectophotometer (Shimadzu, Japan). The experiment was also carried out with 4% w/v cecal contents in the dissolution media.

In vitro cytotoxicity analysis of non-embedded and embedded on HT-29 human colon cancer cell lines

The HT-29 human colon cancer cell lines were purchased from National cell lines facility, Pune and cultured in DMEM (Dulbecco's Modified Eagle Medium) medium supplemented with 10% fetal bovine serum (FBS) at 37 ^{0}C in a 5% CO_2 atmosphere. To examine the effects of non-embedded 5-FU and embedded (chitosan microspheres bearing 5-FU), the cells were treated with 150 mM, 100 mM, 50mM of 5-FU and similar concentrations of embedded 5-FU.

MTT assay

The MTT [3, (4, 5-Demethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay was performed as per standard protocol. In brief, HT-29 human colon cancer cells were cultured in 24 well plates at a density of 5x 10^4 cells per well**.** The cells were treated with varying concentrations of 5-FU and embedded 5-FU. After 48 h, the cells were washed and treated with MTT. Plates were incubated in dark for 4 h, and the absorbance was measured at 570 nm using a microtitre plate reader. To determine the cell viability, percent viability was calculated [(absorbance of drug-treated) sample) / (control absorbance)] x 100.

RESULTS AND DISCUSSION

Effect of various process variables on particle size and entrapment efficiency

The development of a drug carrier made of a bioadhesive and biodegaradable polymer is receiving increasing attention in the field of pharmaceutical technology. [10] These systems offer a number of advantages over the classical drug delivery systems. eg.(i) by selecting the appropriate drug/polymer combination it is possible to achieve the encapsulation hydrophilic and hydrophocbic drugs simultaneously; (ii) the bioactive molecule can be conveniently isolated and protected in the microcavity and (iii) the desired release rate of drug can be easily achieved by selecting a suitable polymer. In the present investigation, a microparticulate system consisting of a microcore of a chitosan (hydrophilic swellable polymer) was used to microencapsulate a water-soluble drug, 5-Fluorouracil. This design was used for colon delivery of 5-Fluorouracil combining two approaches of colon drug delivery: timed release and biodegradation in the colon environment.

The results of entrapment efficiency and particle size measurement due to various core to coat ratio are shown in table 1. The two-way analysis of variance (ANOVA) has shown the concentration of drug had significant effect on the entrapment efficiency and particle size ($p<0.05$). The results show that the increase in drug concentration increases the entrapment efficiency and particle size respectively. There was a linear increase in entrapment efficiency of the drug in chitosan microspheres as a function of concentration. However, the stirring speed also affected the particle size. Increase in stirring speed decreased the particle size significantly.

The cross-linked microspheres of chitosan were subjected *in vitro* drug release rate studies in SGF (pH 1.2) for 2 h and SIF (pH 7.5) for 3 h in order to investigate the capability of the formulation to withstand the physiological environment of the stomach and small intestine. The amount of the 5-FU released during 5 h studies was found to be 15.27 0.56%, which attests the ability of the chitosan to remain intact in the physiological environment of stomach and small intestine. A little amount of the drug, released during 5 h release rate studies was due to the presence of un-entrapped drug on the surface of the microspheres. It is a well established fact that as the chitosan comes in contact with the dissolution medium it creates viscous gel layer around it which controls the release of the entrapped drug. The initials release of the drug present on the surface was higher during 2 h study, as there was no viscous gel layer around the particles and might have been formed after 2 or 3 h which controlled the further releases of drug. After 5 h of testing in 0.1 M HC1 and pH 7.4 Sorensen's phosphate buffer, 20.96 0.58% of the drug was released due to strong shielding effect of compression coat of chitosan. The *in vitro* drug release studies were performed in SCF (pH 7.0) with and without using rat cecal contents. A significant difference ($p <0.005$) was observed in the amount of the 5-FU released at the end of the 24 h from the dissolution medium containing rat cecal content in comparison to the study conducted without rat cecal content. The amount of the drug released from formulations was found to be 47.72 2.39% with 2% w/v cecal matter after 24 h whereas in control study, (without rat cecal contents in dissolution medium) only 31.23 1.49% of drug was released. In case of dissolution medium with 4% cecal matter, 61.65 2.96% of drug release was observed which is considerably higher in comparison to the study involving no cecal matter. The study reveals that the release of the drug in the physiological environment of the colon is due to the degradation of chitosan by colonic bacteria released from rat cecal content. The release of drug from cross-linked chitosan microspheres was supposed to take place after swelling which resulted in the formation of gel followed by the dissolution of 5-FU and diffusion through the gel.

The gel strength of the chitosan microspheres swelled in the dissolution media may be too high, preventing the release of drug from formulation. The colonic bacteria action of rat cecal content medium (2 and 4% w/v) might not be sufficient to degrade the high strength gel barrier of the swollen microspheres. As a result, only 47.72 2.39 and 61.65 2.96% of drug was released after 24 h with 2 and 4% w/v rat cecal content medium, respectively. Hence, one set of animals were administered with 1 ml of 1% w/v aqueous solution of chitosan for 2 days to induce the enzymes that specifically act on chitosan during passage through the colon. The amount of the drug released from the formulation after 24 h with 2 and 4 %

w/v rat cecal contents medium after 2 days induction was found to be 59.35 2.81 and 76.72 3.52% respectively (Fig. 2). Induction of enzymes for 2 days resulted in improved activity of colonic enzymes, as reflected from the release of higher amount of drug in comparison to those, which involved rat cecal content without induction. The release of the drug was much faster during the 18-24 h study period. It is due to the fact that during the initial period (0-18h), the gel strength of the barrier was too high to be broken and during 18-24 h period the network was somewhat loosened which facilitated the release of drug.

In spite of the release of higher percent of drug after 2 days of induction as compared to those without induction, there was a considerable amount of drug to be released and hence, the rats were treated with 1 ml of 1% w/v aqueous solution of chitosan for 4 and 6 days and the release rate study was repeated with 2 and 4 % w/v of cecal matter. The release of the drug was considerably improved with cecal content obtained after 6 days of enzyme induction in comparison to those without enzyme induction or 2 days induction. In the 12-18 h period after 6 days of induction, there was a relatively faster release of the drug due to reduction in the viscosity of the gel network of swollen chitosan around the particles that was susceptible to attack by colonic enzymes. The percent drug release after 24 h release rate study period was observed to be 67.44 3.15 and 88.75 4.15% respectively, with 2 and 4% w/v rat cecal matter obtained after 4 days of enzyme induction and 75.56 3.75 and 96.24 4.77% after 6 days of enzyme induction (Fig. 3). The release of higher amount of drug in case of microspheres with 4% w/v rat cecal mater obtained after 6 days of enzyme induction is due to the larger surface area of microspheres as compared to matrix tablet, which facilitated the release of the drug. The release of the drug is the combined effect of the swelling behavior of chitosan as well as by the biodegradability of guar gum under the influence of colonic enzymes.

FTIR

As mentioned in fig. 4, there was no significant difference in the FTIR spectra of physical mixtures of 5-FU and blank chitosan microspheres as well as 5-FU, when compared to the spectra of individual components.

DSC

Curves of DSC as shown in Fig. 5, one can conclude that drug-loaded microsphere was not a physical mixture, but the formation of real microsphere. The characteristic exothermic peak of 5-FU at 292 ^{0}C and 290 ^{0}C of blank chitosan microspheres, respectively, disappeared in 5-FU loaded chitosan microsphres curve, in which a new characteristic peak at 294 ^{0}C appeared. The DSC curve of the physical mixture also different from that of 5-FU loaded microspheres.

PXRD

PXRD technique was used to define the nature of drug in the microparticles. The X-ray powder diffraction patterns of 5-FU, blank chtosan microspheres, Physical mixture of 5-Fluorouracil and blank chitosan microspheres and 5-FU loaded chitosan microspheres are shown in Fig. 6. The XRD pattern of 5-FU showed peaks, which were intense and sharp indicating its crystalline nature, whereas blank chitosan microspheres showed few sharp peaks. However, 5-FU loaded chitosan microspheres presented the peaks of diminished intensity, suggesting the amorphous nature of drug present in the chitosan microspheres.

Cell cytotoxicity

The cytotoxicity of 5-FU loaded chitosan microspheres and 5-FU-solution was investigated using HT-29 human colon cancer cell lines by MTT assay studying their effect on cell survival and cell cytotoxicity (Table 2). For survival studies, cells were incubated with 5-FU-solution and 5-FU loaded chitosan microspheres continuously and then washed to remove the drug (Fig. 7). Cell survival was determined following the addition of 150, 100, 50 M equivalent of 5-FU. However, equivalent amount of 5-FU embedded in chitosan microspheres crosslinked with different concentrations of GLA exhibited lower cytotoxicity in comparison with 5-FU-solution. There was 42.22 % cell viability after 48 h with free 100 mM 5-FU whereas encapsulated form showed 48.31% cell viability after 72 h with 5omM 5-FU.

CONCLUSION

Use of differential stirring speed during the preparation of chitosan microspheres by the chemical cross-linking method may help to prepare chitosan microspheres using a chitosan solution by less than 1% wt/vol concentration. The pharmaceutical attributes of microspheres were significantly affected by stirring speed, chitosan

concentration and their interaction. Effect of change in drug concentration on the pharmaceutical characteristics of drug-loaded chitosan microspheres is more prominent for water-soluble drug. Therefore, the present investigation showed the promising results of chitosan microspheres as a matrix for drug delivery and also warrants for in vivo study for scale up the technology.

Table 1. Various process parameters used in optimization of 5-Fluorouracil loaded chitosan microspheres

Formulation Code	Composition (Polymer:Drug) (mg)	Entrapment efficiency (%)	Particle size (μm)
CHF-1	200:50	2.12±1.14	10.32±4.32
CHF-2	200:100	5.20±2.49	12.81±5.64
CHF-3	200:200	12.45±1.98	16.28±6.13
CHF-4	200:300	14.66±4.83	18.64±4.29
CHF-5	200:400	18.23±3.56	22.38±5.21

Table 2. Percent viability of 5-FU in free and encapsulated form at different time intervals

		24h		48h		72h	
Sr. No.	Concentration	Free 5-FU	Encap. 5-FU	Free 5-FU	Encap. 5-FU	Free 5-FU	Encap. 5-FU
1.	150 mM	49.87%	89.12%	26.78%	60.24%	15.70%	42.35%
2.	100 mM	94.99%	98.99%	42.22%	80.14%	18.38%	45.26%
3.	50 mM	96.52%	99.10%	79.15%	89.23%	22.68%	48.31%

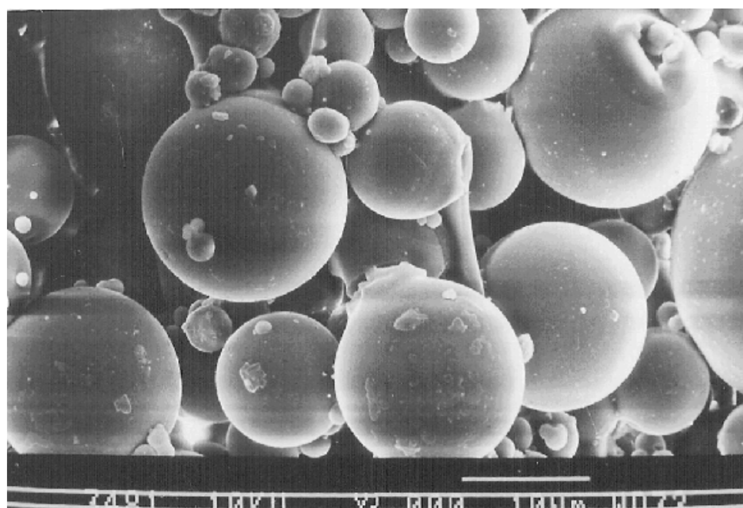

Fig. 1: Scanning electron micrpscopy of drug loaded chitosan microspheres, which indicated the smooth shaped microspheres are formed after loading of 5-fluorouracil

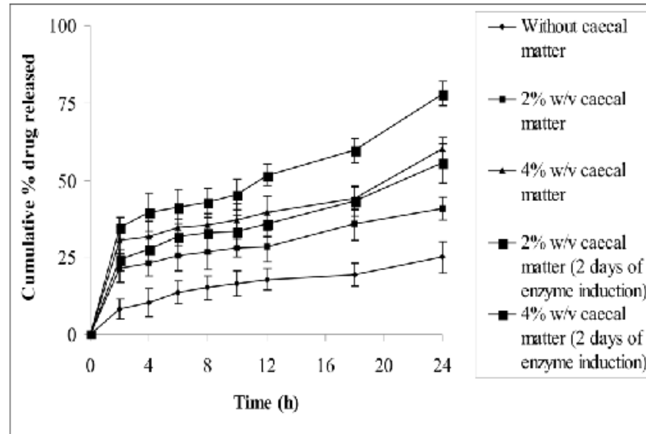

Fig. 2: Cumulative percent drug release in SCF (pH 7.0) with rat caecal matter, without rat caecal matter and after 2 days of enzyme induction. Results indicated that in presence of rat caecal contents, chitosn amicrospheres releases significantly higher amount of 5-FU in comparison of without caecal matter.

