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VIEWS

Formulation, Characterization and Evaluation of Granisetron Buccal Patches

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ABSTRACT

The aim of this investigation was to develop and evaluate mucoadhesive buccal patches of Granisetron(GRN). Permeation of GRN was determined *in vitro* using porcine buccal membrane. Buccal patches were developed by solvent-casting technique using Hydroxypropylmethylcellulose (HPMC) as mucoadhesive polymer. The patches were evaluated for *in vitro* release, moisture absorption, mechanical properties and *ex vivo* permeation studies. The optimized formulation, based on *in vitro* release, *ex vivo* permeation studies and moisture absorption studies, was subjected for *in vitro* residence time using porcine buccal membrane. *In vitro* flux of GRN was calculated to be 3.19±0.27µg.hr^{-1} cm^{-2}. *In vitro* drug release and moisture absorbed was governed by HPMC content. Increasing concentration of HPMC delayed the drug release. All formulations followed Zero order release kinetics where as the release pattern was non-Fickian. The mechanical properties, tensile strength (9.58±3.45kgmm^{-2} for formulation F3) and elongation at break reveal that the formulations were found to be strong but not brittle. Formulations showed a significant permeation through porcine buccal membrane and convenient residence time. The results indicate that suitable bioadhesive buccal patches of GRN with desired permeability and suitable mechanical properties could be prepared using HPMC.

Keywords: Buccal patches, Granisetron, Mucoadhesive, Mechanical properties.

Introduction

Absorption of therapeutic agents from the oral mucosa overcomes premature drug degradation within the gastro intestinal tract, as well as active drug loss due to first pass metabolism that may be associated with other routes of administration. Buccal delivery of drugs became an attractive alternate to the oral route of drug administration by providing rich blood supply that drains drug directly into the jugular vein and chance to administer drugs to patients who cannot be dosed orally[1-3]. Various mucoadhesive formulations were suggested for buccal delivery that includes buccal patches[4], adhesive tablets[5] and adhesive gels[6]. Buccal patches overcome some of the drawbacks of other dosage forms. They have unique characteristics including flexibility, relatively rapid onset of drug delivery, sustained drug release and rapid decline in the serum drug concentration when the patch is removed. The patch is confined to the buccal area over which it is attached and therefore the absorption profile may have less inter and intra-individual variability [7,8].

Granisetron is a selective 5-HT$_3$ receptor antagonist used in treatment of chemotherapy-induced, radiation-induced and post-operative nausea and vomiting. The oral route of administration of GRN is also impractical for patients who are vomiting or who have impaired gastric emptying [9,10]. Both parenteral and oral formulations have also been used, but buccal route may obviate the need for repeated injections, repeated oral dosing and also be useful in patients who cannot tolerate oral dosage forms. It is less invasive than IV or subcutaneous administration. Physicochemical (low molecular weight 312.4g/mol, low dose 1-2mg , Log *P* 2.6) and pharmacokinetic ($t_{1/2}$ 4-6hr, absolute bioavailability about 60%) parameters made GRN to be suitable for buccal delivery[11-13].

In this investigation we developed GRN buccal patches with a dissolvable matrix using HPMC E 15, with an insoluble backing membrane. The developed patches were evaluated for *in vitro* release, *ex vivo* permeation through porcine buccal membrane and mechanical properties.

Materials and Methods

Materials

Granisetron was a gift from by Natco pharma, Hyderabad, A.P, India. Hydroxypropyl methylcellulose E

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15(HPMC E 15) was procured from Loba Chemie Pvt. Ltd., India. Phenol red was obtained from Hi Media Laboratories Pvt. Ltd. Mumbai, India. All reagents used were of analytical grade.

Tissue Preparation (Isolation)

Porcine buccal tissue was taken from local slaughter-house. It was collected within 10 minutes after slaughter of the pig and tissue was kept in Kreb's buffer solution. It was bought immediately to the laboratory and was mounted within 2 hours of isolation of buccal tissue. The tissue was rinsed thoroughly using phosphate buffer saline to remove any adherent material. The epithelium was separated from the underlying connective tissue using surgical procedure. Sufficient care was taken to prevent any damage to the buccal epithelium [14].

***In vitro* Drug Permeation Studies**

The buccal epithelium was carefully mounted in between the two compartments of a Franz diffusion cell with internal diameter of 1.1cm (4.15cm^2 area) with a receptor compartment volume of 24ml. Phosphate buffer solution (PBS) pH (7.4) was placed in receptor compartment. The donor compartment contained a solution of 5ml of phosphate buffer pH 6.6 in which 5 mg of GRN was dissolved. The donor compartment also contained phenol red at a concentration of 20µg/ml. This is because phenol red acts as a marker compound and is not supposed to permeate through the porcine buccal membrane. The entire set-up-was placed over magnetic stirrer and temperature was maintained at 37° C by placing the diffusion cell. The samples were collected at predetermined time intervals and stored under refrigerated conditions till the analysis was carried out using UV-Visible spectrophotometer (Elico, India) at 302nm. All the experiments were performed in triplicates.

Fabrication of GRN patches

Buccal patches were prepared using solvent casting technique with HPMC E 15 as polymer and propylene glycol as plasticizer[15].Polymer was added to 20ml of dichloromethane and methanol (1:1) solvent system and allowed to stand for 6hrs to swell. GRN and propylene glycol were dissolved in 5 ml of solvent system and added to the polymeric solution. This was set aside for 2hrs to remove entrapped air, transferred to a petriplate and dried at room temperature for overnight and then in vacuum oven for 8-12 hrs. The formed patches were removed carefully, cut to size and stored in a desiccator. The composition of the patches is shown in Table 1. Patches with any imperfections, entrapped air, differing in weight or GRN content were excluded from further studies.

***In vitro* Release Studies**

The drug release rate from buccal patches was studied using Franz diffusion cells. Patches were supposed to release the drug from one side only; therefore an impermeable backing membrane (aluminium foil) was placed on the other side of the patch. The patch was sandwiched in dialysis membrane (Hi Media molecular weight 5000) and, was further placed between receptor and donor compartments. The entire set up was placed over magnetic stirrer and temperature was maintained at 37°C by placing the diffusion cell. The contents of receptor compartment were stirred with teflon bead at a speed of 500 rpm. One ml sample was collected at predetermined time intervals from receptor compartment and replaced with an equal volume of the buffer. The samples were analyzed using UV-Visible spectrophotometer (Elico, India) at 302 nm. The experiment was performed in triplicates [15].

Table 1
Formulation ingredients of mucoadhesive buccal patches of Granisetron

Formulation code	GRN (mg)	Ingredients HPMCE 15 (mg)	Propylene glycol (µl)
F1	140	2000	300.0
F2	140	2250	337.5
F3	140	2500	375.0
F4	140	2750	412.0
F5	140	3000	450.0

Note: 25ml of solvent system, 1:1 ratio of dichloro methane and methanol was used.

***Ex vivo* Permeation Studies**

Ex vivo permeation [16,17] of GRN from buccal patches through porcine buccal membrane was studied. Porcine buccal mucosa was obtained and buccal membrane was isolated. The membrane was mounted over a Franz diffusion cell and a buccal patch was placed over the membrane. A dialysis membrane was placed over the membrane so as to secure the patch tightly from getting dislodged from the membrane (the buccal patch was sandwiched between the buccal mucosa and the dialysis membrane). The donor compartment of diffusion cell was filled with PBS pH 7.4. The setup was placed over a magnetic stirrer with temperature maintained at 37^{0}C. Samples were withdrawn and replenished immediately from the receiver compartment at predetermined time intervals. They were stored under refrigerated conditions till the analysis was carried out. The content of GRN in the samples was analyzed by UV-Visible Spectrophotometer at the wavelength of 302nm. All the experiments were performed in triplicates.

Moisture Uptake Studies

The moisture uptake studies give an indication about the relative moisture absorption capacities of polymers and

an idea whether the formulations maintain their integrity after absorption of moisture. The study was carried out as per procedure reported earlier [18] . Briefly, agar (5% w/ v) was dissolved in hot water, transferred into petriplates and allowed to solidify. Six patches from each formulation series were placed in vacuum oven overnight prior to the study to remove moisture if any and laminated on one side with water impermeable backing membrane. They were then incubated at 37°C for one hour over the agar surface. The initial and final weights were recorded and percentage moisture absorption was calculated by using the formula.

$$\%\text{Moisture absorption} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Measurement of Mechanical Properties

Mechanical properties of the patches were evaluated using a microprocessor based advanced force gauze equipped with a motorized test stand (Ultra Test, Mecmesin, West Sussex, UK), equipped with a 25 kg load cell. Film strip with the dimensions 60 x 10 mm and without any visual defects were cut and positioned between two clamps separated by a distance of 3 cm. Clamps were designed to secure the patch without crushing it during the test, the lower clamp was held stationary and the strips were pulled apart by the upper clamp moving at a rate of 2 mm/sec until the strip broke. The force and elongation of the film at the point when the strip broke was recorded. The tensile strength and elongation at break values were calculated using the formula [19].

$$\text{Tensile strength (kg. mm}^{-2}) = \frac{\text{Force at break (kg)}}{\text{Initial cross sectional area of the sample (mm}^2)} =$$

$$\text{Elongation at break (\%mm}^{-2}) = \frac{\text{Increase in length (mm)}}{\text{Original length (mm)}} \times \frac{100}{\text{Cross sectional area (mm}^2)}$$

Measurement of *in vitro* Residence Time

The *in vitro* residence time was determined using USP disintegration apparatus. The disintegration medium was 800 ml of PBS (pH 6.6) maintained at 37±2^{0}C. The segments of porcine buccal mucosa, each of 3 cm length, were glued to the surface of a glass slab, which was then vertically attached to the apparatus. Three mucoadhesive films of each formulation were hydrated on one surface using PBS (pH 6.6) and the hydrated surface was brought into contact with the mucosal membrane. The glass slab was vertically fixed to the apparatus and allowed to move up and down. The film was completely immersed in the buffer solution at the lowest point and was out at the highest point. The time required for complete erosion or detachment of the film from the mucosal surface was recorded [20].

Drug-Polymer Interaction Study

To study the possible interaction between Granisetron and Polymeric materials of the patches, infrared (IR) spectroscopy was carried out on pure substances and their physical mixture. The IR spectra were recorded using IR-Spectrophotometer (Perkin Elmer FT-IR, Perkin Elmer Inst.USA) by KBr pellet method.

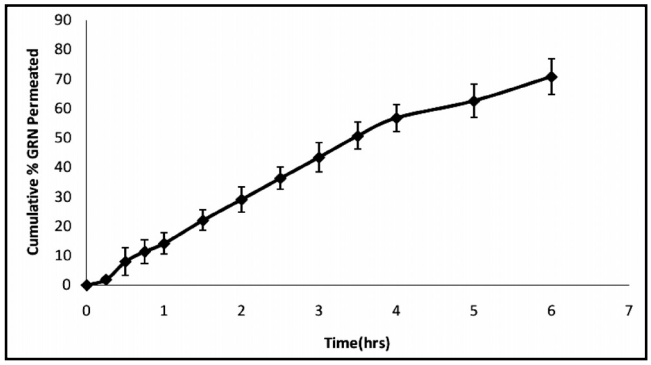
Results and Discussions

Drug Permeation Studies through the Porcine Buccal Membrane

Porcine buccal mucosa has been the most frequently chosen model for *in vitro* permeation studies because of its similarity to human tissue and is available in large quantities from slaughter houses. Cumulative amount of GRN permeated through the porcine buccal epithelium is shown in Figure.1. The isolated membrane was intact as no detectable level of phenol red, which was used as a non-absorbable marker compound, was found in the receiver compartment. The thickness of the isolated membrane, measured with a digital micrometer (Mitutoyo, Japan), ranged from 1040 to 1880 microns. Cumulative amount of GRN permeated in 6 hr was about 71.52 ± 4.12 % and flux was calculated to be 3.19±0.27µg. hr^{-1} cm^{-2}.

Figure 1

***Ex vivo* permeation of GRN (5.0 mg) through porcine buccal mucosa, values represented as Mean±S.D (n=3).**

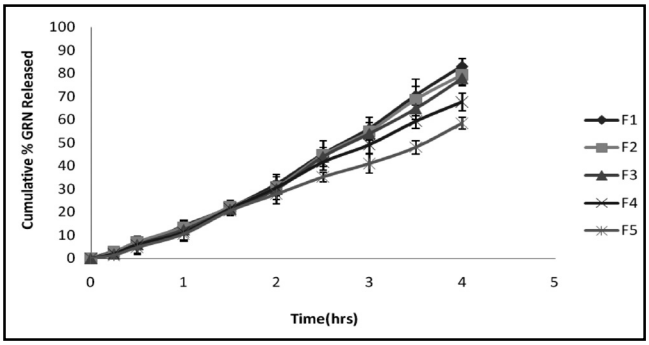


***In vitro* Drug Release Studies**

The drug release profiles of GRN patches were shown in Figure. 2. The drug release was governed by the amount of matrix forming polymer. An increase in polymer concentration causes an increase in the viscosity of the gel as well as formation of a gel layer with a longer diffusional path. This could cause a decrease in the effective diffusion coefficient of the drug and therefore a reduction in the drug release rate. Formulation F1 showed maximum drug release (83.08± 3.36%) where as formulation F5 showed lowest release of (58.43±2.67%), among the series. Data of the *in vitro* release was fit into different equations and

kinetic models to explain the release kinetics of GRN from buccal patches. Zero order model seemed to be the most appropriate model describing release kinetics from all patches (0.986, 0.987, 0.988,0.995, 0.976 for formulation F1 to formulation F5).On other hand 'ç' values indicted that amount of released drug was by non Fickian diffusion[21,22].Increasing the concentration of the polymer in the formulations showed a sustained effect on GRN release. This is because, as the proportion of these polymers in the matrix increased, there was an increase in the amount of water uptake and proportionally greater swelling leading to a thicker gel layer. Zero-order release from swellable hydrophilic matrices occurs as a result of constant diffusional pathlengths. When the thickness of the gelled layer and thus the diffusional pathlengths remain constant, zero-order release can be expected. In this investigation similar behavior was predicted and obtained.

Figure 2
Release profiles of GRN from mucoadhesive buccal patches, values represented as Mean ±S.D (n=3)



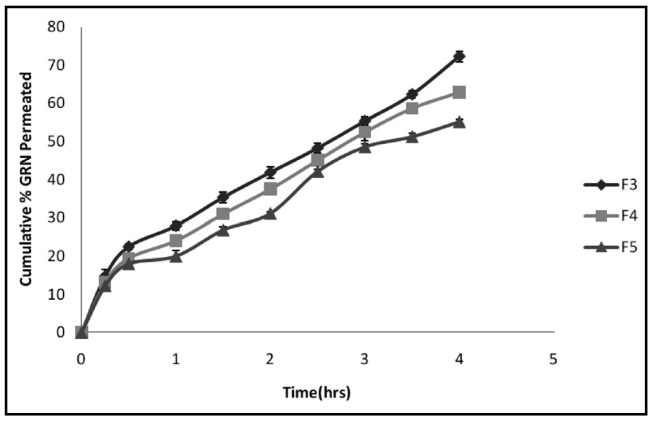
***Ex vivo* Permeation Studies**

Figure 3 shows the graphical representation of cumulative percentage drug permeated from the buccal patches.The results of drug permeation from buccal patches of Granisetron through the porcine buccal mucosa reveal that drug was released from the formulation and permeated through the porcine buccal membrane,hence could possibly permeate through the human buccal membrane. The results indicated that the drug permeation was more in F3 among the last three formulations and about 72.25% of Granisetron could permeate through the buccal membrane in 4 hrs.

Moisture Uptake Studies

Moisture absorption studies evaluate the integrity of the formulation upon exposure to moisture and the results were shown in Table 2. The percentage moisture observed ranged from about 59.6±11.99% to 156.34±9.27 % w/w for different formulations. Formulations F1 and F2 were deformed during the study. The results reveal that, percentage of moisture absorption was increased with increase in polymer content of formulations(Table 2). When the patches were placed without backing membrane complete swelling followed by erosion was observed

Figure 3
***Ex vivo* permeation studies of selected mucoadhesive buccal patches of GRN, values represented as Mean±S.D (n=3).**



indicating that the drug release mechanism involves swelling of the polymer initially, followed by drug release from the swollen matrix by diffusion.

Mechanical Properties of Patches

Ideal buccal film, apart from good bio adhesive strength, should be flexible, elastic and strong enough to withstand breakage due to stress caused during its residence in the mouth. The tensile strength (TS) and elongation at break (E/B) shows the strength and elasticity of the film. A soft and weak polymer is characterized by a low TS and E/B; a hard and brittle polymer is defined by a moderate TS, and low E/B; a soft and tough polymer is characterized by a moderate TS and a high E/B; whereas a hard and tough polymer is characterized by high TS and E/B. It is suggested that an ideal buccal film should have a relatively high TS and E/B. The results of the mechanical properties i.e. TS and E/B were presented in Table 2. TS increased with the increase in polymeric content but E/B values decreased with the increase in polymer content. Maximum TS was exhibited by F5 patch (13.24±2.74kg.mm^{-2}) and minimum was exhibited by F1(3.87±1.28 kg.mm^{-2}). Maximum E/B was seen with F1(117±8.24% mm^{-2}) and the least was observed with F5 (53.6±3.72% mm^{-2}).

***In vitro* residence time**

In vitro residence time was determined for the formulations F3, F4, F5.The formulations F1, F2 were eroded in moisture absorption study. So they were not suitable as buccal patches. The *in vitro* residence time of the formulations was in order of F4> F3> F5.

Drug - Polymer Interaction Study

The IR spectral analysis of Granisetron alone showed that the principal peaks were observed at wave numbers of 1648.15, 1559.88, 1228.95 and 3235.58. In the IR spectra of the physical mixture of Granisetron and HPMC E15 1647.90, 1560.15, 1242.12 and 3448.42 were observed for the Granisetron. However, some additional peaks were

Table 2
Moisture absorption, Mechanical properties of GRN buccal patches, values represented as mean±S.D (n=3).

FORMULATION	Moisture absorbed (%w/w)	TensileStrength ($Kg.mm^2$)	Elongation at break (mm^{-2})
F1	Deformed	3.87±1.28	117±8.24
F2	Deformed	8.74±1.74	94±7.58
F3	59.67±11.99	9.58±3.45	75±6.42
F4	63.72±1.94	11.58±2.42	70±5.24
F5	156.34±9.27	13.24±2.74	53.6±3.72

observed with physical mixtures, which could be due to the presence of polymer. These results suggest that there is no interaction between the drug and polymer used in the present study. It is already well known that the common polymers such as HPMC popular in controlled/sustained release matrix type formulations because of their compatibility with a number of drugs.

Selection of optimized formulation

Based on *in vitro* release and moisture absorption studies formulation F3 was selected as the best formulation. Formulation F1 showed maximum drug release 83.08 ± 3.36 %, where as formulation F2 showed 79.37±4.46% drug release. Formulations F1 and F2 were deformed during Moisture absorption studies, these formulations could not be expected to maintain the integrity after administration. The results indicated that the drug permeation was more in F3 among the last three formulations therefore; formulation F3 was selected as best formulation and subjected for further investigation.

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Assessment Of Neuropharmacological Activities Of *Pandanus Odoratissimus* Root In Mice

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ABSTRACT

The ethanolic extract of the roots of *Pandanus odoratissimus* (PO) pandanaceae was assessed for neuropharmacological activities in mice using a number of experimental models. The ethanolic extract was given at a dose of 100, 200, 400 mg/kg. Spontaneous motor activity, analgesia, grip strength, alertness, immobility, climbing and swimming in forced swimming (FST) and immobility time in tail suspension test (TST) were analyzed. The extract at given doses showed significant dose dependent activity by decreasing the exploratory activity, Spontaneous motor activity, increased immobility time in both FST and TST, decreased climbing and swimming behaviour in FST and did not alter other parameters. The preliminary phytochemical analysis showed the presence of saponins, sterols, glycosides, carbohydrates and flavonoids. The results of the present study indicated that the alcoholic extract may have active constituents with CNS depressant activity and at the given doses they are devoid of memory impairment and neurotoxicity.

Keywords: *Pandanus odoratissimus*, neuropharmacological, CNS depressant activity, neurotoxicity, memory impairment, phytochemical.

Introduction

Pandanus odoratissimus (Pandanaceae) is a perennial shrub (or) slender tree attaining a height of 6m. This plant is distributed in coastal districts of Orissa, Gujarat, Andhra Pradesh, Tamil Nadu and tropical Asia [1]. In Orissa, two morphologically distinct types of Pandanus are found in nature i.e. "Kewda" and "Ketaki" are golden yellow in colour and strongly scented while "Kewda" flowers are cream coloured and sweetly scented [1]. Flower is pungent, bitter and used in pruritus, earache, headache, leucoderma, strengthen the brain (yunani). The fruit is useful in "Vata", "Kapha" and urinary discharges (Ayurveda). Leaves are bright green, pungent, bitter with flavour, useful in leprosy, small pox, syphilis, scabies, pain, leucoderma, diseases of the heart, brain, aphrodisiac and tonic. Oil prepared from the aerial root is used as stimulant, antispasmodic, antirheumatic agent [1], diuretic, depurative and tonic [2]. The chief constituents of oil is methyl ether of phenyl ethyl alcohol, it also contains d-linalool, depentene, phenyl ethyl acetate, citral, caproic acid, stearoptene and pthalic acid. Roots of Pandanus odoratissimus contains 2 phenolic compounds which are reported to possess antioxidant activity [3]. Leaves has lignan and benzofuran [4].

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Materials and Methods

Collection and Extraction of PO root

Plant material (roots) was collected from the wild source in the month of October and identified by the Botanist at the Department of Botany, S.V.University, Tirupati, A.P. The roots were washed under running tap water, shade dried, and crushed to a coarse powder. The powder was passed through sieve no. 40 and used for further studies.

Dried coarse powder of PO root was extracted with petroleum ether and then with alcohol. Ash color powdery extract was obtained after evaporation of solvent the yield was 6.4% w/w. A suspension of the extract was prepared by using 2% v/v tween 80 in distilled water.

Animals

Male Swiss albino mice weighing 25-30g were used. They were housed in groups of five under standard laboratory conditions at temperature 23 ± 1^0C, relative humidity of 55±5%. The animals had access to water and pellet diet ad libitum (Hindustan Lever Foods, Bangalore, India). The animals were deprived of food 12h before experimentation. Control group animals received 2% v/v tween 80 orally and all behavioured parameters were assessed one hour after the oral administration of extract.

Neuropharmacological Tests

Test for locomotor activity

The locomotor activity was measured by using Actophotometer (Inco, Ambala, India). It consists of cage which is 30 x 30 x 30 cms and has a wire mesh at the bottom. Six lights and 6 photo cells placed in the outer periphery of the bottom in such a way that a single mice blocks only one beam. Photo cell is activated when the rays of light falls on photocells, the beam of light is cut as and when animals cross, number of cut off's were recorded for 10 minutes [5].

Hot Plate Test

The hot plate consisting of a electrically heated surface. with a temperature of 55° to 56° C. The animals were placed on the hot plate and the time for either licking or jumping was recorded by a stop-watch, before and after the oral administration of the test compound [6].

Forced Swimming Test (FST)

Mice were forced to swim individually in a glass jar (25 x 12 x 25 cm^3) containing fresh water of 15 cm height and maintained at 25°C (± 3°C). After an initial 2 min period of vigorous activity each animal assumed a typical immobile posture. A mouse was considered to be immobile when it remained floating in the water without struggling, making only minimum movements of its limbs necessary to keep its head above water. The total duration of immobility was recorded during the next 4 min of a total 6 min test. The changes in immobility duration were studied after administering drugs in separate groups of animals [9].

Test for Alertness: Hole Board Test

This test was done using Hole Board. The hole Board consisted of a 0.5m^3 wooden board with 16 holes (3cm in diameter). The mice was placed at the corner of the board and allowed to move freely. First two minutes were allowed for adaptation and the number of head dippings in next four minutes was counted [7].

Tail Suspension Test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by steru et al [12]. Mice were suspended on the edge of a table 50 cm above the floor by the adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility was recorded during the next 4 min of a total 6 min test [9].

Motor Co-ordination Test (Rota rod Test)

Motor Co-ordination test was conducted using a Rota rod apparatus (Inco Ambala, India). The animals were placed on the moving rod prior to the treatment and the mice stayed on the rod without falling for 120 seconds were chosen for the study. The fall of time of animals before and after the extract was noted [6,8].

Phytochemical Analysis

Preliminary phytochemical analysis was carried out according to standard protocol [10,11].

Statistical Analysis

All values are expressed as Mean ± SEM. The data was analyzed using one way ANOVA followed by Dunnet's 'T' tests, in all tests the criteria for statistical significance was $p<0.05$.

Results

Preliminary phytochemical analysis was carried out on the crude alcoholic extract the results indicated the presence of glycosides, sterols, saponins, flavonoids and carbohydrates (Table 1).

No lethal effect was observed in groups of mice during the 24 h period after oral administration at a dose upto 1600 mg/kg. The animals treated with 100, 200, 400 mg/kg po exhibited significant ($p<0.01$) and dose dependent decrease in spontaneous motor activity as compared to control vehicle group (Fig. 1).

Alcoholic extract at a dose of 100, 200, 400 mg/kg po showed no significant change in reaction time in comparison to control group (Fig. 2) in Eddys hot plate test.

Alcoholic extract at given doses decreased motor coordination significantly ($p<0.01$) and dose dependently (Fig. 3).

The results of forced swimming test showed that there was significant increase ($p<0.01$) in immobility and significant decrease ($p<0.01$) in swimming and climbing behaviour of animals at 100, 200, 400 mg/kg in comparison to control vehicle group (Fig. 4, 5, 6).

Table 1:
Preliminary Phytochemical tests of *Pandanus odoratissimus* root

Extract	Sterols	Glycosides	Saponins	Carbo hydrates	Alkaloids	Flavonoids	Tannins	Proteins
Ethyl Alcohol	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve

* +ve – Postive Test; * -ve - Negative Test

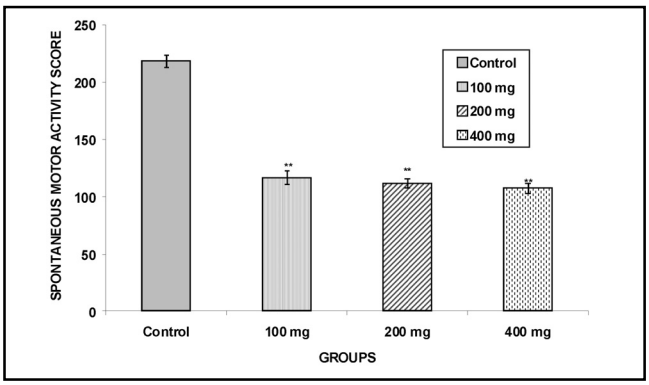

Fig.1: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on the Spontaneous Motor Activity

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

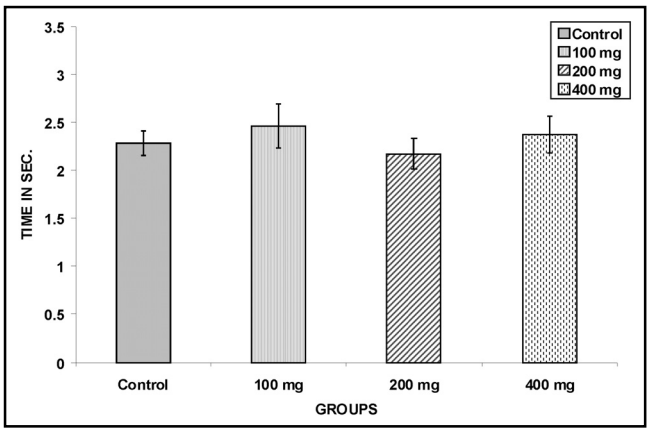

Fig.2: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Analgesia (Eddys Hot Plate Test)

Values are expressed as Mean ± SEM of 8 animals

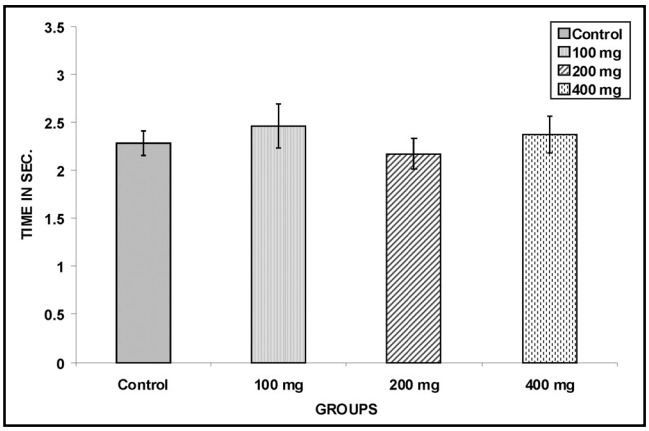

Fig.3: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Motor Co-ordination (Rota Rod Test)

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

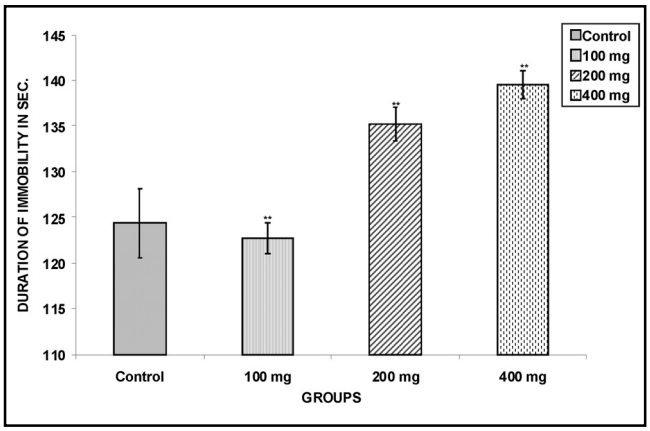

Fig.4: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Immobility Time in Forced Swimming Test

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

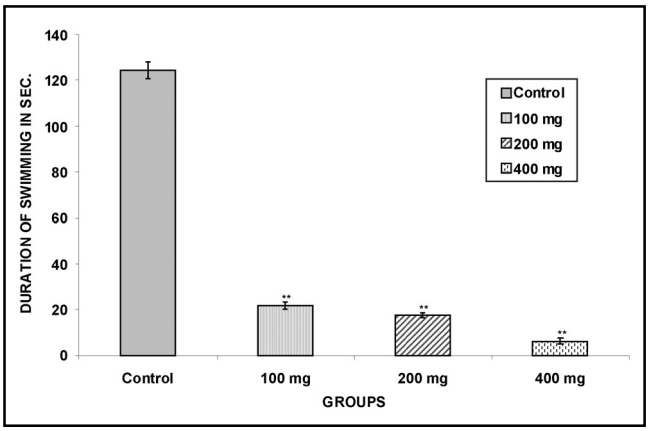

Fig.5: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Swimming Time in Forced Swimming Test

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

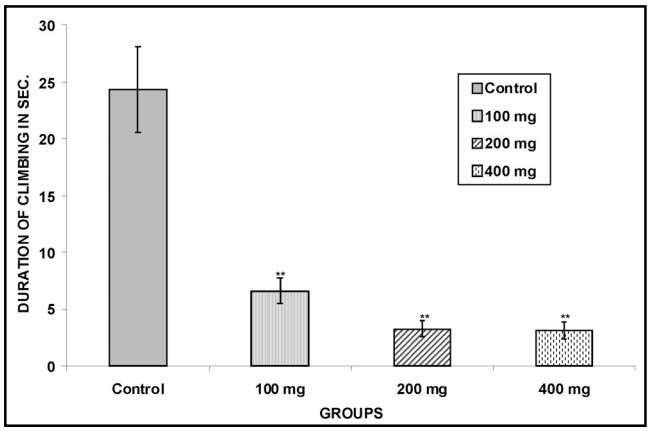

Fig.6: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Climbling Time in Forced Swimming Test

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

At given doses alcoholic extract exhibited significant ($p<0.01$) and dose dependent increase in the immobility time as compared to vehicle group in tail suspension test (Fig. 7).