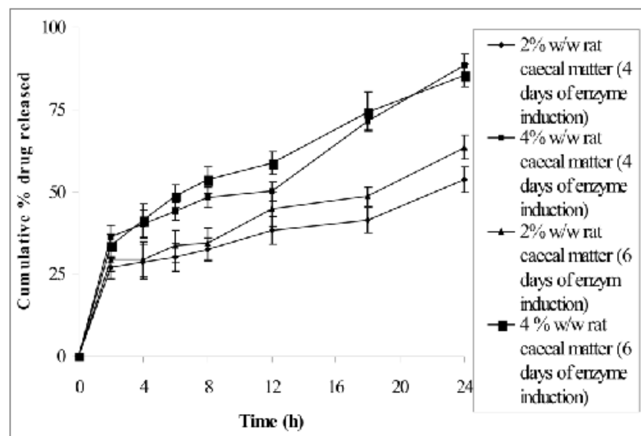

Fig 3: In vitro drug release in presence of rat caecal contents after 4 and 6 days of enzyme induction, which indicated that 6 days of enzyme induction significantly enhanced the release rate.

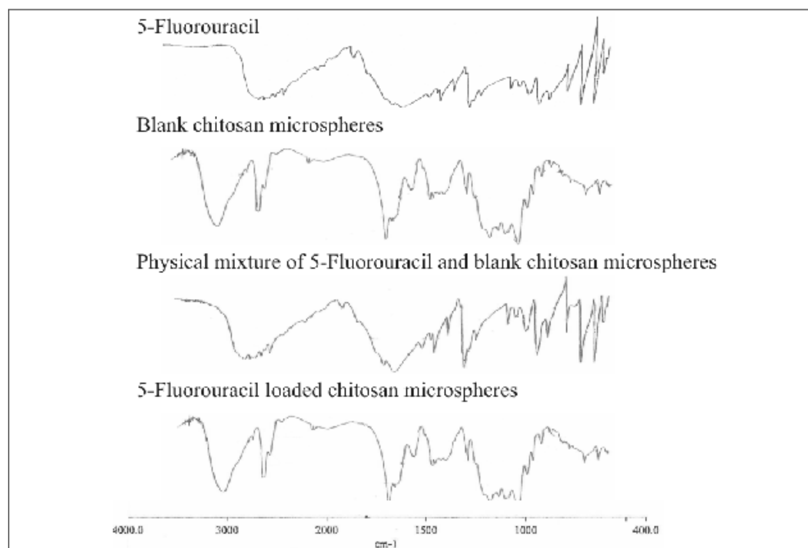

Fig. 4: FTIR spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

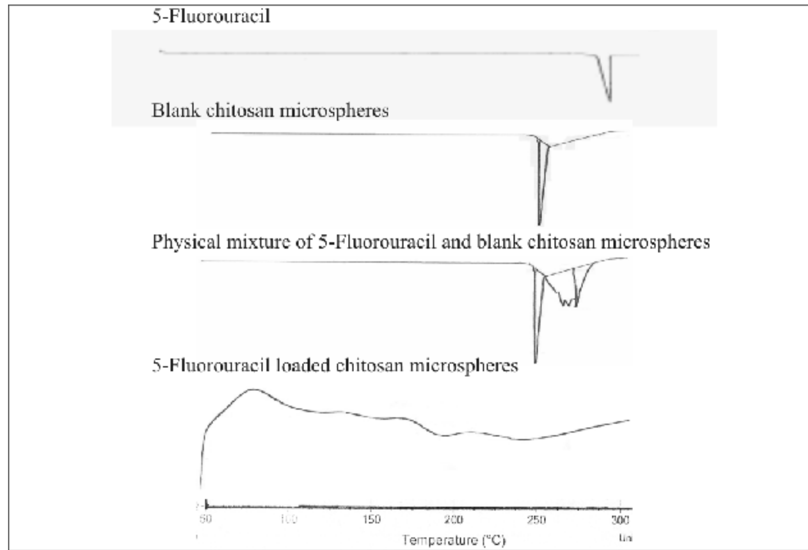

Fig. 5: DSC spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

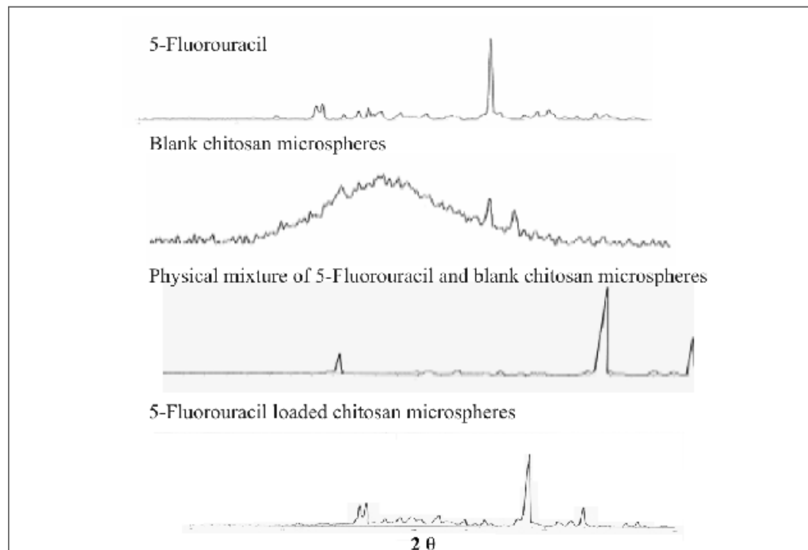

Fig. 6: X-ray diffraction pattern spectra of 5-fluorouracil, blank chirosan microspheres, physical mixture of 5-fluorouracil and blank chitosan microspheres and 5-fluorouracil loaded chitosan microspheres

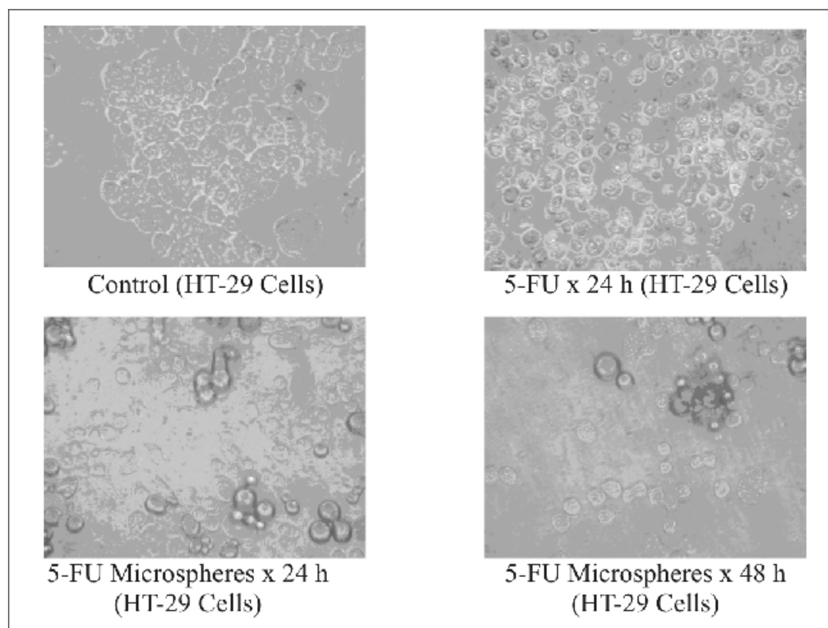

Fig. 7: Photomicrographs of cytotoxicity analysis of 5-fluorouracil and 5-fluorouracil loaded chitosan microspheres

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Depressant and Anticonvulsant Effect of Methanol Extract of *Swietenia Mahagoni* in Mice

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Abstract

The present study was undertaken to evaluate the sleep potentiation (depressant) and anticonvulsant effect of methanol extract from bark of Swietenia mahagoni L. Jacq. (MESM) (Meliaceae) in Swiss male albino mice. The sleep potentiation effect of MESM (25 and 50 mg/kg, i. p.) significantly increased pentobarbitone (45 mg/kg, i. p.) induced sleeping time in a dose dependent manner. The anticonvulsant effect of MESM at the doses of 25 and 50 mg/kg, i. p. was examined against pentylenetetrazole (PTZ, 80 mg/kg, i. p.) and strychnine (STR, 2.5 mg/kg, i. p.) induced seizures and significantly delayed ($p < 0.05$) the onset and also antagonized these seizures in a dose dependent manner. Diazepam (2.0 mg/kg, i. p.) was used as reference drug

Keywords: *Pentobarbitone, Anticonvulsant, Pentylenetetrazole, Strychnine, Swietenia mahagoni.*

INTRODUCTION

Seizure is associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain and status epilepticus is characterized by repeated episodes of epilepsy without the patient having recovered from the previous attack.[1]

A large number of synthetic antiepileptic drugs are currently available to treat various types of seizures but unfortunately these drugs not only fail to control seizure activity in some patients, but they frequently cause side effects. Traditional medicine involves the use of herbal medicine, animal parts and minerals and about 80% of the world population is dependent (wholly or partially) on plant-based drugs.[2]

The *Swietenia mahagoni* L. Jacq. (Meliaceae) is a medium to large evergreen medicinally and economically important timber tree native to the West Indies and Central America and bark is grey-black in colour.[3, 4] The seeds and bark of this plant are used for the treatment of hypertension, diabetes and malaria as a folk medicine in Indonesia and India.[4, 5] The bark contains tannin, and may serve as an antipyretic, tonic and astringent.[6] Traditionally the bark decoction of *S. mahagoni* is used to treat anemia, diarrhea, dysentery, fever, loss of appetite and toothache. The Leave decoction of *S. mahagoni* is used against nerve disorders, seeds infusion against chest pain and leaves or roots poultice against bleeding.[7]

The pentylenetetrazole (PTZ)-induced seizures are similar to the symptoms observed in the absence seizures and drugs useful in the treatment of absence seizures suppress PTZ-induced seizures.[1, 8] The objective of the present study was to find out sleep potentiation effect of the methanol extract of *S. mahagoni* (MESM) on pentobarbitone-induced Swiss albino mice and also to investigate anticonvulsant activity against the seizures induced by PTZ and strychnine (STR).