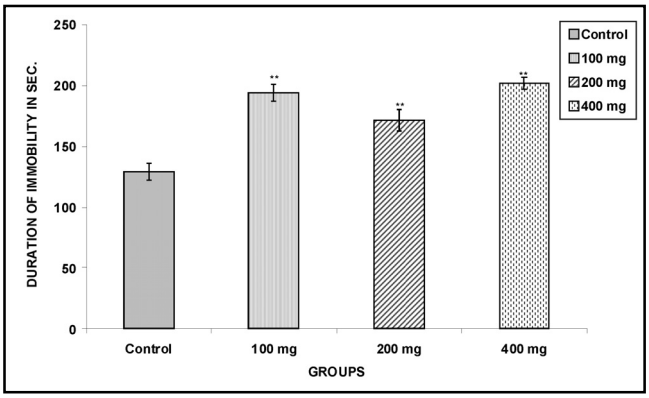

Fig.7: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Alertness (Hole Board Test)

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

The results of the hole board test revealed that there was a significant ($p<0.01$) decrease in the number of head dipping as compared to control vehicle group (Fig. 8).

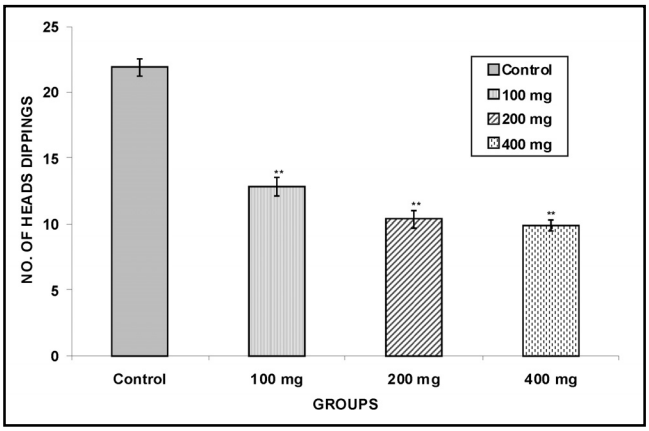

Fig.8: Effect of Alcoholic Extract of Root of *Pandanus Odoratissimus* on Alertness (Hole Board Test)

Values are expressed as Mean ± SEM of 8 animals, ** P<0.01 Vs Control Group

Discussion

In the present study, alcoholic extract of Pandanus odoratissimus was evaluated for its effect on central nervous system. The PO showed significant CNS depressant actions such as reduced alterness and locomotion in a dose dependent manner, which could be attributed to the sedative effect of the extract. Extract also showed the significant loss of muscular coordination. This could be due to loss of muscular strength. PO extract increased immobility time in both FST and TST and decreased swimming and climbing behaviour in FST. This further confirms the presence of chemical constituents having CNS depressant action.

Conclusion

Assessment of neuropharmacological activities of the alcoholic extract of PO root indicates that the root extract may have chemical constituents with CNS depressant action. Further work is required on identification of phytochemicals involved in the CNS depressant action from the root extract of *Pandanus odorotismus*.

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Evaluation and Permeation Studies of Transdermal Patches of Ketorolac Tromethamine

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ABSTRACT

Transdermal drug delivery was carried out by membrane permeation controlled type transdermal patches using different polymers such as PVP, hydroxy propyl methyl cellulose, methyl cellulose, ethyl cellulose in varying proportions and combinations. Transdermal Delivery of Ketorolac a non-steroidal agent with potent analgesic and moderate anti-inflammatory activity certainly appears to be alternative route of administration as a non-invasive mode of drug delivery and to maintain the drug blood levels for an extended period of time. Results from clinical studies indicate that Ketorolac tromethamine transdermal patch was significantly more effective than oral administration. After carrying out the in-vitro diffusion studies for the formulations, the formulation of HPMC-2% & MC-3% was selected for the physico-chemical parameters, ex-vivo, in vivo drug release studies & pharmacodynamic studies. A well designed transdermal controlled drug delivery system is expected to provide the following benefits like limitation of hepatic first pass metabolism, absolute reduction of gastro intestinal side effects, enhancement of therapeutic efficacy, minimisation of the needs of frequent dose intake, maintenance of steady plasma level of the drug, reduced side effects due to optimization of the blood concentration time profile.

Key Words: Transdermal Patches, Ketorolac Tromethamine, Permeation Studies.

Introduction

Transdermal delivery is the delivery of drugs through intact skin to reach the systemic circulation in sufficient quantity to administer a therapeutic dose. Skin is the most extensive and readily accessible organ in the body [1]. Its chief functions are concerned with protection, temperature regulation, control of water output & sensation. In an average adult it covers an area about 1.73m² and receives one third of circulating blood throughout the body at any given time. The potential of using intact skin as the site of administration for dermatological preparations to elicit pharmacological action in the skin tissue has been recognized for several years. Until the turn of the century, the skin was thought to be impermeable. However the view has changed & the progress achieved in this area clearly demonstrates that the skin is a complex organ and allows the passage of chemicals into and across the skin [2]. The Permeation of chemicals, toxicants and drugs are much slower across the skin when compared to other biological membrane in the body. The understanding of these complex phenomena has lead to the development of transdermal drug delivery systems, in which the skin serves as the site for the administration of systemically active drugs [3].

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Materials and Methods

Materials

Ketorolac Tromethamine (Cipla Ltd Goa); Hydroxyl propyl methyl cellulose; Ethyl cellulose; Polyvinyl pyrolidone; Methyl cellulose ; (Strides Arco Labs); were used as procured from the manufactures. All other reagents were analytical grade and used as such.

Experimental

The films were casted on a mercury surface. Required amount of drug was dissolved in ethanol or water and the polymer (HPMC-2%) were added to the drug solution .To this plasticizer dibutylphthalate (30%) was added and stirred to get homogenous solution. The volume was made upto 5 ml and poured on the mercury surface. After 48 hours, the dried films were taken out.

Fabrication of Rate Controlling Membrane with Methyl Cellulose

Accurately weighed quantities of Methyl cellulose-

3% were dissolved in water. Then the solution was mixed with occasional stirring to get clear solution. Glycerin was added as plasticizer. Then it was poured on the mercury surface and dried.

Physico chemical evaluation of transdermal films

1) Percent Moisture Absorption [4]

The percent moisture absorption was carried out to check the physical stability and integrity of the films at highest humidity conditions. The films were accurately weighed and placed in the dessicator containing saturated solution of aluminium chloride, which maintain 79.50% RH After 3 days the films were taken out and weighed.

$$\text{Percntage Moisture Absorbtion} = \frac{\text{Final Weight–Initial Weight}}{\text{Initial Weight}} \times 100$$

2) Percent Moisture Loss

The percent moisture loss was carried out to check the integrity of the films at dry condition. The films were accurately weighed and placed in the dessicator containing saturated solution of anhydrous calcium chloride. After 3 days the films were taken out and weighed. The moisture loss was calculated using the formula

$$\text{Percntage Moisture Loss} = \frac{\text{Final Weight–Initial Weight}}{\text{Initial Weight}} \times 100$$

3) Water Vapour Transmission Rate [5]

For water Vapour transmission studies glass vials of equal diameter were used as transmission cells. About 1 gm of Anhydrous Calcium Chloride was taken in cells and the polymer film was fixed over the brim with the help of the solvent. The cells were accurately weighed and kept in a closed dessicator containing saturated solution of potassium chloride to maintain a humidity of 84%RH.The cells were taken out and weighed after 6,12,24,36,48,78 hrs of storage. The amount of water transmitted were found by

$$\text{Water Vapour Transmission Rate} = \frac{\text{Final Weight–Initial Weight}}{\text{Time X Area}}$$

Water Vapour transmission rate usually expressed as the number of grams of moisture gain/hrs/sq.cm.

4) Thickness

Thickness of the film was measured at 3 different points using a screw gauge and average thickness was found out.

5) Drug Content

A transdermal film was cut into small pieces and put in the buffer solution and it is shaken in a mechanical shaker for 3 ½ hrs to get a homogenous solution and filtered. The filtrate was withdrawn and made up the volume. The drug content was analysed at 313nm [6].

6) Folding Endurance [7]

It was determined by repeatedly folding a small strip of film at the same place till it breaks. The number of times, the film could be folded at the same place without breaking gives the value of folding endurance.

Ex-Vivo Permeation Studies

1) Guinea Pig skin [3]

Full Thickness of guinea pig skin was taken and the patch was placed over it and this was mounted on the donor compartment. The permeation study was carried out in a similar manner as that with artificial membrane.

2) Rat Skin [8]

Male rats weighing 105-120 gm free from any visible signs of disease were selected. Abdominal skin of full thickness was excised from the rat and it was mounted on the donor compartment. The transdermal Patch was placed over it. The permeation study was carried out in a similar manner as that with artificial membrane.

3) Pig Ear Skin

Superficial skin of the pig was taken from the back of its ear and mounted on the donor compartment. The transdermal patch was placed over it and the permeation study was carried out.

In-vivo Studies On Rabbits

Skin Irritation Test using Rabbits [9]

A Primary skin Irritation test was performed since skin is a vital organ through which drug is transported. The test was carried out on 6 healthy rabbits weighing 2-3kg. Formulation (HPMC-2%: MC-3%) was subjected to the study and plain polymer films were used as control. The dorsal surface of rabbits were cleared well and the hair was removed. The skin was cleared with rectified spirit. The patches were placed over skin and it is removed after 24 hrs. The skins were examined for erythema and oedema.

***In-vivo* Drug Release study**

A Set of male healthy Rabbits (corytolagus cuniculus) 10-12 weeks old weighing 2-3 kgs were selected. The temperature, Relative humidity conditions were $28^0C \pm 1^0C$ and $60^0C \pm 15$ % respectively. The dose of Ketorolac tromethamine was calculated according to the body weight [10].The patch was placed on the dorsal surface. At specific intervals of time, the films were removed carefully and analysed for the remaining drug content. The drug content obtained was substracted from the initial content in the film. The value obtained denotes the amount of drug released in the rabbits [11].

Drug release at any interval time = Initial drug content - final drug content

(Before placing the film) -
(After removal of the film)

Pharmacodynamic Study

Comparative Anti-inflammatory study

Method: Carrageenan induced acute paw oedema method [12]. The animals were divided into 3 groups each comprising of 6 albino rats. The first group kept as control. The second group was administered orally with the pure drug solution of Ketorolac tromethamine. The third group was administered with transdermal patches of Ketorolac tromethamine. After 30 minutes all animals were injected with 1%w/v carrageenan in the plantar region of the left paw. The right Paw of all animals was considered as reference. Non- inflammed paw for comparison. The paw volumes for both legs of all the 3 group rats were noted at 1,2,3,4 hrs after carrageenan administration.

The mean paw volume in right and left leg of animals of each group was calculated and the percent oedema inhibition was calculated for every time interval

The difference in paw volume between right and left leg of each group was calculated and the percent oedema inhibition was calculated as follows.

$$\text{Percent Oedema inhibition} = 100\text{-}X$$

$$X = \frac{\text{Difference in mean paw volume in treated group}}{\text{Difference in mean paw volume in treated group}}$$

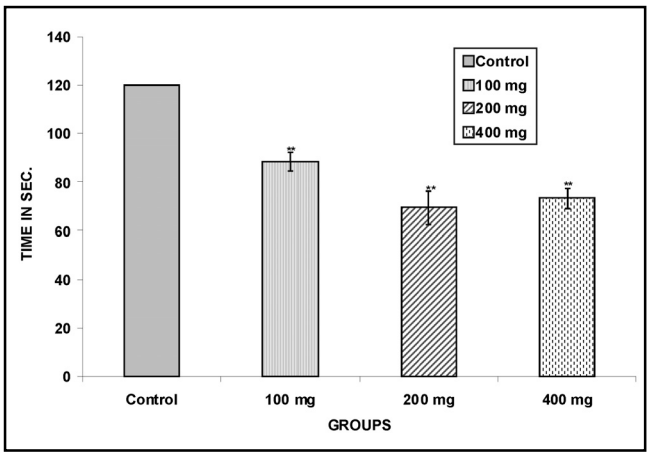

Fig.1: Ex Vivo Permeation Studies

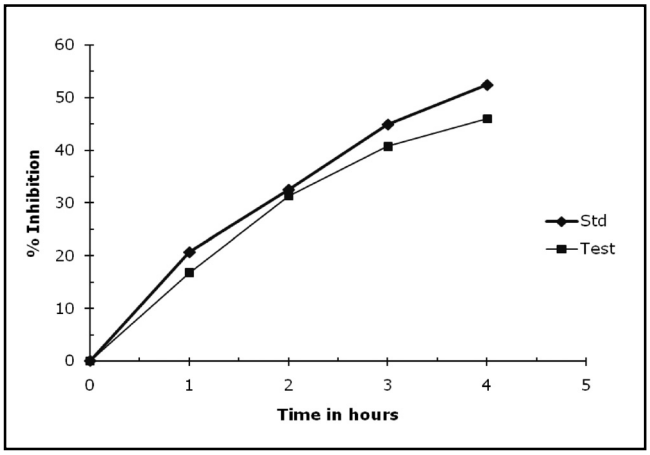

Fig.2: Comparative Anti-inflammatory Studies

Table-1
Comparative Anti-Inflammatory Study

Groups	Dose	%Inhibition	Time in hours			
			1 hr	2 hr	3 hr	4 hr
Control (Carrageenan) 1%w/v Soln	0.1ml		0.52 ±0.02	0.54 ±0.02	0.57 ±0.08	0.6 ±0.03
Standard (Pure drug solution)		0.42 ±0.11	0.37 ±0.11		0.31 ±0.10	0.28 ±0.03
	13.5 mg	%Inhibition	20.67 ±0.11	32.53 ±0.11	44.94 ±0.10	52.46 ±0.03
Test (Transdermal Patch)		0.44 ±0.11	0.37 ±0.11		0.34 ±0.10	0.32 ±0.03
	13.5 mg	%Inhibition ±0.02	16.8 ±0.13	31.33	40.78 ±0.12	46.03 ±0.02

Results and Discussion

After carrying out the Invitro diffusion studies, the formulation HPMC-2% with rate controlling membrane (MC-3%) has shown release upto 21 hrs to the extent of 84.99%and it was selected for ex-vivo permeation studies. The study was carried out in Pig ear skin, Guinea pig skin & Rat skin, inorder to select the best biological system which has good correlation with invitro release. When the study was carried out in Pig ear skin the drug release has shown the drug diffusion for 22 hrs upto the extent of 65.75%.The studies were carried out in guinea pig skin & Rat skin, the drug release has shown for 22 hrs to the extent of 75.73% and 80.42% respectively

The variations among the used biological membranes could be attributed to the the fat content & thickness of the membrane used. As the pig ear skin has more fat distribution and thickness, it might have hampered the drug release through the membrane. As earlier studies indicate that the human skin has best correlation with the diffusion rate of rat skin, the results were analysed on this point of view. As rat skin has shown good correlation with invitro release of formulation and this was considered for further studies.

Skin Irritancy Test

Skin Irritation studies revealed that the formulation has no erythema and oedema.

***In-vivo* Release studies**

In-vivo release studies were carried out in rabbits revealed that the consistent in-vitro release pattern of formulation was reproducible even in biological membrane. At the end of 22 hrs, the in-vivo drug release has shown 72.75% release. To ensure the correlation between the Invitro and in-vivo release pattern, the regression analysis was carried out. It has followed the predicted zero order kinetics in biological systems also.

Pharmacodynamic study

Comparative anti-inflammatory study was carried out with the transdermal formulation and pure Ketorolac tromethamine drug solution(orally).The results obtained in this study showed that the percentage paw oedema inhibition was 46.03% for animals treated with transdermal patch and 52.46% for animals treated with pure drug solution(orally).

The study revealed that the animal treated with orally reduced the inflammation (52.46%) within 4 hrs.But this dosage form requires frequent administration. The animals treated with transdermal patch reduced the inflammation (46.03%) in a sustained manner. The patch will release the drug for 24 hrs.So the frequency of administration will be reduced & the gastro intestinal disturbances can be avoided in case of transdermal mode of treatment. This may be advantageous in case of long-term therapies.

Conclusion

Ex-vivo permeation studies evinced that the in-vitro dissolution data correlated with the ex-vivo data observed in rat skin rather than of pig ear skin and guinea pig skin. In-Vivo studies, carried out using rabbit affirmed the reliability of the in-vitro evaluation methods followed and the adaptability of the delivery system to the biological environment. Pharmacodynamic study revealed that the anti-inflammatory activity of the transdermal formulation was better when compared with the drug given through oral. The formulation has achieved the objectives of extended release, reduced frequency administration, avoids the first pass effect and thus may improve the patient compliance

As an extension of this work pharmacokinetic studies, in vivo studies on higher animals and controlled clinical studies on human beings can be carried out in future.

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In Vitro Dissolution Studies on Solid Dispersions of Mefenamic Acid in Pregelatinised Starch Alone and with PVP

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ABSTRACT

Solid Dispersions of mefenamic acid (MA), with a water soluble polymer (PVP) and a super disintegrant namely, pregelatinised starch(PGS), were prepared by common solvent and solvent evaporation methods employing methanol as solvent. Solid Dispersions prepared were evaluated for dissolution rate and dissolution efficiency in comparison to the corresponding pure drugs. Solid dispersions of mefenamic acid showed a marked enhancement in dissolution rate and dissolution efficiency. The order of increasing dissolution rate was observed with increase in pregelatinised starch ratio. At 1:4 ratio of drug: carrier a 2.83 fold increase in the dissolution rate of mefenamic acid was observed with mefenamic acid-PGS (1:4) solid dispersion. The solid dispersions in combined carriers gave much higher rates of dissolution than super disintegrants alone. MA-PGS-PVP solid dispersion gave a 5.63 fold increase in the dissolution rate of mefenamic acid. Super disintegrants alone or in combination with PVP could be used to enhance the dissolution rate of poorly soluble drug mefenamic acid.

Key Words: Mefenamic Acid, Solid Dispersions, Dissolution rate, Solubility, polyvinyl pyrrolidine, pregelatinised starch.

Introduction

Mefenamic acid, an anthranilic acid derivative, is a non-steroidal anti-inflammatory drug (NSAID) [1]. It is used in mild to moderate pain including headache, dental pain, postoperative and postpartum pain, dysmenorrhoea, osteoarthritis. The usual dose by mouth is 500 mg three times daily. Mefenamic acid is absorbed from the gastro intestinal tract. Peak plasma concentrations occur about 2 to 4 hours after ingestion. Most of the NSAIDs belong to class II category under Biopharmaceutical classification system (BCS) i.e., they are inherently highly permeable through biological membranes, but exhibit low aqueous solubility. Rate of absorption and / or extent of bioavailability for such insoluble hydrophobic drug are controlled by rate of dissolution in gastro-intestinal fluids[2]. The present study aims at enhancing the dissolution rate of MA. In the present investigation solid dispersions[3] were prepared by employing common solvent and solvent evaporation methods. Studies were carried out on mefenamic acid with an objective of enhancing their dissolution rates and bioavailability. Water dispersible super disintegrants, a new class of tablet excipients were evaluated as carriers, alone and in combination with PVP, for enhancing the dissolution rate and bioavailability of mefenamic acid.

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Materials and methods

Mefenamic acid was a gift sample from M/s.Sigma Laboratories, Mumbai, methanol (qualigens) and polyvinyl pyrrolidone (PVP K_{30}) was a gift sample from M/s. Sun Pharma Ind. Ltd., Mumbai. All other materials used were of pharmacopoeial grade and were procured from commercial sources.

Preparation of Solid Dispersions

Preparation of Solid Dispersions Employing Soluble Carriers (PVP)

Solid Dispersions of Mefenamic Acid were prepared by common solvent method employing methanol as solvent for mefenamic acid solid dispersions. The required quantities of drug and carrier were weighed and dissolved in the corresponding solvent in a round bottom flask to get a clear solution. The solvent was then removed by evaporation under reduced pressure (vacuum) at 60^0 C with constant mixing. The mass obtained was crushed pulverized and shifted through mesh no.100. In each case solid dispersions were prepared in the ratio of drug carrier namely 8:2.

Preparation of Solid Dispersions Employing Superdisintegrants

Solid dispersions of mefenamic acid (MA) in superdisintegrants (pregelatinised starch) were prepared by solvent evaporation method. The required quantities of drug were dissolved in methanol to get a clear solution in a dry mortar. The super disintegrant (passed through 120 mesh) was then added to clear drug solution and dispersed. The solvent was removed by continuous trituration. Trituration was continued until a dry mass was obtained. The mass obtained was further dried at 50^0 C for 4 hours in a oven. The product was crushed, pulverized and shifted through mesh no.100. In each case solid dispersions in the superdisintegrants were prepared at three different ratios of drug excipient namely 1:1, 1:2 and 1:4 respectively.

Preparation of Solid Dispersions Employing Combined Carriers

The required quantities of drug and water soluble carrier (PVP) were dissolved in the solvent to get a clear solution in a dry mortar. The super disintegrant was then added to the drug solution and dispersed. The solvent was then evaporated by continuous trituration. Trituration was continued until a dry mass was obtained. The mass obtained was further dried at 50^0 C for 4 hours in an oven. The product was crushed, pulverized and shifted through mesh N0.100. Various solid dispersions prepared with their composition are listed in Tables 1.

Table-1

Various solid dispersions prepared and their composition

Sl. No.	Drug	Composition	
		Carriers	SD Code
1.	Mefenamic Acid (8)	PVP (2)	MA-PVP, 82
2.	Mefenamic Acid (1)	PGS (1)	MA-PGS, 11
3.	Mefenamic Acid (1)	PGS(2)	MA-PGS, 12
4.	Mefenamic Acid (1)	PGS(4)	MA-PGS, 14
5.	Mefenamic Acid (1)	PGS(3.2) PVP (0.8)	MA-PGS-PVP MA-PGS-PVP

Estimation of Mefenamic Acid

A spectrophotmetric method based on the measurement of absorbance at 279 nm in phosphate buffer pH 7.4 was used in the present study for the estimation of mefenamic acid [4]. The method was validated for reproducibility, accuracy, precision and linearity by analyzing six individually weighed samples of mefenamic acid. The stock solution of mefenamic acid was subsequently diluted to a series of dilution containing 5, 10, 15 and 20 *mg/ml* of solution, using phosphate buffer of pH 7.4. The absorbance of these solutions was measured in UV-VIS spectrophotometer (ELICO SL-159). The method obeyed Beer's law in the concentration of 0-20 *mg/ml.*

Estimation of Mefenamic Acid Solid Dispersions Prepared

From each batch, 4 samples of 50 mg each were taken and analyzed for the drug mefenamic acid. 50 mg of dispersions were weighed into a 100 ml volumetric flask. Methanol was added and mixed the contents thoroughly to dissolve the drug from the dispersion. The solution was then filtered and collected carefully into another 100 ml volumetric flask. The solution was made up to volume with the solvent. The solution was suitably diluted with phosphate buffer of pH 7.4 and assayed at 279 nm for mefenamic acid. The results are given in Tables 2.

Table 2
Mefenamic Acid Content of Various Solid Dispersions Prepared

Sl. No.	SD Code	Percent Mefenamic Acid Content ($\bar{x}$ ± s.d.,)
1.	MA-PVP, 82	79.5 ± 0.74 (0.93)
2.	MA-PGS, 11	48.8 ± 0.37 (0.76)
3.	MA-PGS, 12	32.6 ± 0.19 (0.59)
4.	MA-PGS, 14	19.9 ± 0.31 (0.65)
5.	MA-PGS-PVP	19.8 ± 0.17 (0.86)

Dissolution Rate Studies on Solid Dispersions

Dissolution rate of mefenamic were studied using an USP XXIII six station dissolution rate test apparatus (Electro Lab). Paddle stirrer at a speed of 50 rpm and temperature of 37^0 ± 1^0C were used in each test. Drug or solid dispersion of drug equivalent to 100 mg of mefenamic acid was used in each dissolution rate test. Samples of dissolution medium i.e., phosphate buffer pH 7.4 (5ml) were withdrawn through a filter (0.45 m) at different time intervals, suitably diluted, and assayed for mefenamic acid. The dissolution experiments were conducted in triplicate. The results are given in Table 3.

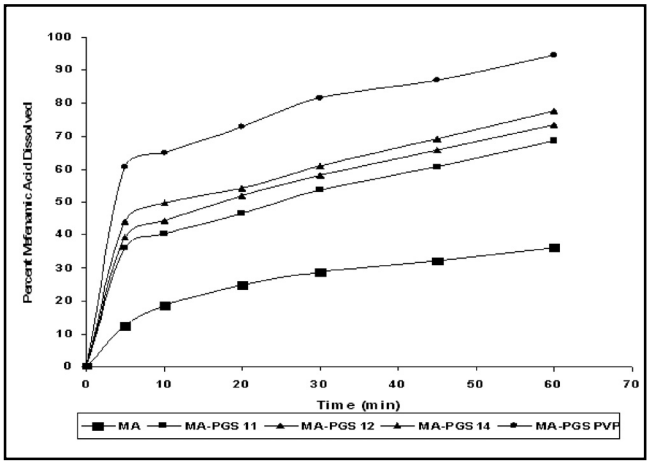

Fig.1: Dissolution Profiles of Mefenamic acid and its solid dispersions

Table 3

Dissolution Profiles of Mefenamic Acid Solid Dispersions

Time (min)	Percent Mefenamic Acid Dissolved ($\bar{x}$ ± s.d., n = 3)					
	MA	MA-PGS 11	MA-PGS12	MA-PGS 14	MA-PGS PVP	MA-PVP 82
5	12.39±0.63	36.23±0.93	39.20±1.67	44.02±0.93	60.84±1.48	21.75±1.88
10	18.66±0.38	40.39±1.43	44.35±1.59	49.71±1.48	64.92±1.45	26.22±1.67
20	24.2±0.56	46.61±1.29	51.93±1.70	54.16±1.70	72.96±2.10	31.41±1.86
30	28.83±0.69	53.54±2.10	58.16±0.93	61.08±1.49	81.74±2.04	36.98±1.85
45	32.25±0.71	60.83±1.70	65.91±1.30	69.25±2.10	86.93±1.66	40.94±2.04
60	36.05±0.54	68.75±1.86	73.33±2.23	77.78±2.04	94.72±1.67	46.01±1.70

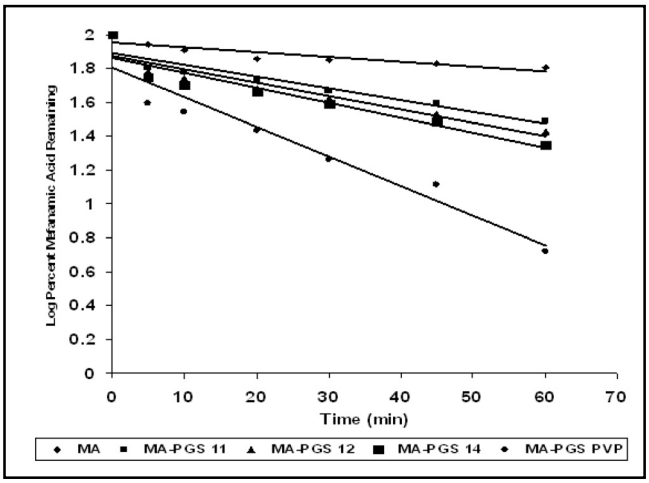

Fig.2: First Order Dissolution Plots of Mefenamic Acid and its Solid Dispersions

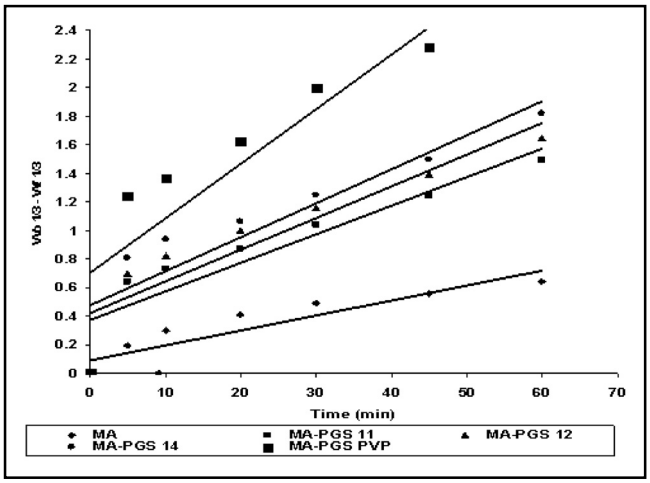

Fig.3: Hixson-Crowell Dissolution Plots of Mefenamic Acid and its Solid Dispersions

Table 4

The Correlation Coefficient (r) values in the Analysis of Dissolution Data of Mefenamic Acid Solid Dispersions as per Zero order, First Order and Hixson-Crowell Cube Root Models