MATERIALS AND METHODS

Plant material

The bark of *S. mahagoni* was collected in the month of October 2007 from the hill region of Midnapore, West Bengal, India. The bark was authenticated by M. S. Mondal, Botanical Survey of India, Kolkata, India and the voucher specimen (PMU-3/JU/2007) has been preserved in Pharmacology Research Laboratory, Jadavpur University, Kolkata for future reference.

Preparation of extract

The bark of *S. mahagoni* was shade dried and powdered with a mechanical grinder. The powder (750 g) was defatted with petroleum ether 60-80°C in a soxhlet extraction apparatus and then extracted with methanol.

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The solvents were completely removed under reduced pressure to obtain a dry mass. The yields of the petroleum ether and methanol extracts were found to be 5.20 and 12.00% w/w respectively. The extracts were stored in a vacuum dessicator for further use. Preliminary phytochemical analysis showed that the triterpenoid and flavonoid are the major components of the extract.

Animals used

Male Swiss albino mice weighing (20-27g) were maintained in identical laboratory conditions and fed with commercial pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All procedures described were reviewed and approved by the university animal ethical committee.

Chemicals

Pentobarbitone Sodium (Ranbaxy, Mumbai), Pentylenetetrazole (PTZ), Strychnine (STR) (HiMEDIA Laboratories Pvt. Ltd., Mumbai) and Diazepam were used for the study.

Pentobarbitone-induced sleeping time in mice [9]

18-Male Swiss albino mice weighing 20-28 g were randomly divided into 3 groups (n=6). Group I received pentobarbitone sodium (45 mg/kg, *i.p*) and served as positive control. Group II and III received MESM (25 and 50 mg/kg, *i.p*) 30 min prior to the administration of pentobarbitone. The time between the loss of the righting reflex and the regain of this reflex measured as the sleeping time.

Assessment of anticonvulsant activity

Pentylenetetrazol (PTZ)-induced seizure [9]

30-Male Swiss albino mice weighing 20-27g were randomly divided into 5 groups (n=6). Group I served as saline control (5 ml/kg, *i.p*). Group II received a convulsive dose of PTZ (80 mg/kg, *i.p*) and served as PTZ-control. Group III, IV and V received MESM (25 and 50 mg/kg, *i.p*) and reference drug diazepam (2.0 mg/kg, *i.p*) respectively, 30 min prior to the administration of PTZ. The animals were observed for onset of myoclonic spasm and clonic convulsion upto 30 min after PTZ injection. The percentages of protection were observed and recorded.

Strychnine (STR)-induced seizure [10]

30-Male Swiss albino mice weighing 20-27g were randomly divided into 5 groups (n=6). Group I served as saline control. Group II received STR nitrate (2.5 mg/kg, *i.p*) and served as STR-control. Group III, IV and V received MESM (25 and 50 mg/kg, *i.p*) and reference drug diazepam (2.0 mg/kg, *i.p*) respectively, 30 min prior to the administration of STR.The animals were observed for onset of myoclonic spasm and clonic convulsion up to 30 min after STR injection. The percentages of protection were observed and recorded.

Statistical Analysis

All results are expressed as the mean ± SEM. The results were analyzed for statistical significance ($p<0.05$, $p<0.01$) by one-way (ANOVA) followed by Dunnett's test using computerized Graph Pad InStat version 3.05, Graph pad software, U.S.A.

RESULT

The total sleeping time induced by pentobarbitone sodium increased significantly from 55.75 ± 2.17 min in the control group to 67.25 ± 2.56 and 96.25 ± 4.80 min in the extract treated group at the doses of 25 and 50 mg/kg respectively (Table 1).

PTZ and STR produced tonic seizures in all mice except saline control. The MESM (50 mg/kg) significantly delayed the onset of seizures from 1.64±0.24 to 5.4±1.28 min for PTZ ($p<0.05$) and 3.6±0.24 to 7.4±0.24 min for STR ($p<0.01$) induced seizures. The results of treated group are comparable with that of reference drug diazepam. (Table-2 and 3).

DISCUSSION AND CONCLUSION

Pentobarbitone sodium is a short to intermediate acting barbiturate, to produce quick onset of sleep.[9] Pentobarbitone-induced sleep potentiation test has been used widely as an animal model in sleep studies and CNS depressant effects.[11] In the present neuropharmacological screening it was observed that the methanolic extract of barks of *S. mahagoni* at the concentration 50 mg/kg prolonged the total sleeping time as compared to that of control group. This suggested that the MESM possess CNS depressant property.

Since many antiepileptic agents induce CNS depression, motor incoordination and ataxia[1], we therefore assessed the spectrum of anticonvulsant activity of MESM against PTZ and STR induced seizures. The PTZ test represents a valid model for human generalized myoclonic seizures and also generalized seizures of the petitmal (absence) type.[12] STR is a potent spinal convulsant and the convulsions produced by strychnine are reflex, tonic-

clonic and symmetrical.[8] The MESM (50 mg/kg) significantly delayed ($p<0.05$) the onset and antagonized the PTZ and STR-induced seizures, which are comparable with the reference drug diazepam. Thus our present results suggested that the methanol extract of *S. mahagoni* may be effective against human generalized myoclonic seizures and also absence seizures.

Preliminary phytochemical analysis performed in this study showed that the triterpenoid and flavonoid are the major components of the extract. There are some evidences about anticonvulsant effect of some flavonoid compounds.[13, 14] Salgueiro *et al*., showed anxiolytic effects of some natural and synthetic flavonoids in rats and found that these compounds exerted their effects through the central benzodiazepine receptors.[15] Some researchers have reported anticonvulsant activity of monoterpene. The SL-1, a synthetic monoterpene homologue of GABA, demonstrated anticonvulsant activity in PTZ-induced seizures.[16] Therefore, it seems that the anticonvulsant effect of *S. mahagoni* may be related in part to flavonoid and /or triterpenoid compounds present in the extract.

It has been shown that PTZ enhances the basal activity and the sensitivity of dopaminergic neurons to PTZ in rat brain and the nigrostriatal dopaminergic neurons contribute to the central alterations associated with experimental epilepsy.[17] The blockade of D_1 and D_2 receptors by haloperidol inhibited (-) bicuculline-induced seizures. [18] Thus the blockade of DA receptor may have some protective effect in epilepsy.

The methanol extract of *S. mahagoni* was however more efficacious against PTZ induced seizure where protection was observed in all of the mice, an effect which indicates that the extract produce its central nervous system depressant action as consequence of its GABAergic and less importantly, glycinergic transmission, since PTZ is a selective $GABA_A$ receptor antagonist [8, 19] while Strychnine antagonizes the inhibitory spinal cord and brainstem reflexes of glycine.[20]

From such informations it may be stated primarily that the methanol extract of bark of *S. mahagoni* may contain some biomolecule(s) that produce CNS depression and anticonvulsant action after blocking D_1 and D_2 receptors or facilitating GABA transmission.

In conclusion, the data of our study suggests that *S. mahagoni* may have beneficial effects in epilepsy that holds the hope of new generation of anticonvulsant drugs. However, comprehensive chemical and pharmacological research is required to find out the exact mechanism of this extract for its anticonvulsant effect and to identify the active constituent(s) responsible for this effect.

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Table 1: Effect of methanol extract of Swietenia mahagoni on pentobarbitone-induced sleeping time in mice (n=6).

Group	Treatment	Dose (mg/kg)	Onset of sleep in minute (Mean±SEM)	Duration of sleep in minute (Mean±SEM)
I	Pentobarbitone 45	2.5 ± 0.57	55.75 ± 2.17	
II	Pentobarbitone+MESM	45+25	1.5 ± 0.57	67.25 ± 2.56
III	Pentobarbitone+MESM	45+50	1.5 ± 0.57	96.25 ± 4.80*

*P<0.01 when compared with control group; statistical analysis by Dunnett ' s vs. control.

Table 2: Effect of methanol extract of Swietenia mahagoni (MESM) on pentylenetetrazole (PTZ)-induced seizures (n=6).

Group	Treatment	Dose (mg/kg)	Onset of sleep in minute (Mean±SEM)	Duration of sleep in minute (Mean±SEM)	Mortality (%)	Protection (%)
I	Saline Control	5ml	0.0	0.0	0.0	100
II	PTZ	80	1.6±0.24	1.2±0.20	100	0.0
III	MESM	25	3.4±0.50	10±3.36*	80	20
IV	MESM	50	5.4±1.28*	9±1.18*	0.0	100
V	Diazepam	2.0	5.2±1.02**	10.2±1.02**	0.0	100

*P<0.05, **P<0.01 when compared with control group; statistical analysis by Dunnett ' s vs. control

Table 3: Effect of methanol extract of Swietenia mahagoni (MESM) on strychnine (STR)- induced seizures (n=6).

Group	Treatment	Dose (mg/kg)	Onset of sleep in minute (Mean±SEM)	Duration of sleep in minute (Mean±SEM)	Mortality (%)	Protection (%)
I	Saline Control	5ml	0.0	0.0	0.0	100
II	Strychnine	2.5	3.6 ± 0.24	11.6 ± 2.11	100	0.0
II	MESM	25	5 ± 0.54	11.8±1.96	60	40
V	MESM	50	7.4±0.24**	5.6±0.74*	0.0	100
V	Diazepam	2.0	7.6±0.67**	7.4±0.92	0.0	100

*P<0.05, **P<0.01 when compared with control group; statistical analysis by Dunnett ' s vs. control.

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Free Radical Scavenging Activity of *Nyctanthes arbortristis* in Streptozotocin-Induced Diabetic Rats

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Abstract

Nyctanthes arbortristis is reported to have a wide range of biological activities such as antidiabetic, antipyretic, anthelmintic, antibilous, expectorant, laxative and is used for treatment of arthritis, obstinate, sciatica, malaria, intestinal worms and also as tonic. The qualitative test of the crude extract shown the presence of alkaloids and flavonoids. The study was aimed to find out the protective effect of Nyctanthes arbortristis on lipid peroxidation (LPO) and activity of both enzymatic and non-enzymatic antioxidants in streptozotocin (STZ) induced diabetic rats. The oxidative stress was measured in liver homogenate LPO, Superoxide dismutase (SOD) and Catalase (CAT) levels; blood serum levels of SGPT, SGOT, Alkaline phosphatase (Alk Phos) and cholesterol, triglyceride levels. The significant elevation in LPO, SGPT, SGOT, Alk Phos and cholesterol, triglyceride levels and decreased enzymatic activity of SOD, CAT were the salient features observed in diabetic control rats. Administration of Nyctanthes arbortristis leaves and flower chloroform extracts (50, 100 and 200 mg/kg) orally for 27 days caused a significant reduction in LPO, SGPT, SGOT, Alk Phos, cholesterol and triglyceride levels on extracts treated STZ diabetic rats, compared to diabetic control rats. Further more Nyctanthes arbortristis extract treated diabetic rats showed significant increase in SOD and CAT enzymatic antioxidant activity when compared to diabetic control rats. The administration of the extracts and glibenclamide (10 mg/kg) improved the activity of both enzymatic and non-enzymatic antioxidants and lipid profile in STZ-induced diabetic rats.