Sl. No.	Solid Dispersion	Correlation coefficient (r) value		
		Zero order	First order	Hixson-Crowell
1.	Pure Drug	0.9875	0.9940	0.9920
2.	MA -PVP, 82	0.8763	0.9075	0.9061
3.	MA- PGS 11	0.8599	0.9379	0.9174
4.	MA –PGS 12	0.8486	0.9376	0.9163
5.	MA –PGS 14	0.8299	0.8829	0.9074
6.	MA –PGS PVP	0.7769	0.9547	0.9192

Table 5

Dissolution Parameters of Mefenamic Acid and its Solid Dispersions in Superdisintegrants

Sl. No.	Solid Dispersion	Dissolution Parameter			
		T_{50} (min)	% Dissolved in 10 min	DE_{30} (%)	K_1 (min^{-1})
1.	Mefenamic Acid	> 60	10.63	19.60	0.0072
2.	MA-PGS 11	25	35.85	40.59	0.0160
3.	MA-PGS12	18	39.41	44.62	0.0184
4.	MA-PGS 14	16	43.18	48.01	0.0204
5.	MA-PGS PVP	3.50	64.92	64.48	0.0405
6.	MA-PVP 82	> 60	26.20	26.81	0.0087

Dissolution rates of mefenamic acid and its solid dispersions followed first order kinetics.(Table 4)

Analysis of Dissolution Data of Solid Dispersions as per Hixson-Crowell's cube root law

The dissolution data of mefenamic acid and their solid dispersions were also analyzed as per Hixson-Crowell's [5] cube root equation. Hixson-Crowell introduced the concept of changing surface area during dissolution and derived the "cube-root law" to nullify the effect of changing surface area and to linearize the dissolution curves. Hixson-Crowell's cube root law is given by the following equation. $(W_o)1^{/3} - (W_t)^{1/3} = Kt$, where W_o is initial mass and W_t is the mass remained at time 't'. The cube root equation is applicable to the dissolution of monodisperse powder consisting of uniform sized particles. A plot of $(W_o)^{1/3} - (W_t)^{1/3}$ versus time will be linear when dissolution occurs from monodisperse particles of uniform size. Hixson-Crowell plots of the dissolution data were found to be linear (Fig.3) with all solid dispersions. This observation indicated the drug dissolution from all the solid dispersions is occurring from discretely suspended or deposited (monodisperse) particles. This might have also contributed to the enhanced dissolution rate of the solid dispersions.

The correlation coefficient (r) values of the first order release model are found to be (0.9075 to 0.9940) slightly higher when compared to the Hixson-Crowell's cube root model. Hence the release of drug from the preparations followed predominantly first order kinetics compared to Hixson-Crowell cube root law.

Another parameter suitable for evaluation of *in vitro* dissolution has been suggested by Khan [6] by a parameter Dissolution efficiency (DE). DE is defined as the area under the dissolution curve up to a certain time 't' expressed as percentage of the area of the rectangle described by 100% dissolution in the same time.

$$\text{Dissolution Efficiency (DE)} = \left[\frac{\int_o^t y.dt}{y_{100}.t} \right] 100$$

The index DE_{30} would relate to the dissolution of drug from a particular formulation after 30 minutes and could be compared with DE_{30} of other formulations. Summation of the large dissolution data into a single figure DE enables ready comparison to be made between a large numbers of formulations. First order dissolution rate constants (K_1) calculated from the slopes of the first order liner plots, dissolution efficiency (DE_{30}) values, T_{50} (time for 50% dissolution) and percent dissolved in 10 minutes are given in Table 2.

Results and Discussion

All the dissolution parameters given in Table 2 indicated rapid and higher dissolution of mefenamic acid from all solid dispersions when compared to mefenamic acid pure drug. Mefenamic acid-PVP (8:2) solid dispersion gave rapid and higher dissolution than the pure drug. A 1.21 fold increase in the dissolution rate of mefenamic acid was obtained with this solid dispersion when compared to pure drug. Water dispersible superdisintegrants gave much higher enhancement in the dissolution rate of mefenamic acid than water soluble carriers. Solid dispersions of superdisintegrants gave rapid and higher dissolution of mefenamic acid when compared to pure drug as well as its solid dispersion in water soluble PVP. In each case, the K_1 and DE_{30} values were increased as the concentration of carrier (superdisintegrant) in the solid dispersion was increased. At 1:4 ratio of drug:carrier, the order of increasing dissolution rate with various superdisintegrant ratios was 1:4>1:2>1:1. A 2.83 fold increase in the dissolution rate of mefenamic acid was observed with mefenamic acid-PGS (1:4) solid dispersion. All the solid dispersions in combined carriers gave much higher rates of dissolution, several times higher than the dissolution rate of pure drug. PVP combined super disintegrants gave higher dissolution rates than superdisintegrants alone. MA-PGS-PVP solid dispersion gave a 5.63 fold increase in the dissolution rate of mefenamic acid whereas solid dispersion of mefenamic acid in PGS lone (MA-PGS 14 solid dispersion) gave only 2.83 fold increase. Thus combination of superdisintegrants with water soluble carrier PVP resulted in a greater enhancement in the dissolution rate of mefenamic acid.

Conclusion

Thus superdisintegrant pregelatinised starch was found to be useful as a carrier in mefenamic acid solid dispersions alone and in combination with PVP to enhance their solubility, dissolution rate and dissolution efficiency.

Acknowledgements

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Simultaneous Estimation of Lamivudine, Zidovudine and Nevirapine by RP-HPLC in Pure and Pharmaceutical Dosage Form

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ABSTRACT

A new, simple, accurate, precise, reproducible, economical RP-HPLC method was developed for the determination of lamivudine, zidovudine and nevirapine in pure and pharmaceutical dosage forms. A Phenomenix ODS C18 column (4.6 mm × 25 cm i.d., 5mm particle size) in isocratic mode ,with the mobile phase containing 0.1M sodium dihydrogen orthophosphate (adjusted to pH 3.5 using diluted ortho phosphoric acid (1 in 100), Acetonitrile and methanol in the ratio of (2:1:1) (V/V/V) was used for the separation. The flow rate was set at 0.6 ml/min and effluent was monitored at 271 nm. The run time was 15 min. The retention times for lamivudine, zidovudine and nevirapine were 5.408, 7.417, and 10.125 min respectively. The linearity for lamivudine, zidovudine and nevirapine were in the range of 12-84, 24-160 and 16-112 µg/ml respectively.

Key Words: RP-HPLC, Lamivudine, Zidovudine, Nevirapine.

Introducton

Zidovudine [1, 2] (AZT), was the first drug approved for the treatment of AIDS and HIV infection. Jerome Horowitz first synthesized AZT in 1964. It is from the class of nucleoside reverse transcriptase inhibitor. Chemically, 3'- azido- 2', 3'-dideoxythymidine. The mode of action is by terminating the growth of the DNA chain and inhibiting the reverse transcriptase of HIV.

Lamivudine, [1, 2] (3TC) also belongs to the class of nucleoside reverse transcriptase inhibitor. Chemically lamivudine is L-2', 3'- dideoxy -3'-thiacytidine. It acts by competing with deoxycytidine tri-phosphate for binding to reverse transcriptase and incorporation into DNA results in chain termination.

Nevirapine, [1, 2] (NEV) belongs to the class of non-nucleoside reverse transcriptase inhibitor (NNRTI) .Chemically, it is 11-cyclopropyl -5, 11-dihydro-4-methyl-6H-dipyrido [3,2-b: 2',3'-e][1,4]diazepin-6-one. It acts by binding reverse transcriptase adjacent the catalytic site and terminates the DNA chain.

The litrerature survey [3-7] reveals that the analytical methods like UV, HPLC and HPTLC for determination of these drugs individually and other combination in pharmaceuticals and biological preparations. In the present investigation an attempt was made to develop a simple, new, accurate, sensitive and economical HPLC method for the simultaneous estimation of lamivudine, zidovudine and nevirapine in pure sample and tablet dosage forms. The proposed method was validated as per ICH guidelines [8].

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Experimental

High performance liquid chromatography Shimadzu LC 2010 CHT series equipped with quaternary constant flow pump, auto injector with injection volume of 20 ìl, UV-visible detector and LC 10 software, Phenomenix ODS C18 column (4.6 mm i.d × 25cm , 5ìm particle size) forms the stationary phase, a calibrated electronic single pan balance (SARTORIUS AG), Labindia pH con meter, and Ultra sonicator. The reference standards of lamivudine, zidovudine and nevirapine were collected from Cipla Laboratory. The tablet was purchased from the local market. All chemicals and reagents used were of AR/HPLC grade and HPLC water was prepared from Milli-Q in the lab.

Preparation of mobile phase and standard stock solution:

The mobile phase was prepared by mixing 0.1M sodium dihydrogen orthophosphate [adjusted to pH 3.5 using diluted ortho-phosphoric acid (1 in 100)] buffer,

acetonitrile and methanol in the ratio of (2:1:1) (V/V/V). An accurately weighed quantity of lamivudine (150 mg), zidovudine (300mg) and nevirapine (200 mg) were transferred to 100 ml volumetric flask, which was then dissolved and made up to volume with mobile phase. From the above stock solution 0.4, 0.8, 1.2, 1.6, 2, 2.4, 2.8 ml of lamivudine, zidovudine, nevirapine were diluted to 50 ml with mobile phase to give final concentration 12, 24, 36, 48, 60, 72, and 84 µg/ml of lamivudine 24, 48, 72, 96, 120, 144 an 168 µg/ml of zidovudine and 16, 32, 48, 64, 80, 96 and 112 µg/ml of nevirapine. The solutions were injected and chromatograms were recorded.

Optimized chromatographic conditions:

HPLC analysis was performed by isocratic elution with flow rate of 0.6 ml/min. The mobile phase containing 0.1M sodium dihydrogen orthophosphate [adjusted to pH 3.5 using diluted ortho phosphoric acid (1 in 100)] buffer, Acetonitrile and methanol in the ratio of 2:1:1 (V/V/V) separated to obtain well resolved peaks of Lamivudine (Rt = 5.408 min), Zidovudine (Rt = 7.417 min) and Nevirapine (Rt = 10.125 min), respectively as shown in Fig No.1. Overlay Chromatograms of mixture of standard Lamivudine, Zidovudine, and Nevirapine is shown in the Fig No.3.

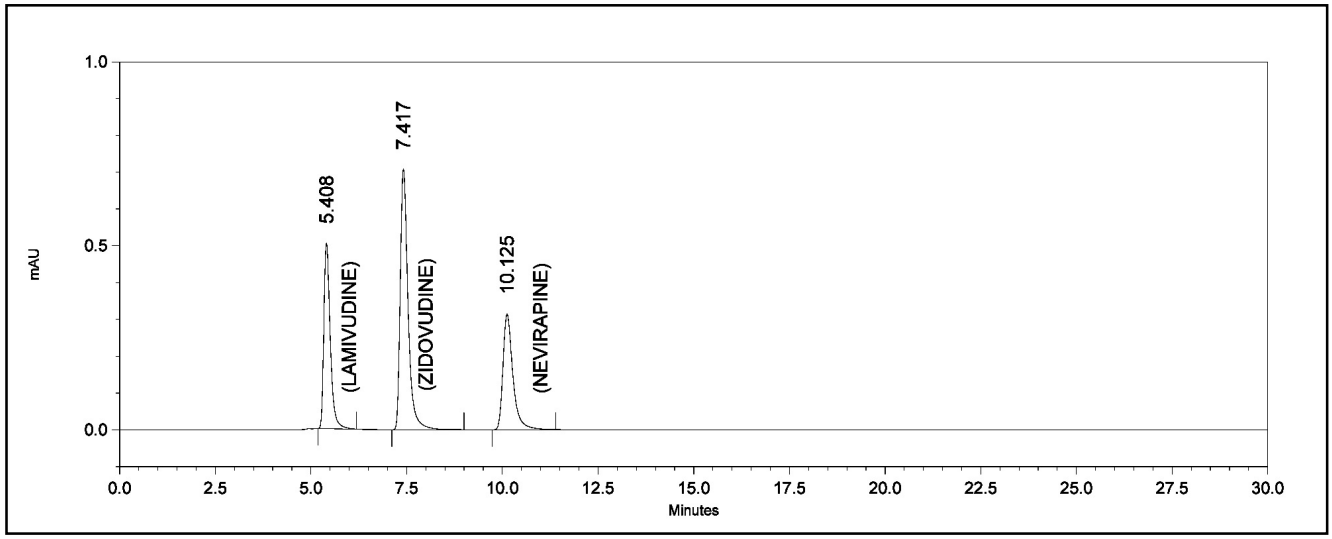

Fig No: 1. Typical chromatograms of mixture of standard Lamivudine, Zidovudine and Nevirapine.

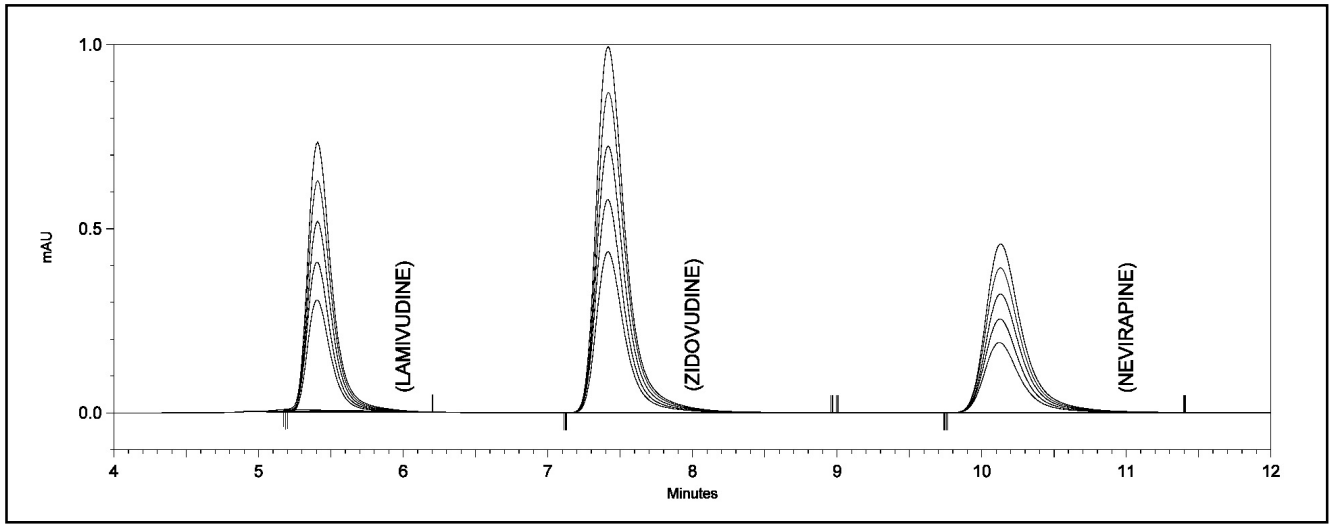

Fig no.3. Overlay Chromatograms of mixture of standard Lamivudine, Zidovudine, and Nevirapine.

Wavelength of maximum absorption was selected by UV spectrum which was recorded by UV-Visible spectrophotometer Pharmaspec-1700. All the three components showed good response at 270 nm.

Calibration curves for Lamivudine, Zidovudine and Nevirapine

Linearity was assessed by injecting 20 ìl of seven different standard concentrations obtained by diluting standard stock solution with mobile phase under optimized

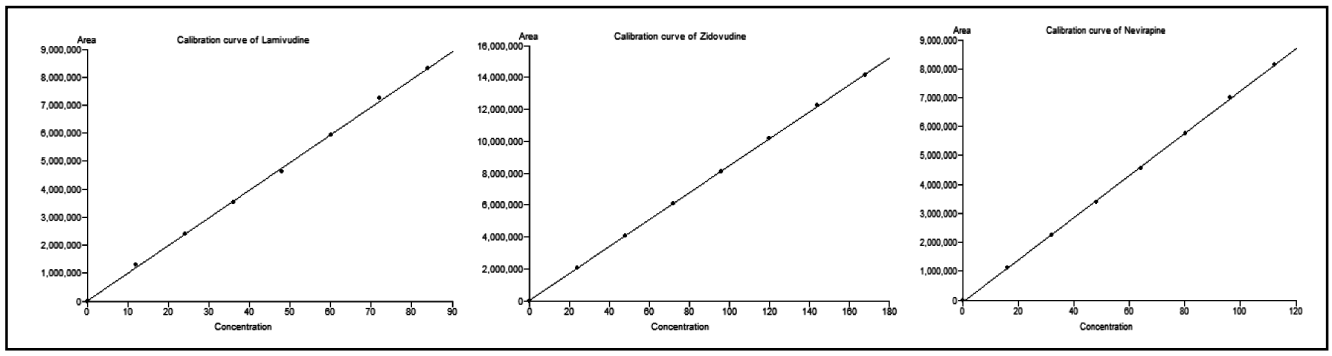

Fig.2: (a), (b) and (C) The calibration curves of Lamivudine and Zidovudine and Nevirapine

chromatographic conditions, which provides 12, 24, 36, 48, 60, 72, and 84 µg/ml lamivudine, 24, 48, 72, 96, 120, 144 and 168 µg/ml of zidovudine and 16, 32, 48, 64, 80, 96 and 112 µg/ml of nevirapine. The chromatograms were recorded and using peak area of individual drugs vs. respective concentrations linearity graph was plotted. The calibration curves of Lamivudine and Zidovudine and Nevirapine are shown in Fig NO.2. (a), (b) and (c) respectively.

Validation of the method:

The developed method was validated in terms of linearity, accuracy, specificity, limit of detection and limit of Quantitation, intraday and interday precision and repeatability of measurements.

Analysis of marketed formulations:

Twenty tablets were weighed accurately and crushed to the fine powder .An accurately weighed quantity of powder equivalent to 150 mg of lamivudine, 300 mg of zidovudine and 200 mg of nevirapine were then transferred to a 100 ml volumetric flask , sonicated for 15 min and made up to volume with mobile phase . Then the solution was filtered through 0.45 am filter paper. Two ml aliquot from the above solution was transferred into 50ml volumetric flask and volume was adjusted with the mobile phase up to mark to get sample solution. Working sample solution (n=5) were injected into column at above chromatographic conditions and peak areas were measured. The quantification was carried out by keeping these values to the straight line equation of calibration curve. The results of tablet analysis are shown in Table 1:

Table 1
Analysis of commercial tablet (n=5)

Analyte	Label claim (mg/tablet)	Amount found (mg/tablet)	C.I.	SD	%RSD	t
Lamivudine	150	150.178	100.1186±0.5088	0.4098	0.4093	0.6476
Zidovudine	300	299.902	99.9673±0.2780	0.2239	0.2239	0.3263
Nevirapine	200	200.334	100.167±0.6760	0.5444	0.5434	0.6859

Results and Discussion

Method Development:

The method was chosen after several trials with various proportions of 0.1M sodium dihydrogen orthophosphate (adjusted to pH 3.5 using diluted ortho phosphoric acid (1 in 100)) buffer, Acetonitrile and methanol in the ratio of (2:1:1) was selected to achieve maximum separation and sensitivity. The flow rate was 0.6 ml/min and effluent was monitored at 271 nm and a Phenomenix ODS C18 column and an injection volume of 20 µl and 25°C temperatures for the HPLC system were found to be the best for the analysis. The System suitability results are as follows.

Parameter	Lamivudine	Zidovudine	Nevirapine
Retention time(min)	5.408	7.417	10.125
Resolution factor	0	6.1125	6.6475
Capacity factor	539.6225	741.085	1011.5
Asymetric Factor	1.48	1.485	1.475
Theoretical plates	5174.3225	6852.8675	7918.2475
Lower limit of detection (µg/ml)	0.03217	0.00160	0.00697
Lower limit of Quantitation (µg/ml)	0.09747	0.00486	0.02112

Method Validation:

The proposed method has been validated for the simultaneous determination of Lamivudine, Zidovudine and Nevirapine in bulk as well as tablet dosage form using following parameters

Specificity:

The peak purity of Lamivudine, Zidovudine and Nevirapine were assessed by comparing the retention time (R_t) of standard Lamivudine, Zidovudine and Nevirapine. Good correlation was found between the retention time of standard and sample of Lamivudine, Zidovudine and Nevirapine.

Linearity:

Linearity was studied by preparing different concentration levels. The linearity ranges for Lamivudine, Zidovudine and Nevirapine were found to be 12-84µg/ml, 24-168µg/ml, and 16-112 µg/ml respectively. The regression equations for Lamivudine, Zidovudine and Nevirapine were found to be y = 9,106.3026 x + 19,911.4167, y = 84555.8289 x + 33183.0, and y = 72998.1607 x - 48915.50 and Correlation- coefficient (r^2) 0.9993, 0.9999 and 0.9998 respectively. Table-2

Precision:

The precision of the method was demonstrated by repeatability studies. Precision study was performed to find out intraday and inter-day variations in the estimation of different concentrations with the proposed method. Percentage relative standard deviation (%RSD) was found to be less than 1% for within a day and day to day variations over a period of one week, which proves that method is precise. Results are shown in Table 3 and Table 4.

Accuracy:

It was found by recovery study using standard addition method. Known amounts of standard Lamivudine, zidovudine and Nevirapine were added to pre-analyzed samples at a level from 80% upto 120% and then subjected to the proposed HPLC method. Results of recovery studies are shown in Table 5.

Robustness:

It was done by making small changes in the chromatographic conditions and found to be unaffected by small changes like ±0.1 changes in pH and 2% change in volume of the mobile phase.

Conclusion

The modalities adopted in experimentation were successfully validated as per analytical procedures laid down in routine. The proposed method was validated by preliminary analysis of standard sample and by recovery studies. The percentage of average recoveries was obtained in the range of 99 to 100. The results of analysis of average recoveries obtained in each instance were compared with the theoretical value of 100 percent by means of Student's't' test. As the calculated't' values are less than theoretical' values (Table 5), it is concluded that the results of recoveries obtained in agreement with 100 percent for each analyte. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the tablets. This demonstrates that the developed HPLC method is new, simple, linear, accurate, sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of bulk and tablet dosage forms.

Acknowledgments

The Authors are thankful to the Director of Startech Pvt Ltd, Hyderabad for providing the necessary facilities to carry out this work.

Table 2:
Linearity of Lamivudine, Zidovudine and Nevirapine

3TC		AZT		NEV	
Concentration(µg/ml)	Area	Concentration(µg/ml)	Area	Concentration(µg/ml)	Area
12	1303328	24	2096650	16	1131979
24	2419809	48	4101648	32	2274168
36	3534323	72	6107466	48	3416325
48	4648807	96	8113264	64	4558462
60	5943136	120	10204268	80	5765789
72	7267957	144	12305826	96	7001740
84	8341649	168	14157859	112	8163389

Table 3
Intraday precision of Lamivudine, Zidovudine and Nevirapine

3TC			AZT			NEV		
Conc.(µg/ml)	Area	%RSD	Conc.(µg/ml)	Area	%RSD	Conc.(µg/ml)	Area	%RSD
36	3534031	0.105	72	6117657	0.329	48	3427936	0.253
48	4650188	0.119	96	8142684	0.273	64	4554229	0.195
60	5940981	0.134	120	10239424	0.246	80	5777715	0.277
72	7269244	0.108	144	12339070	0.192	96	6997762	0.213
84	8337291	0.159	168	14197529	0.192	112	8156711	0.281

Table 4
Inter- day precision of Lamivudine, Zidovudine and Nevirapine

3TC			AZT			NEV		
Conc.(µg/ml)	Area	%RSD	Conc.(µg/ml)	Area	%RSD	Conc.(µg/ml)	Area	%RSD
36	3552826	0.325	72	6116968	0.329	48	3420662	0.262
48	4662300	0.290	96	8121353	0.138	64	4566016	0.151
60	5940719	0.162	120	10222629	0.122	80	5759016	0.272
72	7234947	0.381	144	12299546	0.264	96	7030650	0.507
84	8354959	0.270	168	14180449	0.270	112	8168819	0.314

Table 5
Recovery study of 3TC, AZT, and NEV

Analyte	Level of % Recovery	Formulation (µg/ml)	Amount of standard drug added (µg/ml)	Amount recovered (µg/ml)	C.I.	SD	% RSD	% SE	t
3TC	80%	36	28.8	28.848	100.1668±0.3189	0.2568	0.2564	0.1148	1.4523
	100%	36	36	36.06	100.1664±0.3658	0.2946	0.2941	0.1318	1.2631
	120%	36	43.2	43.194	99.9862±0.1717	0.1383	0.1385	0.0619	0.2231
AZT	80%	72	57.6	57.556	99.9238±0.2851	0.2295	0.2297	0.1027	0.7422
	100%	72	72	71.892	99.8500±0.2988	0.2406	0.241	0.1076	1.3936
	120%	72	86.4	86.4122	100.0140±0.1811	0.1457	0.1456	0.0651	0.2146
NEV	80%	48	38.4	38.422	100.0574±0.3386	0.2728	0.2726	0.122	0.4707
	100%	48	48	47.964	99.9252±0.3575	0.2879	0.288	0.1287	0.5809
	120%	48	57.6	57.618	100.0321±0.1195	0.0963	0.0962	0.043	0.7248

SD: Standard deviation, % SE: Percent standard error, C.I.: Confidence Interval within which true value may be found at 95% confidence level = R ± ts/√n, R: Mean percent result of analysis of Recovery study (n = 5). Theoretical't' values at 95% confidence level for n - 1 degrees of freedom t (0.05, 4) = 2.776.

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Synthesis, Anticonvulsant Activity of some Novel 1, 2, 4-triazol-3-yl-thioacetohydrazides Derived from Benzimidazole and Benzoxazole

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ABSTRACT

A series of novel 1, 2, 4-triazol-3-ylthiols were synthesized from base catalyzed dehydrative cyclization of thiosemicarbazide intermediates. These triazoles were further treated with ethylbromoacetate to yield their respective thioethylacetates. The thioacetohydrazides were prepared from these esters by reaction with hydrazine hydrate. The compounds synthesized were screened for *in vivo* anticonvulsant activity in albino mice against pentylenetetrazole (PTZ) induced convulsions. The neurotoxicity of the compounds was evaluated by rotarod method in mice.

Key words: - 1, 2, 4-triazol-3-ylthiol; Anticonvulsant activity.

Introduction

Epilepsy is a neurological disorder characterized by unprovoked seizures that affects at least 50 million people worldwide. There is continuing demand for new anticonvulsant agents as it has not been possible to control all kinds of seizure with the currently available antiepileptic drugs. The anticonvulsant drugs like, Loreclezole- a positive modulator of Gamma Amino Butyric Acid (GABA) receptors and Estazolam- a Benzodiazepine agonist, possess triazole ring system [1-2]. The fact that several 1,2,4-triazol-3-ylthiol derivatives, substituted benzimidazoles and benzoxazoles have been proven to exhibit anticonvulsant properties [1–5], led us to synthesize some new compounds possessing analogous structures in order to obtain more potent anticonvulsant agents. In this paper we describe the synthesis of novel 1,2,4-triazol-3-ylthioacetohydrazide derivatives and their anticonvulsant & neurotoxicity activities.

Materials and Methods

Melting points were determined in open capillary tube and are uncorrected. The purity of the compounds was checked by TLC using ethyl acetate and toluene (7:3) as solvent system and iodine vapors as visualizing agent. IR spectra were recorded on Schimadzu 8400-S by KBr pellet method. ^{1}H-NMR spectra were recorded on AMX 400 NMR Spectrophotometer at 400 MHz using deuteriated dimethyl sulfoxide (DMSO-d_6) as solvent and Tetra methyl silane (TMS) as internal standard. The chemical shifts are expressed in ppm.

The scheme of the synthesis for the present study is depicted in Fig 1.

General procedure for the synthesis of 1-(substituted)-4-aryl-thiosemicarbazide (3-12)

The hydrazides that served as starting materials were prepared by following the method described earlier [6]. An equimolar quantity of 2-(1*H* benzimidazol-2yl-thio) acetohydrazide (**1**) or 2-(benzoxazol-2yl-thio) acetohydrazide (**2**) and appropriate arylisothiocyanate were separately dissolved in minimum quantity of absolute ethanol. The solution of isothiocyanate was poured into the solution of hydrazide with continuous stirring. The reaction mixture was refluxed for 8 h. The white solid obtained on cooling the reaction mixture to room temperature, was filtered and recrystalized from dichloromethane to obtain the corresponding thiosemicarbazides.

General method for the synthesis of 5-(substituted)-4-aryl-4H-1,2,4-triazol-3-ylthiols (13-22).

The triazole 3-thiols were prepared by base catalyzed dehydrative cyclization of thiosemicarbazides [7]. Appropriate thiosemicarbazide (0.004 mol) was added portion wise to 15ml of 2M NaOH solution and the resulting solution was refluxed for 6h. After the completion of the reaction (monitored by TLC), the mixture was allowed to cool and filtered. The filtrate was acidified with 2M HCl. The solid obtained by acidification was filtered,

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SCHEME

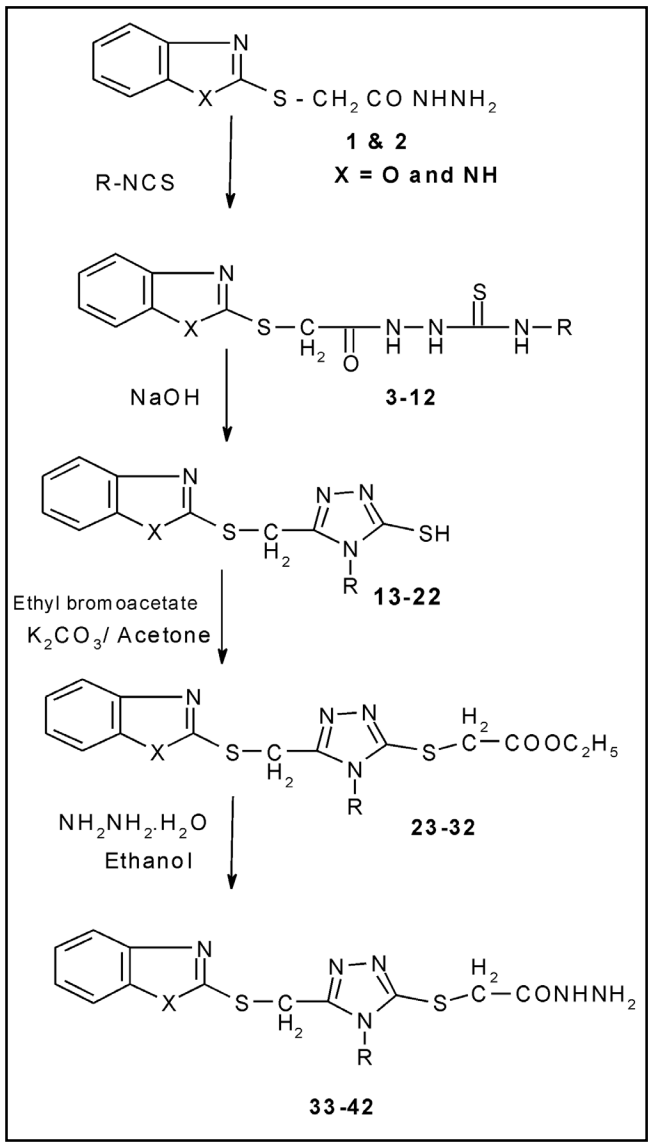


Fig.1

washed with water, dried and recrystalized from dichloromethane to obtain the respective 1, 2, 4-triazol-3-ylthiol.