Keywords: *Diabetes, Nyctanthes arbortristis, lipid profile, Oxidative stress.*

INTRODUCTION

Oxidative stress plays an important role in chronic complication of diabetes and is postulated to be associated with increased lipid peroxidation[1]. The streptozotocin (STZ) is frequently used to induce diabetes mellitus in experimental animals through its toxic effect on pancreatic β-cells. The cytotoxic action of STZ is associated with the generation of reactive oxygen species causing oxidative damage. Diabetes manifested by experimental animal's model exhibits high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense systems. Increased oxidative stress and change in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the etiology of diabetic complication[2]. The disturbances of antioxidant defense systems in diabetes were shown: alteration in antioxidant enzyme such as Superoxide dismutase (SOD) and Catalase (CAT) Glutathione peroxidase (GPx) Glutathione reductase (GR) and impaired glutathione (GSH) metabolism. Anti-oxidants provide protection to living organism from damage caused by uncontrolled production of ROS concomitant lipid peroxidation, protein damage and DNA strand breaking. Ethnomedical literature contains a large number of plants including, *Nyctanthes arbortristis* that can be used against diabetes, Insulin resistance[3-4] in which reactive oxygen species and free radicals play a major role. Many minor components of foods, such as secondary plant metabolites, have been shown to alter biological processes, which may reduce the risk of chronic diseases in diabetic humans[5]. Recently, there has

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been an increasing interest in the use of medicinal plants. The plant kingdom has become a target for the search of drugs and biological active lead compounds. Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes[6]. Hence, the present study was under-taken to explore free radical scavenging activity of *Nyctanthes arbortristis* on STZ-induced diabetic rats. In recent years, considerable focus has been given to an intensive search for novel type of antioxidants from numerous plant materials[7]. The Management of diabetes without any side effects is still a challenge to the medical system. There is an increasing demand from patients to use the natural products with antidiabetic activity, because insulin and oral hypoglycemic drugs possess undesirable side effects[8] .

Nyctanthes arbortristis Linn. (Division: Magnoliophyta; Class: Magnoliopsida; Order: Lamiales; Family: *Oleaceae*), commonly known as Harsinger or Night jasmine, is a well documented plant. It is a native of India, distributed wild in sub-Himalayan region and also found in Indian garden as ornamental plant. The indigenous people of Chittoor district Andhra Pradesh (India) widely use the whole plant for treatment of cancer, root for fever, sciatica, anorexia; bark as expectorant, Leaf for control fever, diabetes and as cholagogue, diaphoretic and anthelmintic. The decoction is used to treat arthritis, malaria, intestinal worms, tonic and laxative[9-11]. Antitrypanosomal[12], anti-inflammatory and antioxidant activity has also been exhibited by the various extracts of the plant[13-15]. The *Nyctanthes arbortristis* were tested against Encephalomyocarditis virus (EMCV) and Semliki forest virus (SFV)[16]

Previously isolated constituents:

Earlier workers have reported the isolation of polysaccharides, iridoid glycosides, phenypropanoid glycoside, ß-sitosterol, ß-amyrin, hentri-acontane, benzoic acid, glycosides, nyctanthoside-a iridoid, nyctanthic acid, Friedelin and lupeol and oleanolic acid and 6ß-hydroxylonganin and iridoid glucosides-arborsides A, B and C, alkaloids, Phlobatanins, terpenoids and cardiac glycosides[17]. Iridoid glucosides (arbortristosides-A [I], B [2], C [3], and 6ß-hydroxyloganin [4] isolated from the traditional plants *Nyctanthes arbortristis* show Antileishmanial activity in both has been exhibited *in vitro* and *in vivo* test systems[18].

MATERIALS AND METHODS

Drugs and chemicals:

Streptozotocin was obtained from Sigma chemical Co (St Louis, USA). Petroleum ether (40-60 °C) benzene, chloroform, ethyl acetate and methanol (Nice Pvt Ltd, India) and all other chemical are obtained from Sigma and HiMedia laboratories Pvt Ltd.

Preparation of plant:

Nyctanthes arbortristis leaves and flower were collected from widely growing plants from the region of North Karnataka in the months of Sept-October 2005. The plants were identified and confirmed by the Taxonomist of the Basaveshwar Science College, Bagalkot, Karnataka.

The plant material was dried in shade and uniformly powdered by passing through the sieve #. 44 and extracted with petroleum ether (40-60 °C) to defat the preparation, followed by benzene, chloroform, ethyl acetate and methanol by solvent extraction 24 h/cycle. The extract was concentrated under rotary evaporator and dried in lyophilizer (Mini Lyotrap, Serial No. J8199/5, LET Scientific LTD, UK). The extracts were formulated as suspension in distilled water using 5% Tween-80, as suspending agent[19]. The petroleum ether extraction is known only to defat the plant preparation therefore, the chloroform extract of leaves and flower were selected for the present study. Henceforth, the leaves and flowers extract of *Nyctanthes arbortristis* refers to chloroform extracts of *Nyctanthes arbortristis* leaves and flower

Experimental animals:

Animals

Wistar albino rats (150-200 g) of either sex were used in this study. After one week acclimatizing to laboratory used for investigation. The animals were housed under standard environmental condition of temperature (21±2 °C), humidity (51±10%) and a 12 h light-dark cycles with standard pellet diet (Amrut Lab) and water *ad libitum*. All the experiments animals were carried out as per the guidelines of Institutional Animals Ethics Committee (RGE No. 821/a/CPCSEA) of College, after approval (HSK/IAEC.Clear/2004-2005) dated 27/12/04.

Induction of diabetics in rats:

The streptozotocin freshly prepared was dissolved in

citrate buffer (pH 4.5) and rats were made diabetic by injection of a single dose (55 mg/kg) intraperitonally. They were given 5% of glucose in drinking water for the first 24 h to encounter any initial hypoglycemia. On the third day the animals were checked for serum blood glucose levels, those with higher than 300 mg/dl were used for the experiments[19-20].

In our study, a total 54 rats (48 diabetic surviving rats, 6 normal rats) were used. These rats were randomly divided into 9 groups of six rats, after induction of STZ diabetes. Group No.1 (diabetic control) received distilled water in 5% (Tween-80). Group No. 2 received glibenclamide (positive control) at an oral dose (10 mg/kg). Group No. 3 (normal) received distilled water in 5% (Tween-80). Group No. 4, 5 and 6 received chloroform extract of *Nyctanthes arbortristis* leaves (50, 100 and 200 mg/kg). Group No. 7, 8 and 9 received chloroform extract of *Nyctanthes arbortristis* flower oral dose (50, 100 and 200 mg/kg) respectively. The treatment was continued orally daily for 27 days.

Preparation of tissue homogenate:

The tissues were weighed and 10% tissue homogenate was prepared with 0.1 m phosphate buffer (pH. 7.0). After centrifugation at 1000 rpm for 15 m. The supernatant was used to measure protein, thiobarbituric acid reactive substance (TBARS), SOD and CAT.

Analytical procedure:

The blood samples were collected by retro-orbital plexus under anesthesia and used for estimation of blood serum; SGPT, SGOT, Alk Phos, cholesterol and triglyceride were estimated by using commercial diagnostic kit. (Tecodiagnostics USA) on star-21plus semi-autoanalyser.

Protein:

The protein content of the liver homogenate was estimated by the following method[21]. 2.25 ml of 90% alcohol was added to 1 ml of liver homogenate, centrifuged for 3000 rpm for 10 m. The supernatant was discarded and precipitate that settled down was dissolved in 1 ml of 0.1N NaoH, alkaline mixture was added, left for 10 m, 0.5 ml of folin reagent (Phenol reagent) was added and further left for 10 m for colour development, and the absorbance was measured at 610 nm. The protein levels were calculated using standard Bovine serum solution, 200 mg in 100 ml of distilled water.

Lipid peroxidation:

The LPO of the liver homogenate was estimated by the following method[22]. The reaction mixtures 1 ml liver homogenate, 100 µl of 8.1 % Sodium dodecyl sulfate (SDS) and 600 µl acetic acid solution was left for 2 m at room temperature. Than added 600 µl TBA solution was added. The above solution was boiled for 60 m in water at 95 °C, then cooled with ice cooled water at 4 °C. The mixtures of *n*-butanol and pyridine (15:1, v/v) were added, and the mixture was shaken vigorously and centrifuged at 10.000 rpm for 5 m. The absorbance of the organic layer (upper layer) was measured at 523 nm. The enzyme activity was expressed as units per mg of protein.

Catalase:

The CAT was estimated by the following method[23]. The reaction mixture test tube contained 1 ml of Phosphate buffer 0.01 M (pH 7.0) and 0.1 ml of 10% liver homogenate. The reaction was started by addition of 0.4 ml of 2M H_2O_2. The tube was incubated at 37 °C for 10 m. The reaction was stopped by the addition of 2.0 ml of 5% of dichromatic–acetic acids reagent (5% potassium dichromate and glacial acetic acids were mixed in 1:3 ratio). The control was carried out without addition of H_2O_2. The absorbance was read at 620 nm. The CAT activity was expressed as µM H_2O_2 consumed/min/mg of protein. The enzyme activity was expressed as units/mg protein.

Superoxide dismutase:

The SOD was estimated by the following method[24-25]. The assay based on the reduction of Nitro blue tetrazolium (NBT) to water insoluble blue formazan. The liver homogenate 0.5 ml was taken, and 1 ml of 50 mM sodium carbonate and 0.4 ml of 24 µm NBT and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Absorbance was measured at 560 nm at zero time followed by second measurement after 5 m at 25 °C. The control was simultaneously run without liver homogenates. Units of SOD activity were expressed as the amount of enzymes required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

RESULTS

The Table No.1 & 2 depicts the effect of rats with STZ and

treatment of chloroform extracts of *Nyctanthes arbortristis* leaves and flower (50, 100 and 200 mg/kg) on the levels of LPO, SOD, CAT activity in liver homogenates. The treatment of rats with a single dose of STZ at (55 mg/kg) of body weight significantly increased ($P<0.001$) in liver LPO, and blood serum SGPT, SGOT, Alk Phos, and cholesterol, triglyceride in diabetic control rats, on the other hand, SOD, CAT decreased significantly ($P<0.001$) as compared to normal groups of rats due to STZ treatment.

However, treatment of the rats with the chloroform extracts of *Nyctanthes arbortristis* leaves and flower (50, 100 and 200 mg/kg) significantly ($P<0.05$-0.001) reduced lipid peroxidation, SGPT, SGOT, Alk Phos, cholesterol and triglyceride, (except in leaves extract 50 mg/kg in Alk Phos) of which are comparable with the positive control valued. The treatment of the rats with *Nyctanthes arbortritis* extract (50, 100 and 200 mg/kg) respectively, preserves significantly ($P<0.05$-0.001) CAT and SOD activity, which are comparable with glibenclamide was used as positive control.

DISCUSSION

To establish a scientific basis for the utility of this plant in the treatment of diabetes, it was decided to evaluate free radical scavenging activity in STZ-induced diabetic rats. This demonstrates the protection provided by feeding of chloroform extracts of *Nyctanthes arbortristis* leaves and flower (50, 100 and 200 mg/kg) to rats by maintaining the levels LPO, SOD and CAT, biomarker enzymes, cholesterol and triglycerides in STZ-induced diabetic rats. The lipid peroxidation values have been shown to be restored showing antilipid peroxidation effects of the components of chloroform extracts of *Nyctanthes arbortristis* leaves and flower (50, 100 and 200 mg/kg) and glibenclamide (10 mg/kg). Oxidative stress in diabetes is coupled to a decrease in the antioxidant status, which can increase the deleterious effects of free radicals[26]. The SOD and CAT are the two major scavenging enzymes that remove radicals *In vivo*. A decreased activity of these antioxidants can lead to an excess availability of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which in turn generate hydroxyl radicals ($^{\cdot}OH$), resulting in initiation and propagation of LPO. The SOD can catalyze dismutation of O_2^- into H_2O_2, which is then deactivated to H_2O by catalase or SOD works in parallel with selenium-dependent glutathione peroxidase, which plays an important role in the reduction of H_2O_2 in the presence of reduced glutathione forming oxidized glutathione, and it protects cell protein and cell membranes against oxidative stress[26].