Preparation of ethyl 2-(5-((1H-benzo[d]imidazol-2-ylthio) methyl)-4-phenyl-4H-1,2,4-triazol-3-ylthio)acetate (23)

A mixture of **13** (0.001mol), ethyl bromoacetate (0.0015mol) and 1g of anhydrous potassium carbonate in 50ml dry acetone were refluxed for 6h on a water bath. The resulting solution was filtered and the acetone was evaporated on a water bath. The cooled reaction mixture was poured into ice-cold water. The crude precipitate obtained was recrystallized from dichloromethane to obtain the ester **23**.

Similar procedure was employed to prepare the ethyl esters (**24-32**) using corresponding triazlole-3-ylthiols.

Preparation of 2-(5-((1H-benzo[d]imidazol-2-ylthio) methyl)-4-phenyl-4H-1,2,4-triazol-3-ylthio) acetohydrazide (33)

A mixture of **23** (0.001mol) and hydrazine hydrate 99 % (0.003mol) in 25 ml of absolute ethanol was refluxed for 7h on a water bath. After the completion of the reaction (monitored by TLC), the mixture was concentrated, cooled and poured into the crushed ice. The solid separated was filtered, dried and recrystallized from dichloromethane to obtain the hydrazide (**33**). Similar procedure was employed to prepare the hydrazides (**34-42**) using corresponding triazlole-3-ylthioesters (**24-32**). The physical and the spectral data of the synthesized compounds are summarized in **Table-1** and **Table-2** respectively.

Biological Activities

ANTICONVULSANT ACTIVITY:

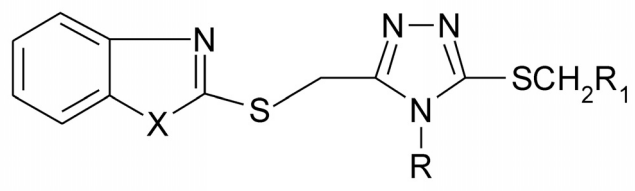
The *in vivo* anticonvulsant activity was carried out in albino mice against pentylenetetrazole (PTZ) induced convulsions [8]. The subcutaneous PTZ seizure threshold test (sc-PTZ) was carried out by an intraperitoneally administration of pentylenetetrazole (85 mg/kg) 30 minutes after the drug treatment. Each compound was administered orally at three dose levels (30, 100, and 300 mg/kg). The compounds were suspended in 0.5% methylcellulose. Animals were observed over 4hr. Failure to observe the generalized clonic seizure is defined as protection. Minimal neurotoxicity was measured by the rotarod test. Mice were placed on a 1-in.diameter plastic rod rotating at 6 rpm after the administration of the drug, and their ability to maintain their balance was tested. Neurological deficit was indicated by the inability of the animal to maintain its equilibrium for 1 min on the rotating rod in each of three trials. Both the animal experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC). The anticonvulsant activity and neurotoxicity data of the synthesized compounds is presented in the **Table 3.**

Result and Discussion

Chemistry:

A series of novel 1,2,4-triazol-3-ylthiols were synthesized from thiosemicarbazide intermediates from base catalyzed dehydrative cyclization. Absence of the peak corresponding to the C=O str between 1600-1700 cm^{-1} in the IR spectrum of **13** suggests the cyclization of the corresponding thiosemicarbazide. The triazole thiol proton has resonated at 12.7 ppm in the proton NMR spectrum. This high down field value is attributed to tautomeric thiol-thione system of the triazole. These triazoles were further converted to thioethylacetates by the treatment with ethylbromoacetate. The IR spectrum of the **23** indicates the presence of esteric carbonyl group with the characteristic absorption at 1645 cm^{-1}. The proton NMR spectrum of the compound **23** shows a quartet at 4.1 ppm and a triplet at 1.04 ppm due to the ethyl group of the ester. The

Table-1:
Physical Data of the Compounds



Compd	X	R	R_1	Melting point (^{0}C)	Rf Value*	% Yield
23	NH	phenyl		125	0.62	55
24	NH	4-tolyl	-do-	131	0.75	60
25	NH	3-tolyl	-do-	128	0.83	70
26	NH	4-methoxy phenyl	-do-	121	0.64	65
27	NH	4-chloro phenyl	-do-	145	0.76	60
28	O	phenyl	-do-	152	0.63	70
29	O	4-tolyl	-do-	142	0.65	65
30	O	3-tolyl	-do-	135	0.82	60
31	O	4-methoxy phenyl	-do-	160	0.83	60
32	O	4-chloro phenyl	-do-	152	0.73	70
33	NH	phenyl		194	0.64	75
34	NH	4-tolyl	-do-	188	0.68	65
35	NH	3-tolyl	-do-	192	0.52	65
36	NH	4-methoxy phenyl	-do-	205	0.47	60
37	NH	4-chloro phenyl	-do-	210	0.53	70
38	O	phenyl	-do-	225	0.60	75
39	O	4-tolyl	-do-	248	0.56	60
40	O	3-tolyl	-do-	235	0.45	65
41	O	4-methoxy phenyl	-do-	212	0.50	75
42	O	4-chloro phenyl	-do-	195	0.64	65

* Mobile phase: ethyl acetate and toluene (7:3)

Table 3:
Anticonvulsant and Toxicity Screening Data in Mice

Compound	Anticonvulsant activity (Sc PTZ[a,b])		Rotarod toxicity[a,c]	
	0.5h	4h	0.5h	4h
33	++	++	+	+
34	++	++	+	+
35	++	++	+	+
36	+++	+++	+	-
37	+++	+++	+	+
38	++	+	+	+
39	++	+	+	-
40	++	+	+	-
41	+++	+++	+	+
42	++	+++	+	+

[a] Key: (+++) activity at 30 mg/kg, (++) activity at 100 mg/ kg, (+) activity at 300 mg/kg,

(-) no activity or no toxicity at 300 mg/kg.

[b] Subcutaneous pentylenetetrazole seizure test. [c] Neurologic toxicity (rotarod) test.

disappearance of the thiol proton indicates the position of the substitution. The IR spectrum of the **33** indicates the presence of amide carbonyl group with the characteristic absorption at 1725 cm^{-1}. The hydrazide **33** derived from the ester **23** shows characteristic broad singlet at 9.2 ppm corresponding to CONH group. The absence of signals due to ethoxy group also confirms the formation of hydrazide.

Anticonvulsant activity: Compounds **36** and **37** from the benzimidazole series and compounds **41** and **42** from the benzoxazole series exhibited significant anticonvulsant activity. However, all the synthesized compounds were

Table-2
Spectral data of the compounds

Compd	IR and ^{1}H-NMR Spectral Data
3	Chemical name: 1-(2-(1H-benzo[d]imidazol-2-ylthio)acetyl)-4-phenyl thiosemicarbazide. IR: 3345 (NH), 3309 (NH); 3010 (Ar-CH); 2362 (SCH_2); 1675 (C=O);1155 (C=S)^{1}H-NMR: 10.1 (s,1H,NH); 9.3 (s,1H,NH); 8.9 (bs, 1H, CONH); 6.85-7.40 (m, 9 H, ArH); 5.10 (bs,1H, NH benzimidazole); 3.60 (s, 2H, SCH_2).
8	Chemical name: 1-(2-(benzo[d]oxazol-2-ylthio) acetyl)-4-phenyl thiosemicarbazide. IR: 3335 (NH); 3010 (Ar-CH); 2360 (SCH_2); 1685 (C=O);1158 (C=S)^{1}H-NMR: 10.05 (s,1H,NH); 9.2 (s,1H,NH); 8.70 (bs, 1H, CONH); 6.90-7.32 (m, 9 H, ArH); 3.76 (s, 2H, SCH_2).
13	Chemical name: 5-(1H-benzo[d]imidazol-2-ylthio) methyl)-4-phenyl-4H-1,2,4-triazole-3-thiol. IR: 3309 (NH); 3025 (Ar-CH); 2990 (CH); 2362 (SCH_2)^{1}H-NMR: 12.7 (bs, 1H, NH of triazole); 6.90-7.46 (m, 9H, ArH); 5.1(bs,1H, NH benzimidazole); 3.80 (s, 2H, SCH_2).
18	Chemical name: 5-(benzo[d]oxazol-2-ylthio)methyl)-4-phenyl-4H-1,2,4-triazole-3-thiol. IR: 3021 (Ar-CH); 2950 (CH); 2362 (SCH_2)^{1}H-NMR: 12.5 (bs, 1H, NH of triazole); 6.90-7.4 (m, 9 H, ArH); 3.75 (s, 2H, SCH_2).
23	Chemical name: ethyl 2-(5-(1H-benzo[d]imidazol-2-ylthio)methyl-4-phenyl-4H-1,2,4-triazol-3-ylthio)acetate IR: 3312 (NH); 3010 (Ar-CH); 2362 (SCH_2); 1645 (C=O)^{1}H-NMR: 6.90-7.50 (m, 9H,ArH); 5.01 (bs,1H, NH benzimidazole);4.1 (q,2H, OCH_2); 3.92 (s, 2H, SCH_2); 3.86 (s, 2H, SCH_2); 1.04 (t, 3H, CH_3)
28	Chemical name: ethyl 2-(5-(benzo[d]oxazol-2-ylthio)methyl)-4-phenyl-4H-1,2,4-triazol-3-ylthio)acetate IR: 3309 (NH); 3015 (Ar-CH); 2362 (SCH_2); 1640 (C=O)^{1}H-NMR: 6.85-7.40 (m, 9H, ArH);); 4.2 (q,2H, OCH_2); 3.92 (s, 2H, SCH_2); 3.86 (s, 2H, SCH_2); 1.04 (t, 3H, CH_3)
33	Chemical name: 2-(5-(1H-benzo[d]imidazol-2-ylthio)methyl)-4-phenyl-4H-1,2,4-triazol-3-ylthio)acetohydrazide IR: 3350 (NH); 3015 (Ar-CH); 2360 (SCH_2); 1665 (C=O)^{1}H-NMR: 9.2 (s, 1H, CONH); 6.90-7.50 (m, 9H,ArH); 5.0 (bs,1H, NH benzimidazole);4.3 (bs,2H, NH_2); 3.92 (s, 2H, SCH_2); 3.86 (s, 2H, SCH_2).
38	Chemical name: 2-(5-(benzo[d]oxazol-2-ylthio)methyl)-4-phenyl-4H-1,2,4-triazol-3-ylthio)acetohydrazide IR: 3342 (NH); 3045 (Ar-CH); 2362 (SCH_2); 1650 (C=O)^{1}H-NMR: 9.3(s, 1H, CONH); 6.85-7.40 (m, 9H, ArH);); 4.2 (bs,2H, NH_2); 3.92 (s, 2H, SCH_2); 3.86 (s, 2H, SCH_2).

well tolerated at the tested doses as indicated by the toxicity data.

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Characterization and Screening of Antimicrobial Activity of Lactic Acid Bacterium Isolated from A Traditional Beverage Marcha of Sikkim

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ABSTRACT

A strain DDM5 was isolated from traditionally fermented beverage *marcha* of Sikkim, showed broad range of antagonistic activity against some member of Gram positive *viz. Staphylococcous aureus*, *Staphylococcus epidermis, Pediococcous pentosaceus, Leuconostoc citreum* and Gram negative bacteria *viz Escherichia coli*, *Alcaligenes feacalis*. The isolate was found to be vancomycin and norfloxacin resistant like other antimicrobial substance producing lactic acid bacteria such as *Pediococcus pentosaceus* and *Streptococcos spp*. The isolate was also able to ferment glucose, sucrose, xylose, trehalose, mellibiose, galactose, and fructose. The purpose of this study was to evaluate the potential of DDM5 as a bioprotective culture by inhibiting the food spoilage microorganism.

Keywords: Antagonistic activity, lactic acid bacteria, carbohydrate fermentation

Introduction

One of the major challenges for the current food industry is to reduce the cost of food processing and avoiding the economic losses due to the food spoilage. Many researches have been focused on naturally produced antimicrobials that can reduce the usage of chemical preservatives as well as increase the nutritional properties of the food. Lactic acid bacteria (LAB) are usually associated with fermented dairy products or used as starter cultures but they also play an important role in food preservation due to the production of antimicrobial substance such as organic acids, diacetyl compounds, hydrogen peroxide, reuterin and bacteriocins [1]. Recently many studies have focused on natural antimicrobial substances secreted by LAB in order to inhibit spoilage microorganism pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* [2]. Marcha was prepared from soaked glutinous rice mixed with various parts from locally grown plants and used as starters for preparation of various indigenous alcoholic beverages in Sikkim in India [3]. The aim of this research was to isolate and identify of LAB strains from Marcha that could be useful as bioprotective cultures. As a result of these screening, isolate DDM5 was found to produce bacteriocin like substances.

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Materials and Methods

Isolation of LAB

Samples of Marcha was collected aseptically in sterile tube from local market in Gangtok city and transported to laboratory for analyses. The isolation of lactic acid bacteria from Marcha was done by dissolving 1g of Marcha into 9 ml 0.85% sterile NaCl solution [4]. Serial dilution up to 10^{-7} was done and all dilutions were plated on MRS medium with 2% glucose and supplemented with 2% agar (pH-6.4) by spreading and the plates were incubated at 30ÚC for 48 h. Randomly ten distinct colonies from 10^{-6} plates and ten distinct colonies from 10^{-7} plates were picked and inoculated into MRS medium supplemented with 2% glucose (pH-6.4) for 24 h at 30ÚC. Purity of each single colony was checked by streaking again it into MRS agar plate and incubated for 24 h at 30ÚC and then sub-cultured in MRS broth.

Preparation of cell free supernatant

100 µl of culture was taken from 18-20 h old culture of MRS medium and inoculated in 5 ml of fresh MRS medium supplemented with 2% glucose (pH-6.4) and incubated at 37°C for 20 h. The culture was then centrifuged at 10,000g and 4°C for 15 min. The cell free supernatant was collected and pH was adjusted to 6.0 using sterile 4N NaOH. The supernatant was filtered through 0.2 µm filter membrane and the filtrate was used for checking the

antimicrobial activity on the growth of the indicator strains [5].