In this study the SOD and CAT activity significantly decreased ($P<0.001$) in diabetic control group of rats, which may be due to inactivation caused by radicals. However, administration of *Nyctanthes arbortristis* leaves and flower chloroform extracts (50, 100 and 200) mg/kg) and glibenclamide (10 mg/kg) could reverse progress of the disease. The above observations may clearly suggest that increased levels of SOD and CAT of *Nyctanthes arbortristis* extracts has free radical scavenging activity, which may exert a beneficial effect against pathological alterations caused by reactive oxygen species.

The elevation of biomarker enzymes such as SGOT, SGPT, and Alk Phos was observed in diabetic control rats and indicates the hepatic damage[27]. The hepatic damage was restored hepatocytes and the elevated transaminase was significantly reduced by *Nyctanthes arbortristis* extract. The diabetic complications such as increased gluconeogenesis and ketogenesis may be due to elevated transaminase activity[28]. From this point of view *Nyctanthes arbortristis* leaves and flower chloroform extracts (50, 100 and 200 mg/kg) may act as a hepatoprotective agent[29]. Diabetic rats showed an important lipolytic activity, due to the insulinopenic state which contributes to maintain the abnormally elevated serum cholesterol and triglyceride levels on STZ diabetic rats[30].

The results demonstrated that the *Nyctanthes arbortristis* leaves and flower chloroform extracts (50, 100 and 200 mg/kg) exhibited a potent hypocholesterolemic effect; the possible underlying mechanism is not elucidated at this stage of the study. The previous studies have reported that administration of *Momordica charantia* lead to decrease in cholesterol levels probably by two mechanisms a) by decreasing absorption of cholesterol from intestine by binding with bile acids within intestine and increasing the extraction of faecales bile acids b) by

biosynthesis of cholesterol especially by decreasing the activity of 3-hydroxyl-3-methly-glutaryl coenzymes A reductase (HMG CoA reductase) an enzyme of cholesterol biosynthesis[30]. Same mechanism may be appropriate to explain the observed cholesterol and triglycerides lowering activity by *Nyctanthes arbortristis* extracts. Its anti-leishmanial activity has been attributed to its constituent iridoid glucoside, arbortristosides A, B and C and 6β-hydroxyloganin. Given some of the medicinal properties of the plant might be attributed to its free radical scavenging ability of *Nyctanthes arbortristis* extracts.

CONCLUSION

It was concluded from this study that *Nyctanthes arbortristis* extracts has free radical scavenging activity and improved antioxidant effect was observed. The precise mechanism(s) and site(s) of action as well as constituents of *Nyctanthes arbortristis* will be further determined, including their toxicological effects.

Table 1: Effect of Nyctanthes arbortristis leaves and flowers on LPO, SOD, CAT, levels in STZ-induced diabetic rats liver

Treatment	TBARS (nmol/ mg protein)	SOD (Unit/ mg protein)	Catalase (ng/ mg protein)
Control	19.73 ± 2.05***	9.81 ± 0.24***	149.4 ± 14.54***
Gliben 10 mg/kg	9.12 ± 2.23**	14.52 ± 0.70***	314.5 ± 24.10***
Normal	7.48 ± 1.04	18.42 ± 1.59	321.1 ± 22.56
NLCH 50 mg/kg	12.71 ± 0.95*	12.88 ± 0.7140**	281.6 ± 30.95**
NLCH 100 mg/kg	12.85 ± 1.33*	13.81 ± 1.09**	284.4 ± 34.62**
NLCH 200 mg/kg	12.19 ± 1.00**	13.35 ± 0.68***	266.6 ± 24.11**
NFCH 50 mg/kg	12.01 ± 1.07**	13.51 ± 0.61***	254.5 ± 22.55**
NFCH 100 mg/kg	12.07 ± 1.20**	14.85 ± 0.79***	286.2 ± 33.60**
NFCH 200 mg/kg	11.85 ± 1.29**	14.17 ± 0.43***	295.2 ± 27.41***

Control: STZ (Tween-80); Gliben: Glibenclamide; NLCH: Nyctanthes arbortristis leaves chloroform; NFCH: Nyctanthes arbortristis flower chloroform extract.

Values are mean ± SEM, n=6 in each group *P <0.05), **P <0.01 ***P <0.001, when (Unpaired *t* test) compared to control.

Table 2: Effect of Nyctanthes arbortristis leaves and flower on serum SGPT, SGOT, Alk Phos, Cholesterol, Triglyceride levels in STZ-induced diabetic rats

Treatment	SGPT (U/dl)	SGOT (U/dl)	Alk Phos (U/dl)	Cholesterol (U/dl)	Triglyceride (U/dl)
Control	256.8 ± 21.29***	318.0 ± 26.7***	238.5 ± 9.00***	114.5 ± 4.25**	168.0 ± 9.43**
Gliben 10 mg/kg	68.57 ± 6.52***	119.8 ± 6.14***	186.9 ± 10.85**	94.71 ± 2.91**	122.2 ± 9.27**
Normal	60.9 ± 2.32	64.68 ± 4.67	127.4 ± 2.28	84.36 ± 3.86	115.1 ± 6.82
NLCH 50 mg/kg	81.67 ± 6.31***	154.5 ± 13.91***	226.8 ± 14.89ns	98.52 ± 2.99*	130.6 ± 4.41**
NLCH 100 mg/kg	65.87 ± 3.10***	172.5 ± 13.21***	181.6 ± 8.99**	94.47 ± 4.39**	131.3 ± 1.94**
NLCH 200 mg/kg	68.29 ± 4.34 ***	184.5 ± 18.26**	184.1 ± 8.81**	97.59 ± 1.17**	117.7 ± 5.94**
NFCH 50 mg/kg	75.96 ± 6.83***	163.8 ± 10.47***	155.8 ± 13.38***	97.89 ± 4.29*	123.9 ± 4.37**
NFCH 100 mg/kg	65.76 ± 4.44***	184.8 ± 13.70**	176.2 ± 10.77**	98.56 ± 2.81*	124.2 ± 7.92**
NFCH 200 mg/kg	57.42 ± 3.62***	191.1 ± 16.01**	184.9 ± 11.17**	91.34 ± 4.42**	131.7 ± 2.36**

CHO: Cholesterol; TRI: Triglyceride; Control: STZ (Tween-80); Gliben: Glibenclamide; NLCH:Nyctanthes arbortristis leaves chloroform; NFCH:Nyctanthes arbortristis flower chloroform extracts.

Values are mean ± SEM, n=6 in each group *P <0.05), **P <0.01 ***P <0.001, when (Unpaired *t* test) compared to control.

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Quantitative Determination of Fexofenadine in Human Plasma by UPLC

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Abstract

A selective, rapid and sensitive reverse phase ultra-performance liquid chromatography method was developed for the quantitative determination of fexofenadine in human plasma. With carbamazepine as internal standard, sample pretreatment involved a one-step extraction with ethyl acetate from 980µl plasma. The sample was analyzed using 10mM KH_2PO_4 buffer pH 2.5 and acetonitrile (70:30 v/v) as mobile phase. Chromatographic separation was achieved on an ACQUITY UPLC™ BEH (C-18) column (1.7 µm, 2.1mm x 100mm) using isocratic elution (at a flow rate of 0.25 ml/min). The peak was detected using UV-PDA detector set at 210 nm and the total time for a chromatographic separation was 10 min. Linear calibration curves were obtained in the concentration range of 30.09-1805.39 ng/ml with a lower limit of quantification of 30.09 ng/ml. The inter- and intra- day precision (RSD) values were below 15% and accuracy (RE) was from 1.55 to 5.51 % at all QC levels. The mean recoveries for fexofenadine at high, middle and low quality control samples was obtained 74.3%, 73.2% and 64.8% respectively and for internal standard was 82.8%. Developed method was found to be accurate, precise, selective and rapid for estimation of fexofenadine in plasma and can be used for pharmacokinetic and bioequivalence studies.

Keywords: Fexofenadine, UPLC, Human plasma, Liquid-liquid extraction.

INTRODUCTION

Fexofenadine [2-{4-{1-hydroxy-4-{4-(hydroxy-diphenylmethyl)-1-piperidyl}-butyl}phenyl}-2-methyl-propanoic acid] is a active metabolite of terfenadine, is a selective histamine H_1 receptor antagonist and is clinically effective in the treatment of seasonal allergic rhinitis and chronic idiopathic urticaria as a first-line therapeutic agent, such as loratadine and cetirizine. In clinical trials fexofenadine did not prolong the QT interval or decrease the heart rate, unlike terfenadine, astemizole and ebastatine.[1,2]

The literature survey revealed that some HPLC methods are available for determination of fexofenadine in human plasma involving solid phase extraction, precipitation or liquid-liquid extraction[3-8]. HPLC-MS/MS methods describe the quantitative analysis of fexofenadine in human plasma using 96-solid phase extraction[9]. However, the reported methods are not ideal for large number of sample determination as either methods are expensive[4, 6, 9] involving sophesticated instrument like LC-MS and solid phase extraction or time consuming[3, 8] as requiring derivatization step, arduous sample preparation and long chromatographic run time of 14min and 60min.

Compared to HPLC, UPLC is recently developed technology and provides a higher peak capacity, greater resolution, increased sensitivity and high speed of analysis[10, 11].

In this work, a new, rapid, simple, precise, economical, sensitive and accurate isocratic reverse phase UPLC method was developed for determination of fexofenadine in human plasma using liquid-liquid extraction for sample preparation.

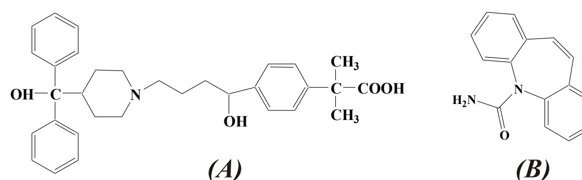

Fig.1: Chemical Structures of (A) Fexofenadine (B) Carbamazepine (I.S.)

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EXPERIMENTAL

Chemicals and reagents:

Fexofenadine hydrochloride (99.6% of purity) and carbamazepine (Internal standard, 99.3% of purity) drug substances were obtained as gift samples from Ind-swift Laboratories Ltd (Patiala, India). The structures of fexofenadine and carbamazepine (I.S.) are given in Fig.1. Acetonitrile, methanol and ethyl acetate (HPLC grade) were purchased from J. T. Baker Ltd. U.S.A. Potassium dihydrogen ortho phosphate was purchased from Merck Chemical Division Ltd., Mumbai. All other chemicals were of analytical grade. Water was purified by re-distillation and filtered through 0.22µm membrane filter before use.

Chromatography:

For chromatographic analysis, Waters-Acquity UPLC equipped with binary solvent manager, column manager, autosampler, multiple wavelength UV-PDA detector with chromatography data empower 2 software was used. The separation of the compounds was made on an ACQUITY UPLC™ BEH (C-18) column (100mm x 2.1mm i.d.,1.7 µm) with an eCord technology which tracks column usage such as date of installation, number of injection, number of sample sets, maximum temperature and pressure. A mixture of acetonitrile: 10mM KH_2PO_4 buffer pH 2.5 in the ratio of 30:70% v/v was used as mobile phase and was filtered through 0.45 µ membrane filters before use and degassed in an ultrasonic bath. All analysis was performed under isocratic condition at a flow rate of 0.25ml/min and the sample volume injected was 10µl. Detection was carried out at 210 nm at 25 ±1°C.

Preparation of standards and quality control samples:

Standard stock solution of fexofenadine and carbamazepine were prepared in a mixture of methanol and water (50:50% v/v) at concentration of 1000 µg/ml and 100 µg/ml respectively. The internal standard solution was further diluted to prepare the working solution containing 7 µg/ml of carbamazepine. The fexofenadine stock solution was then serially diluted with methanol and water (50:50% v/v) to provide working standard solution of desired concentration. Calibration standards were prepared by spiking 980µl of blank human plasma with working standard solutions of fexofenadine. The effective concentration in standard plasma samples were 30, 60, 90, 270, 902, 1263, 1534 and 1805 ng/ml. A calibration curve was constructed on each analysis day using freshly prepared calibration standards. The quality control sample (QCs) was prepared with blank plasma at low, middle and high concentration of 90.27, 902.70 and 1534.18 ng/ml. The standards and quality controls were extracted on each analysis day with the same procedure for plasma samples as described below.

Plasma sample preparation:

Extraction of drug and internal standard from plasma was done by employing optimized liquid-liquid extraction procedure. To 980µl of plasma in a hook tube, 20µl of methanol: water (1:1)/Std. drug solution in methanol: water (1:1), 20µl an aqueous internal standard solution carbamazepine (7µg/ml) and 30µl of 1M sodium hydroxide solution were added. 5.0 ml of ethyl acetate was added to each hook tube and vortexed in multipulse vortexer for total 8 minutes (4 min-with pulse+4 min-without pulse) and then centrifuged at 3500 rpm for 5 minutes at 10C. The organic layer was separated and evaporated to dryness at 40°C under the stream of nitrogen gas for 15 min. Separated residue was reconstituted with 100 µl of methanol: water (50:50v/v) and vortexed for 30 seconds. The contents were transferred into micro centrifuge tube (pre-labeled). Then re-centrifuged the content at 12000 rpm for 5 minutes at 5C. Finally the content was transferred into appropriate pre-labeled vials. 10 µl of the test and standard solutions were injected separately and chromatogram was recorded at 210 nm on the basis of spectral characteristics. This wavelength showed no interference to the selected solvent system and provides highly consistent results.

Method validation:

Validation runs were conducted on three consecutive days. Each validation run consisted of a minimum of one set of calibration standards and six sets of QC plasma samples at three concentrations.

Selectivity

Selectivity was studied by comparing chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with fexofenadine and carbamazepine.

Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by assaying standard

plasma samples at eight concentrations of fexofenadine ranging 30.09-1805.39 ng/ml. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of fexofenadine to carbamazepine (I.S.) versus the nominal concentration (x) of fexofenadine. The calibration curves were constructed by weighted ($1/x$) least square linear regression. The limit of detection was defined, as analyte responses are at least five times the response compared to blank responses. The lowest standard on the calibration curve was defined as the limit of quantification as an analyte peak was identifiable, discrete and reproducible with a precision of less than or equal to 20% and accuracy of 80–120%.

Precision and accuracy

The method precision and accuracy were evaluated by using replicate analysis of quality control samples at three concentrations. The intra-day precision and accuracy were determined by analysis of five QC samples on the same day. The inter-day precision and accuracy were assayed by analyzing three batches of QC samples on three consecutive days. The precision was expressed as the relative standard deviation (RSD %) and the accuracy as the relative error (RE %).

Extraction recovery and matrix effect

The extraction recovery of fexofenadine was determined by calculating the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. According to the guidance of USFDA[12], recovery experiments were repeated for five replicates at three concentrations (low, medium and high) of 90.27, 902.70 and 1534.18 ng/ml.

To evaluate the matrix effect, three concentration levels of fexofenadine (90.27, 902.70, 1534.18 ng/ml) were added to the dried extracts of 980µl of blank plasma sample from five different lots, then dried and reconstituted with 100µl mobile phase. The corresponding peak areas (A) were compared with those of the fexofenadine standard solutions dried directly and reconstituted with the same volume of mobile phase (B). The ratio ($A/B\times100\%$) was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

Stability

The bench top stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for approximately 27 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of fexofenadine was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 24 h at 10±1C. For dry extract stability, six aliquots of each low and high concentration QC samples were stored at −30±5C for 24 days. Stability of fexofenadine in human plasma was tested after storage at approximately −70C for 60 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of fexofenadine after each storage period was related to the initial concentration as determined for the samples.

RESULTS AND DISCUSSION

Method development:

The separation of fexofenadine and carbamazepine were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. In experiment, different ratio (50:50, 40:60, 35:65 and30:70) of acetonitrile: 10mM KH_2PO_4 buffer pH 2.5 was used as mobile phase, 30% acetonitrile and 70% 10mM KH_2PO_4 buffer pH 2.5 (v/v) in mobile phase was considered suitable in view of satisfactory retention time and peak shapes in the chromatogram.

A very narrow chromatographic peak with a peak width about 5 s, produced by UPLC™ indicated an increase in the chromatographic efficiency which produced fast separation. Both fexofenadine and carbamazepine were rapidly eluted with retention times less than 10 min (Fig. 2). The analysis time for fexofenadine in the literatures[3-9] by HPLC–MS and HPLC–MS/MS were in the range from 13-15 min. The short analysis time may meet the requirement for high sample throughput in bioanalysis. The method is highly economic in terms of solvent consumption as flow rate is 0.25 ml/min.

Liquid–liquid extraction was chosen as the sample preparation method, as it could produce purified as well as concentrated samples. Several extraction solvents such as *tert*-butyl methyl ether, chloroform, diethyl ether, ethyl acetate and the mixed solvent of *n*-hexane-isopropanol (60:40 v/v) were investigated. It was found that ethyl

acetate extracted the analyte more efficiently. Moreover, the I.S. carbamazepine solution which was dissolved in ethyl acetate was used as extraction liquid. This extraction method is more convenient than the reported method[3-9].

Fig. 2 shows the representative chromatograms of (A) blank plasma, (B) mobile phase, (C) plasma spiked with fexofenadine at 90.27 ng/ml (Low QC), (D) plasma spiked with fexofenadine at 902.70 ng/ml (Mid. QC), (E) plasma spiked with fexofenadine at 1534.18 ng/ml (High QC). The analytes were well separated from co-extracted material under the present chromatographic conditions at retention time of 9.58± 0.3 min and 4.69± 0.3 min for fexofenadine and carbamazepine respectively. The peaks were symmetrical and completely resolved one from another. The chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte or I.S.

Method validation:

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig.2, no interference from endogenous substance was observed at the retention time of fexofenadine and carbamazepine.

Calibration curves were linear over the concentrations range from 30.09-1805.39 ng/ml for fexofenadine (r = 0.999 or better). The best-fit calibration curve were achieved with linear equation $y = mx + c$ with $1/x2$ weighting factor. The mean linear equation of calibration curve were achieved with linear equation $y = mx + c$ with $1/x2$ weighting factor.

Data for intra- and inter-day precision and accuracy of the method for fexofenadine are given in Table 1. The intra- and inter day RSDs were less than 8.5% and 13% and RE was from 1.55% to 5.51% at all QC levels, indicating acceptable precision and accuracy of the present method. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of USFDA[12] where the precision (RSD) determined at each concentration level is required not exceeding 15%.

The extraction recoveries of fexofenadine from human plasma were 64.8 %, 73.2 % and 74.3 % at concentrations of 90.27, 902.70 and 1534.18 ng/ml respectively. The mean extraction recovery of I.S. was found to be 82.8 % as mentioned in Table 2.

Fexofenadine was found to be stable through all validation parameters, % changes of quality control (low & high) samples were found below 15%. The results of stability were shown in Table 3.

CONCLUSION

The developed method was checked for its applicability and validity by analyzing laboratory samples of spiked plasma containing fexofenadine having known concentration and was found to be suitably applicable and validated. UPLC is the most advanced technology introduced in LC to work more efficiently with higher speed, sensitivity and resolution. With HPLC retention time of fexofenadine was found to be 15 min with poor resolution but with UPLC, it was found to be 10 min with sharp resolution, Hence the present method is adaptable for routine analysis on account of its cost effectiveness, rapidity, simplicity and sensitivity.

Table1: Precision and accuracy for the determination of fexofenadine in human plasma (Intra-day: n = 5; inter-day: n = 5 series per day, 3 days)

Added Concentration (ng/ml)	Found Concentration (ng/ml)	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy RE (%)
30.09	30.629±0.373	1.2	1.3	1.79
90.27	93.84±7.979	8.5	13.0	3.95
902.70	952.44±20.79	2.18	3.5	5.51
1534.18	1558±45.31	2.9	7.5	1.55

Table 2: Extraction recovery data of fexofenadine and carbamazepine (n=5)

Drug	Nominal Conc. (ng/ml)	(%) Mean recovery + SD	(%) CV
Fexofenadine	90.27	64.8 + 2.1	3.2
	902.70	73.2 + 2.5	3.4
	1534.18	74.3 + 1.8	2.4
Carbamazepine	400	82.8 + 2.3	2.7

Table 3: Stability of fexofenadine in human plasma at three QC levels (n=5)

Stability	% Change	
	LQC	HQC
Bench Top Stability	4.07	-0.03
Freeze Thaw Stability	3.49	0.07
Dry Extract Stability	0.99	-0.99

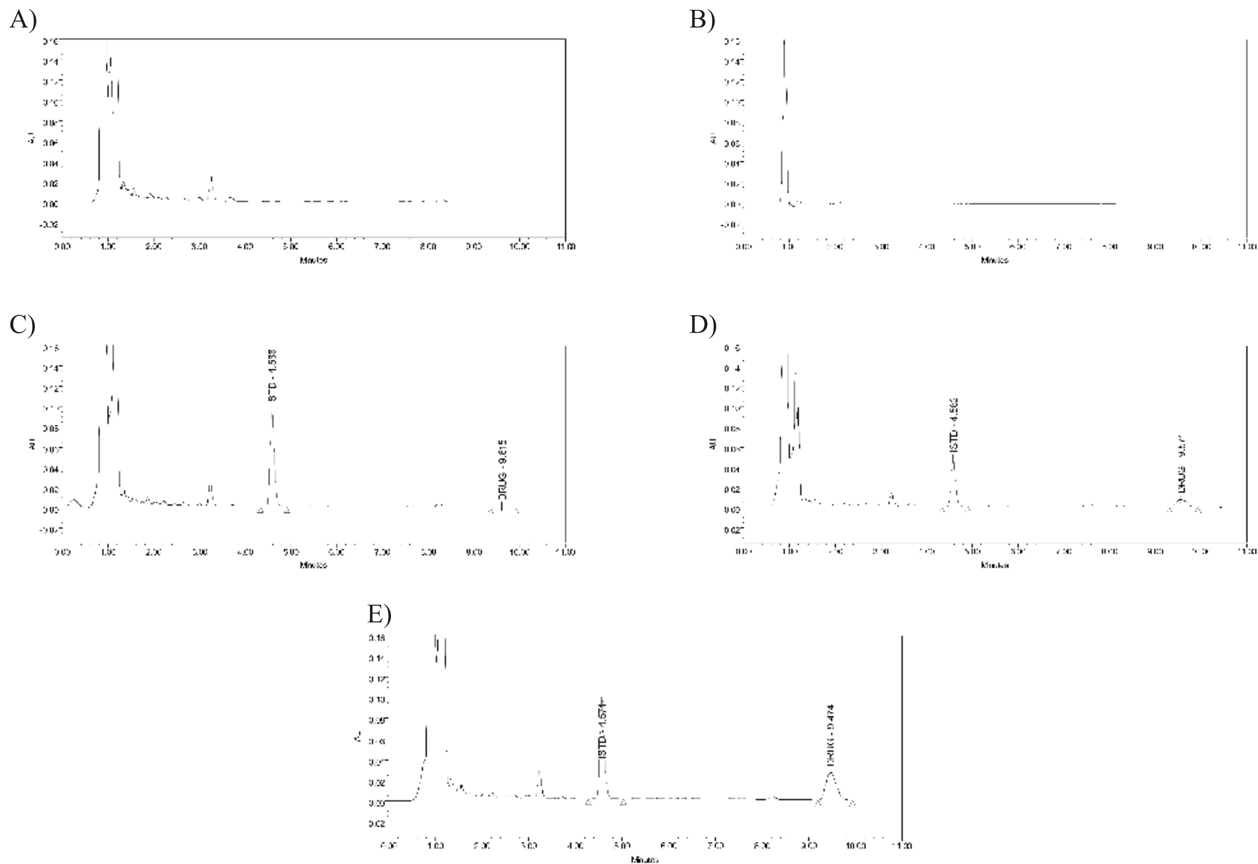

Fig. 2: Chromatograph resulting from
(A) analysis of blank human plasma, (B) mobile phase
(C) human plasma spiked with fexofenadine at 90.27 ng/ml (Low QC)
(D) human plasma spiked with fexofenadine at 902.70 ng/ml (Mid. QC),
(E) plasma spiked with fexofenadine at 1534.18 ng/ml (High QC).

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APTI ijper

Pharmaceutical Impurities: An Overview

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Abstract
The impurities in pharmaceuticals are unwanted chemicals that remain with the active pharmaceutical ingredients (APIs) or develop during formulation or upon aging of both API and formulation. The presence of these unwanted chemicals even in trace amount may influence the efficacy and safety of pharmaceutical product. The control of impurities is currently a critical issue to the pharmaceutical industry. International Conference on Harmonization (ICH) formulated guidelines regarding the control of impurities. This review outlines the description of different types and origins of impurities and degradation routes with specific examples.
***Keywords**: Impurities, formulation, efficacy, degradation.*

INTRODUCTION

In the present era, there is a tremendous upsurge for the impurity profiling of pharmaceutical products. Presence of impurities in trace quantity in drug substance or drug product is inevitable. Therefore, their level should be controlled and monitored. They can reinforce or diminish the pharmacological efficacy of the Active Pharmaceutical Ingredient (API). Sometimes, the effect produced by impurities can be teratogenic, mutagenic or carcinogenic. This can jeopardize the human health by affecting quality, safety and efficacy (QSE) of the product. Therefore, there is an ever-increasing interest in controlling and monitoring impurities present in API / pharmaceutical products. Hence, API impurity profiling (identification, isolation and characterization) is required. Their limits and threshold values should comply with the limits set and specified by official bodies and legislation (Pharmacopoeias and International Conference on Harmonization (ICH) guidelines). This is very important when company files Investigational New Drug Application (IND) or Abbreviated New Drug Application (ANDA). However, monitoring and controlling of impurity is different for different people. Therefore, there must be unified system to ensure that every one speaks the same language when addressing *"Issues related to impurities"*[1].

ICH has published guidelines for validation of methods for analysis of impurities in new drug substances[2a], new drug products[2b], residual solvents[2c] and microbiological impurities[2d, 2e] for registration of pharmaceuticals for human use. ICH defines impurities as *"substances in the API that are not the API itself"*. For pharmaceutical products, impurities are defined as "*substances in the product that are not the API itself or the excipients used to manufacture it"* i.e. impurities are unwanted chemicals that remain within the formulation or API in small amounts which can influence QSE, thereby causing serious health hazards. According to ICH guidelines on impurities in new drug substances and new drug products, identification of impurities below the 0.1% level is not necessary unless the potential impurities expected to be unusually potent or toxic. In all cases, impurities should be qualified. If data related to qualification of the proposed specification level of an impurity is not available then studies were required to obtain such data. According to ICH, the maximum daily dose qualification threshold is as follows[2a],

$\leq$2g / day 0.1% or 1mg/ day intake and $\geq$2g/day 0.05%

As impurity profile received a critical attention from regulatory authorities, different Pharmacopoeias such as British Pharmacopoeia (BP), United States of Pharmacopoeia (USP), European Pharmacopoeia (EP) and Indian Pharmacopoeia (IP) are slowly incorporating limits to allowable levels of impurities present in new drug substances or APIs and formulations[3]. Moreover, a number of articles have stated guidelines and designed approach for isolation and identification of process related impurities and degradation products using Mass spectroscopy (MS), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography

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(HPLC), FT-Ion Cyclotron Resonance MS (FT-ICR-MS) and Tandem MS for pharmaceutical substances. Impurity profiling is a major concern in drug developing and processing[4,5].

CLASSIFICATION OF IMPURITIES[1]:

Impurities have been described sometimes commonly or as specified by ICH guidelines,

1. Common terminology:

Intermediates - The compounds produced during synthesis of the desired material or as part of the route of synthesis.

Penultimate intermediate – It is the last compound in the synthesis chain prior to the production of the final desired compound.

Byproduct – The compounds produced in the reaction other than the required intermediates.

Transformation product – They are theorized or non-theorized products, which produced in the reaction.

Interaction products – These products formed by the interaction of chemicals in reaction either intentionally or unintentionally.

Related products – These are chemically similar to drug substance and may even possess biological activity.

Degradation products – Compounds produced due to degradation by the effect of external factors like light, heat and moisture.

2. Official compendial terminology: According to USP impurities are discussed as;

- Impurities in official articles described as foreign substances, toxic impurities and concomitant components.
- Ordinary impurities
- Organic volatile impurities (OVI) or residual solvents

ICH terminology:

As per the ICH guidelines impurities in the new drug substances and formulations broadly classified as,

1. Organic impurities
2. Inorganic impurities
3. Residual solvents

SOURCES OF IMPURITIES[6,7]:

A detailed elaboration of various sources of impurities given here under:

Pharmaceutical formulations (medicines) or bulk pharmaceutical chemicals (BPC) used in the manufacture of APIs and formulations. Hence the two major broad sources of impurities are:

I. Synthesis related impurities.

II. Formulation related impurities.

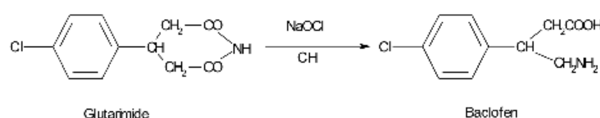
I. SYNTHESIS RELATED IMPURITIES:

Impurities in pharmaceutical compounds or new chemical entities (NCE) arise mainly during synthetic process from raw materials, solvents, intermediates and by products. Hence, the impurities during synthetic process categorized as;

1. Organic impurities – These impurities mainly arise during synthetic process or storage of drug substance. The impurities classified as starting materials, by products, degradation products, reagents and chiral impurities.

- **a) Starting materials or Intermediates:** The impurities from the starting materials and intermediates or by products found in every drug substance if proper care not exercised to remove them in the end – product during multi step synthesis. Though products washed with solvents frequently, there is a chance for the presence of unreacted starting material in the final product. For example,

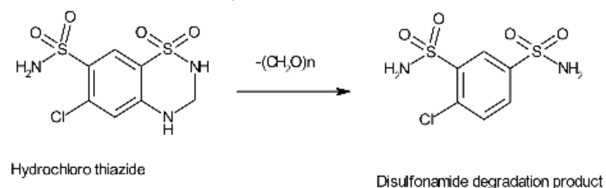
- In the synthesis of amlodipine besylate traces of 4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-2-[(2-phthalimidoethoxy)methyl]*p*-1-4-dihydroxy pyridine is the synthesis related impurity[8].
- In the synthesis of Baclofen, the last step carried out with β-(*p*-chlorophenyl) gutarimide, which on reaction with sodium hydroxide/ sodium hypochlorite solution at room temperature yields a potential impurity *p*-chloro phenyl glutaric acid, which has to be evaluated[9].



b) Degradation products:-During manufacturing of bulk drugs degradation of end products results in the formation of impurities. Degradation products arise from synthetic process, storage, formulation of dosage form and aging[10]. For example, penicillins and cephalosporins are classic examples for impurities from degradation products.

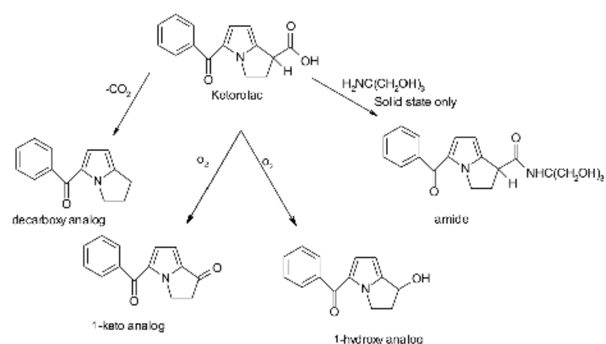
Hydrochlorothiazide has a known degradation pathway

through which it degrades to the starting material as disulfonamide in its synthesis[11].



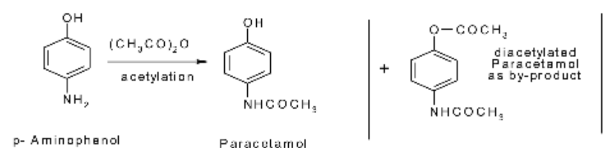
Another example, the rate of hydrolysis of mannitol containing methyl prednisolone sodium is significantly higher than lactose containing methyl prednisolone.

Degradation pathway of ketorolac in solid and solution state[11]



c) By – products: In synthetic organic chemistry, getting a single end – product with 100% yield is seldom. There is always a chance of having by-products. Because they can be formed through variety of side reactions, such as incomplete reaction, over reaction, isomerization, dimerization, rearrangement or unwanted reactions between starting materials or intermediate with chemical reagents or catalysts[12]. In the case of paracetamol bulk production, diacetylated paracetamol may forms as a by-product[3].

Production of paracetamol from intermediate *p* – Aminophenol



2. Inorganic impurities- Inorganic impurities derive from the manufacturing process and excipients. Generally, excipients contain high levels of heavy metals such as arsenic, bismuth, cadmium, chromium, copper, iron, lead, mercury, nickel and sodium. Sometimes they might present in the product during processing or they leached from packing material. For example, excipients such as hydrogenated oils and fats, which produced using metal catalysts, found to contain high concentrations of metals (platinum and palladium). This may be due to leaching from process equipment or storage container.

3. Residual solvents - Residual solvents are potentially undesirable substances. They either modify the properties of certain compounds or may be hazardous to human health. The residual solvents also affect physicochemical properties of the bulk drug substances such as crystallinity of bulk drug, which in turn may affect the dissolution properties, odor and color changes in finished products. As per the ICH guidelines, the solvents used in the manufacturing of drug substances classified in to four types[13, 14].

a) Class I solvents: Class I solvents and their permissible concentration limits given in the Table 1. These solvents not employed in the manufacture of drug substances, excipients and formulations because of their unacceptable toxicity or their deleterious effects. If use of these solvents is unavoidable, then their usage should be restricted.

b) Class II solvents: Class II solvents usage should be limited in pharmaceutical products because of their inherent toxicity. Table 2 lists class II solvents with their daily permissible exposure.

c) Class III Solvents: These are less toxic and possess lower risk to human health than class I or class II solvents[2d]. Long-term toxicity or carcinogenicity not reported, which is evident from the available data for the solvents under this category. The use of class III solvents in pharmaceuticals does not have any serious health hazard.

Some of the solvents are; *Acetic acid, anisole, butanol, 2-butanol, isopropyl acetate, methylacetate, butylacetate, ter-butyl methyl ether, pentene, cumene, Dimethyl sulfoxide, ethanol, ethylacetate, formicacid, heptane, isobutyl ketone, tetrahydrofuran, 1-pentanol, 2-propanol, methyl isobutyl ketone, propylacetate, 3-methyl-1-butanol, methyl ethylketone.*

d) Class IV Solvents: Class IV solvents, adequate toxicological data is not available. The manufacturers should justify the residual levels for these solvents in pharmaceutical products. The solvents under class IV are 1, 1-diethoxy propane, 1-1-dimethoxy propane, 2-2-

dimethoxy propane, methyl isopropyl ketone, isooctane, isopropyl ether, methyl tetrahydrofuran, petroleum ether, trichloro acetic acid.

II. FORMULATION RELATED IMPURITIES

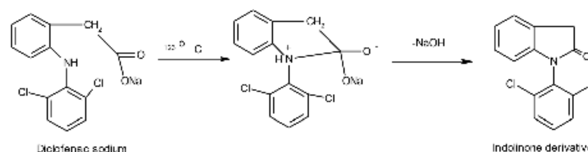
APIs formulated with excipients into solutions, tablets, capsules, semi-solids, aerosols and Novel Drug Delivery Systems. During formulation, excipients added to API to render the product elegant. They can be sometimes heterogeneous mixtures. In such a case, drug – excipient incompatibility may lead to undesirable products which can affect the therapeutic efficacy of the product. Any undesirable reaction produced due to the impurities associated with excipients can provide a ripe source for many potential reactions. The source of these potential reactions may be because of excess amount of water, which is usually present in API or excipients as residue due to use of hygroscopic materials. In addition, other solvents, which used in the synthesis of API or excipients, may also interact with excipients resulting in impurities.

a) Dosage form related impurities: - The impurities in the dosage forms like solutions can be significant. Precipitation of main ingredient can occur due to various factors like pH, environment or leaching. For example, precipitation of imipramine HCl with sodium bisulfite and pH alteration of lidocaine HCl solution in presence of 5% dextrose in saline or normal saline solution and lactated ringer solution have been reported[12]. Following are some of the examples of the excipients that affect the stability of pharmaceutical solutions as shown in Table 3. Although the pharmaceutical companies perform pre-formulation studies, including a stability study, before marketing the products, sometimes the dosage form factors that influence drug stability force the company to recall the product. Fluocinonide Topical solution USP, 0.05% in 60-mL bottles, recalled in the United States because of degradation/impurities leading to sub-potency[15].

Pharmaceutical solids: - In the presence of excipients and moisture, topochemical and nucleation reactions occur, some of these are first order reactions. For example, presence of sodium CMC during granulation of aminopyrine, papverine, theobromine and salicylic acid tablets caused reduced discoloration. Presence of lactose induces discoloration of several drugs in solid dosage forms. Moisture adsorption and tablet expansion occur more readily with α-lactose tablets due to formation of monohydrate.

b) Method related impurity: -

A known impurity, 1-(2, 6-dichlorophenyl) indolin-2-one is formed in the diclofenac sodium ampoules. Formation of this impurity depends on initial pH of the preparation and the condition of sterilization i.e., autoclave method (123±2°C) that enforce the intramolecular cyclic reaction of diclofenac sodium forming indolinone derivative and sodium hydroxide[16].



c) Environmental related impurity:-

1. Temperature: - During formulation of vitamins and antibiotics, especially extreme care should be exercised to prevent them from degradation. Because these classes of compounds are heat liable when subjected to extreme temperature, loss of potency takes place.

2. Light - UV light: - Light is one of the means by which the formulation degrades because of photolytic reaction. Exposure to light is known to be deleterious on a number of pharmaceutical compounds. For example, sunlight having about 8000 foot-candles can destruct nearly 34% of vitamin–B_{12} in 24hrs[17]. It is necessary to control the wavelength and intensity of light and number of photons actually absorbed by material. Photolytic degradation of fumagillin in ethanol was reported as a first order reaction that caused by light of wavelength below 400 nm. The lists of compounds that affected by light or catalyst are given in Table 4. Moreover, several studies have reported that ergometrine as well as ergometrine injection are unstable under tropical condition such as light and heat[18]. The custom-made injection of ergometrine (0.2mg/ml) showed almost complete degradation when kept 42hrs in direct sunlight.

3. Humidity: - Humidity is one of the important key factors incase of hygroscopic compounds. It is detrimental to both bulk powder and formulated solid dosage form. The classic examples are ranitidine and aspirin[3].

Impurities on Aging: -

a) Mutual interaction amongst ingredients: - Most often, vitamins are highly prone to instability on aging in different dosage forms. i.e., degradation of vitamins such as folic acid, thiamine and cyanocobalamines does not yield toxic impurities but lose their potency well below compendial specifications. Moreover, presence of nicotinamide in formulation containing four vitamins (nicotinamide, pyridoxine, riboflavin and thiamine) cause the degradation of thiamine to a substandard level within a one year shelf life of vitamin–B complex injection[19]. The custom-made formulation in a simple distilled water vehicle and in a typical formulated vehicle included with di-sodium edetate, benzyl alcohol also investigated and similar mutual interaction observed.

b) Hydrolysis: - A reaction in which water is the reactant causing precipitation. Well-known examples of such reactions in pharmaceutical compounds are esters and amides. Many drugs are derivatives of carboxylic acids or contain functional groups based on the moiety. For example esters, amides, lactones, lactams, imides and carbamates, which are susceptible to acid base hydrolysis, e.g., aspirin, atropine, chloramphenicol, barbiturates, chlordiazepoxide, oxazepam[12] and lincomycin.

c) Oxidation: - Drugs which prone to oxidation are hydrocortisone, methotrexate, adinazolam, catecholamine, conjugated-dienes (Vitamin–A), heterocyclic aromatic rings, nitroso and nitrite derivatives. In pharmaceuticals, the most common form of oxidative decomposition is auto oxidation through a free radical chain process. For example, auto-oxidation of ascorbic acid studies reveals that cupric ion known to oxidize ascorbic acid rapidly to dehydroascorbic acid and potassium cyanide. As a result, there is a cleavage of chain due to the formation of copper complexes. From the stability investigations on substituted 5-amino-ethyl-1, 3benzenediol sulfate (AEB) revealed that copper effectively catalyses AEB degradation down to 10 ppb level in presence of oxygen, leading to discoloration of product. The effectiveness of metals in terms of AEB degradation follows $Cu^{2+} > Fe^{3+} > Ca^{2+}$.

d) Photolysis: - Photolytic cleavage on aging includes examples of pharmaceutical drugs or products that are prone to degradation on exposure to UV-light. During manufacturing process as solid or solution, packaging or on storage, drugs like ergometrine, nifedipine, nitropruside, riboflavin and phenothiazines are liable to photo oxidation[20-22]. This oxidation involves generation of free radical intermediate, which will degrade the products. For example, the formulation of ciprofloxacin eye drop 0.3% on exposure to UV light induces photolysis thereby resulting in the formation of ethylene di-amine analogue of ciprofloxacin[23].

e) Decarboxylation: - Some of the carboxylic acids such as *p*-amino salicylic acid shown loss of carbon dioxide from carboxyl group when heated. For instance, photo reaction of rufloxacin tablet enteric coated with cellulose acetate phthalate (CAP) and sub-coating with calcium carbonate cause hydrolysis of CAP liberating acetic acid, which on reacting with calcium carbonate produced carbon dioxide, a by product that blew off the cap from the bottle after cap was loosened[24].

f) Packaging material: - Impurities result also from packaging materials i.e., containers and closures[25]. For most drugs the reactive species for impurities consists of;

Water – hydrolysis of active ingredient.

Small electrophiles – Aldehydes and carboxylic acid derivatives.

Peroxides – oxidize some drugs.

Metals – catalyze oxidation of drugs and their degradation pathway.

Extractable or leachables – Emerge from glass, rubber stoppers and plastic materials, in which oxides like NO_2, SiO_2, CaO, MgO are major components leached or extracted from glass.

Some examples of synthetic materials include styrene from polystyrene, diethylhexylpthalate (DEHP) plasticizer in PVC, dioctyltin iso octyl mercaptoacetate stabilizer for PVC, zinc stearate stabilizer in PVC and polypropylene.

Analytical methodology:

In new drug development, impurity profiling (characterization and isolation) plays a vital role. Regulatory bodies such as US FDA, EU mandates to estimate the impurity present above 0.1% level. ICH provided guidance document for evaluate and analytical validation of impurities. Thus, variety of analytical methodologies evolved to monitor impurities present in New Drug substances and new drug products. The

primary criteria of analytical methodology are to differentiate the compounds of interest and impurities. A wide variety of highly sophisticated equipments are available in characterizing the impurities such methods include spectroscopic methods, chromatographic methods and their combinations[26-29].

Remedies:

1. Critical factors for controlling impurities in API-

a) During crystallization, the manufacturer of API should take care to produce finer crystals to prevent entrapment of minute amounts of chemicals from mother liquor, which causes the degradation of drug.

b) Washing the wet cake or powder should be thorough to remove unwanted chemicals including residual solvents.

2. Packaging- Light sensitive pharmaceuticals have to pack in light protective packaging.

3. Production method selection is depending upon the stability studies. For diclofenac sodium injections, the aseptic filtration process has been recently recommended as the alternative to the autoclave method that produces impurity[16].

4. Pharmacopoeias should take measures to incorporate impurity limits for drug products made of raw materials. ICH should lay stringent regulations to incorporate limits for the impurities present in both drug substance and drug products. Diclofenac sodium is an example where an impurity limit is not mentioned in the case of injections.

CONCLUSION:

Identification of impurities is very important task during the synthesis of drug substances and manufacture of dosage forms. It can provide crucial data regarding the toxicity, safety, various limits of detection and limits of quantitation of several organic and inorganic impurities, usually accompany with APIs and finished products. ICH has outlined guidelines with regard to impurities but much more need to be required. There is strong requirement to have unified specifications/standards with regard to impurities.

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The authors are thankful to Chalapathi Educational Society, Guntur for providing the necessary facilities.

Table 1: Class I Residual Solvents

Residual solvent	Concentration limit (ppm)
Benzene	2 (Carcinogenic)
Carbon tetrachloride	4 (Toxic)
1,1 Dichloro ethene	8 (Toxic)
1,2 Dichloro ethene	5 (Toxic)
1,1,1 trichloro ethane	1500 (Environmental hazard)

Table 2: Class II Solvents with Their Permissible Daily Exposure Limits

Solvent	Permissible daily exposure (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,1-Dimethoxyethane	1.0	100
N,N-Dimehtyl acetamide	10.9	1090
N,N-Dimethyl formamide	8.8	880
1,2-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethylene glycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxy ethanol	0.5	50
Methyl butyl ketone	0.5	50
Methyl cyclo hexane	11.8	1180
N-methyl pyrrolidone	48.4	4840
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloro ethane	0.8	80
Xylenes	21.7	2170

Table 3 : - Effect of Pharmaceutical Aids on Stability of Active Ingredients

Active ingredient	Pharamaceutical aid	Effect
Kanamycin	Honey, sugar syrup	Loss of activity at room temperature (RT)
Cholecalciferol	2%polyoxy ethylene ester surfactant, polysorbate	Change in pH resulted, degradation of active ingredient.
Tetracyclines	Calcium or magnesium or metal ions	Complexation
Thiomersal	Bromine, chloride, iodide	Form different soluble halides of cationic mercury compounds.
Adrenaline	Boric acid, povidone	Stabilization
Tryptophan	Sodium pyrosulfite, oxygen	Discoloration, precipitation.

Table 4: Drugs Affected By Light or Catalyst

S.No	API / Drug	Light /catalyst
1	Epinephrine	Sodium metabisulfite
2	Penicillins	Sodium bisulfite
3	Nalidixate sodium	Light
4	Antipyrine	Light
5	Phenothiazine	Light
6	Dihydroergotamine mesylate	Light
7.	Ergometrine	Light
8.	Nifedipine	Light
9.	Ofloxacin	Light
10	Nitropruside	Light
11	Riboflavin	Light
12	Fluroquinolones	Light
13	Penicillin G potassium	Monohydrogen and dihydrogen citrate ions.

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