Screening of the isolates

The isolates were screened for antagonistic activity by agar well diffusion method using *E. coli* DH5α as indicator strain [5]. An overnight culture of the indicator strain (0.5%) was inoculated in Soft agar medium (0.7% agar). 10 ml of this soft agar with the indicator strain was plated over the agar base (1.5% agar). The wells were created with sterile tips and were inoculated with 50 µl of cell free supernatant of the isolate. The plates were then placed at 4°C for 6 h which allowed diffusing the supernatant into the agar. Then the plates were incubated at 37°C and examined after 24 h for zone of inhibition.

Antibiotic sensitivity profile of the isolate

The isolate DDM5 was tested for susceptibility to twenty eight antibiotics using agar disc diffusion test [6]. This method determines the efficacy of the drug by measuring the diameter of the zone of inhibition which results from diffusion of the antibiotic from the disc into the medium. The antibiotic tests were performed using commercially available antibiotic octodiscs from Hi-media Pvt. Ltd., India. The discs were placed over the soft agar inoculated with 1% DDM5 and incubated for 36-48 h at 30°C for observing the clear zone of inhibition around the discs. The results were compared with *Pediococcus pentosaceus* [4].

Carbohydrate fermentation profile of the isolate

The isolate DDM5 was tested for their ability to ferment various carbohydrates using the method of Kandler and Weiss [7]. 50µl of culture was inoculated into 5 ml MRS medium lacking glucose but containing phenol red and 2% other test carbohydrates. Then the test medium was incubated for 48 h at 30°C under static condition. The acid production was observed between 24-48h. The acid production was indicated by changing color of the phenol red indicator dye from red to yellow as a result of carbohydrate fermentation [6]. The results were compared with the published data of *Pediococcus pentosaceus* [4].

***In vitro* inhibition test**

The antimicrobial activity of screened isolate DDM5 was checked with different Gram positive and Gram negative indicator strains using agar well diffusion method. The indicator strains were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh and from the Agricultural Research Service Culture Collection (NRRL), U.S.A. The 50 µl filter sterilized (0.2 µm membrane) cell free supernatant of DDM5 was placed in the wells of soft agar inoculated with indicator strains over the base agar plate. The plates were incubated at the optimum growth temperature of the indicator strain used [5]. The antimicrobial activity was determined by measuring the distance of the clear zone around the wells.

Results and Discussion

Based on the finding of zone of inhibition around the well against *E. coli* DH5α as indicator strain, it was concluded that among the twenty isolates from marcha, the isolate DDM5 was selected, as it showed the maximum zone of inhibition (> 15 mm). The selected isolate was named after discover as DDM5. The antibiotic resistance, carbohydrate fermentation profiles of the isolate DDM5 was carried out for its characterization. The antibiotic susceptibility and carbohydrate fermentation profile of DDM5 was compared with the *Pediococcus pentosaceus* isolated from soil of Assam [4], as *Pediococcus pentosaceus* was also well known for producing antimicrobial substance [5]. The antibiogram of DDM5 has been shown in Table 1. By measuring the zone of inhibition around the octodiscs, the isolate DDM5 was found resistant to vancomycin, ciprofloxacin, norfloxacin, nalidixic acid and sensitive to bacitracin penicillinG, ampicillin like and *Pediococcus pentosaceus* [4] (Table 1). The resistance of lactic acid bacteria to vancomycin is a common characteristic of this group [8]. The antibiogram pattern of DDM5 and *Pediococcus pentosaceus* are in similar with few exceptions. The isolate DDM5 utilized glucose, sucrose, xylose, trehalose, mellibiose, galactose but did not show any activity towards rhamnose (Table 2). The isolate DDM5 could ferment raffinose unlike *Pediococcus pentosaceus* [4] shown in Table 2. The carbohydrate fermentation profiles of the isolate DDM5 and *Pediococcus pentosaceus* are 62% similar. The isolate DDM5 showed broad range of antagonistic activity. It inhibited the growth of Gram positive *Staphylococcous aureus* MTCC 737 and *Staphylococcus epidermis* MTCC 6810, *Leuconostoc citreum* NRRL B-742 and *Pediococcus pentosaceus* as well as Gram negative *Alcaligenes feacalis* MTCC 2952 and *E. coli.* DH5α (Table 3). The isolate DDM5 showed strong inhibitory effect against Gram negative bacteria as compared to *Pediococcus pentosaceus* ACCEL [9] and *Pediococcus acidilactici* LAB 5 [10]. Both *Pediococcus pentosaceus* ACCEL and *Pediococcus acidilactici* LAB 5 did not inhibit the growth of any Gram negative bacteria but were active against Gram positive *Staphylococcus epidermis* and *Staphylococcous aureus* [9, 10]. As the isolate DDM5 had shown clear zone of inhibition (>15 mm) against several major food borne pathogens, such as *E. coli, Staphylococcus epidermis, Alcaligenes feacalis* and *Staphylococcous aureus* in food stuffs, it could be potentially used as bio-preservatives in food systems.

Acknowledgement

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Table 1
Antibiogram of isolate DDM5 using antibiotic octodiscs on MRS agar

Sl. No.	Antibiotic	Concen-traion	DD M5	**Pediococcus pentosaceus*
1	Kanamycin (K)	30 µg	M	R
2	Lincomycin (L)	2 µg	M	S
3	Olaendomycin (OL)	15 µg	S	S
4	Penicillin G (P)	10 U	S	S
5	Tobramycin(Tb)	10 µg	R	R
6	Nalidixic acid (Na)	30 µg	R	-
7	Sulphamethoxazole (Sx)	50 µg	S	-
8	Amoxyclav(Ac)	10 µg	S	M
9	Cephalexin (Cp)	10 µg	M	M
10	Ciprofloxacin(Cf)	10 µg	R	R
11	Clindamycin(Cd)	2 µg	S	M
12	Co-Trimaxazole(Co)	25 µg	S	
13	Erythromycin(E)	15 µg	S	S
14	Tetracycline(T)	30 µg	S	S
15	Ampicillin (A)	10 µg	S	R
16	Carbenicillin	100 µg	S	S
17	Cephotaxime(Ce)	30 µg	S	M
18	Chloramphenicol (C)	30 µg	S	S
19	Norfloxacin (Nx)	10 µg	R	R
20	Oxacillin(Ox)	5 µg	M	M
21	Amikacin (Ak)	10 µg	R	R
22	Amoxycillin (Am)	10 µg	S	S
23	Bacitracin(B)	10 U	S	M
24	Novobiocin(Nv)	30 µg	S	M
25	Vancomycin (Va)	30 µg	R	R
26	Gentamicin (G)	10 µg	S	-
27	Cefexime (Cfx)	5 µg	R	-
28	Ceftazidime (Ca)	30 µg	R	-

R- *Resistant (0-0.1cm);* **M**-Moderate (0.2-0.8 cm); **S**- Sensitive (0.9-2.5 cm)

* The data of antibiogram of *Pediococcus pentosaceus* was taken from earlier report [4]

**Values in centimeter are the distance of zone of inhibition of growth of microorganism.

Table 2
Carbohydrate fermentation profile of isolate DDM5

Sl. No.	Carbohydrate	DDM5	**Pediococcus pentosaceus*
1	Xylose	+	+
2	Trehalose	+++	+++
3	Mellibiose	++	+++
4	Galactose	+++	+++
5	Mannitol	+++	-
6	Raffinose	++	-
7	Cellobiose	++	+++
8	Rhamnose	-	-
9	Maltose	+++	+++
10	Lactose	+++	++
11	Fructose	++	+++
12	Glucose	+++	+++
13	Sucrose	+++	+++

(+++): strongly positive, **(++)**: fairly positive, **(+):** weakly positive, **(-):** negative

* The data of carbohydrate fermentation profile of *Pediococcus pentosaceus* was taken from earlier published report [4].

Table 3

Inhibitory Spectra of DDM5 against some indicator strain

Indicator Strain	Media	Temp.(°C)	Sensitivity
E. coli DH5á	LB	37	+++
Staphylococcous aureus MTCC 737	NB	30	+++
Staphylococcus epidermis MTCC 6810	NB	30	++
Klebsiella oxytoca MTCC 3030	NB	37	-
Alcaligenes feacalis MTCC 2952	NB	30	+
Enterobacter aerogenes MTCC 7016	NB	30	-
L. mesenteroides NRRL B-640	MRS	30	-
L. dextranicum NRRL B-1146	MRS	30	-
L. mesenteroides NRRL B-1149	MRS	30	-
L. citreum NRRL B-742	MRS	30	++
L. mesenteroides NRRL-512	MRS	30	-
Pediococcus pentosaceus	MRS	30	++
Pseudomonas Fluorescens NRRLB-1612	NB	37	-
Wesiella confuse	MRS	30	-

(+++): zone>15 mm, (++): 10 mm<zone< 15 mm, (+): 5mm<zone<10mm, (-): no zone of inhibition

**Values in millimeter are the distance of zone of inhibition of growth of microorganism

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Optimized and Validated Spectrophotometric Methods for the Determination of Aripiprazole Using Ferric Chloride Based on Complexation Reactions

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ABSTRACT

Three simple and sensitive spectrophotometric methods (A-C) were developed for assay of aripiprazole (**APZ**) in pharmaceutical formulations. Methods A and Method B are based on the oxidation of APZ with Fe (III) and the estimation of reduced Fe(III) after chelation with either *o*-phenanthroline (*o*-phen) or bipyridyl (bipy) in the presence of acetate buffer solution. The absorbances of the colored complexes were measured at 508 nm or 519 nm for Method A and Method B respectively. Method C is based on the oxidation of APZ by Fe (III) in acidic medium, and the subsequent interaction of iron (II) with ferricyanide to form Prussian blue, with the product exhibiting an absorption maximum at 796 nm. Beer's law is obeyed over the ranges 0.5-7.0 µg/mL, 0.5-7.0 µg/mL and 0.5-9.0 µg/mL for Methods A, B and C, respectively. The calculated molar absorptivity values are 8.88 $x10^4$, 7.21 $x10^4$ and 7.74 $x10^4$ L/mol/cm for Method A, B and C, respectively, and the corresponding Sandal's sensitivities are $5.0x10^{-3}$, 6.2 $x10^{-3}$and 5.7 $x10^{-3}$ µg/cm^2.The results of the proposed procedures were validated statistically according to ICH guidelines. The proposed methods were applied successfully for the determination of APZ in tablets.

Keywords: Aripiprazole, determination, spectrophotometry, oxidation, tablet forms.

Introduction

Aripiprazole is a quinolinone derivative with the chemical name 7-[4-[4-(2, 3- dichlorophenyl)-1-piperazinyl] butoxy]-3, 4-dihydro-2(1H)-quinolinone (Fig 1). It is a psychotropic agent belonging to the chemical class of benzisoxazole derivatives and is indicated for the treatment of schizophrenia. It is a selective monoaminergic antagonist with high affinity for the serotonin Type 2 (5HT2), dopamine Type 2 (D2), 1 and 2 adrenergic and H1 histaminergic receptors. The different analytical techniques reported so far for the determination of this drug and its metabolites in biological samples as well as in pharmaceutical formulations include LC-MS-MS previously published [1-3]. The determination of aripiprazole in plasma by RP-LC [4] and that in bulk drug, solid dosage forms and related substances by RP-LC [5-8] were also reported. As far as sensitive and economical methods of assay are concerned, very few UV-Visible spectrophotometric methods have been reported for the quantification of aripiprazole based on extractable ion pair complexes [9-10] and UV spectrophotometric method [11]. Literature survey revealed that no attempt was made for the determination of APZ by Fe (III) in the presence of 1, 10-phenanthroline or 2, 2-bipyridyl or potassium ferricyanide. In view of this, we report three simple and sensitive spectrophotometric methods for the assay of APZ in pharmaceutical formulations are reported in this article. The methods are based on the reducing property of the cited drug by iron (III). The formed iron (II) is interacted with 1, 10-phenanthroline, 2, 2'-bipyridyl or ferricyanide to give colored species whose absorbances were measured at 508, 519 and 796 nm respectively. Such reaction schemes have successfully been used for spectrophotometric determination of many pharmaceutical compounds [12-14]. The developed methods are simple, accurate, precise and sensitive for the determination of APZ in tablets.

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Experimental

Apparatus

All absorption spectra were recorded using UV-Vis-

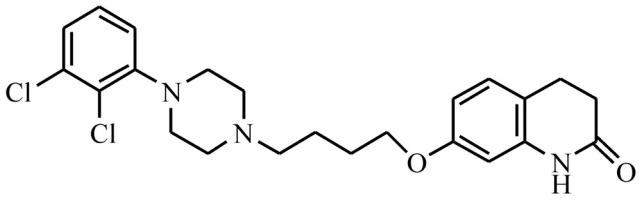


Fig. 1: Chemical structure of Aripiprazole

NIR spectrophotometer (Shimadzu 1601, Japan) equipped with 1 cm matched quartz cells by using a personal computer loaded with the UV-PC 3.9 software package. An electronic microbalance (Sartorius MC 5, Germany) was used for weighing the solid materials. A digital pH meter 802 (Systronic, India) was used for measuring the pH of solutions.

Materials and Reagents

All solvents and reagents used were of analytical grade. Double-distilled water was used throughout the investigation. The aqueous solution of 0.05 M ferric chloride (S.D. Fine Chem., Mumbai, India) was prepared by dissolving 0.8110 g of the chemical in 100 ml of distilled water and stored in a dark bottle. The stock solution was then diluted appropriately with distilled water to get 0.005 M working concentration for Method A, Method B and Method C. The solution was prepared afresh just before the experiment.

o-phenanthroline (0.02M) was prepared by dissolving 0.3960 g of *o*-Phenanthroline (S.D. Fine Chem., Mumbai, India) in 100 mL of distilled water.

2, 2' bipyridyl (0.02M) was prepared by dissolving0.3120 g of 2, 2' bipyridyl (E-Merck., Mumbai, India) in 100 mL of distilled water. Potassium ferricyanide (0.01M) was prepared by dissolving 0.3293 g of potassium ferricyanide (S.D. Fine Chem., Mumbai, India) in 100 mL of distilled water. The stock Potassium ferricyanide solution was then diluted appropriately with distilled water to get 0.002 M working concentration for method C. Sodium acetate- acetic acid buffer of varying pH [3-6.0] values were prepared by mixing appropriate volumes of 0.1M of sodium acetate (S.D. Fine Chem., Mumbai, India) and acetic acid (S.D. Fine Chem., Mumbai, India). The pH was adjusted with the aid of pH-meter.

A gift standard APZ sample was obtained from Inogent laboratories, Hyderabad, India. Three brand tablets were purchased from local medical shop. A stock standard solution containing 500µg $mL^{-1}$of APZ was prepared by dissolving accurately weighed 0.025 g of pure drug in a 50 mL of calibrated flask with methanol. The solution was further diluted with methanol to get working concentrations of 50 µg mL^{-1} APZ for all three methods.

Method A

Different aliquots of APZ solution corresponding to 0.5-7.0 µg mL^{-1} were transferred into a series of 10 mL volumetric flasks. To each flask 1.0ml of acetate buffer (pH 5), 2.0ml of 0.005M ferric chloride and 3.0 mL of o-Phenanthroline (0.02M) were added and kept in a water bath (75 ± 2 ^{0}C) for 20min, then immediately cooled to room temperature (27 ± 1^{0}C) using cold water and the solutions were made up to volume with distilled water. The absorbance of each solution was measured at 508 nm against the reagent blank. The calibration graph was constructed by plotting the absorbance versus concentration of the drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation.

Method B

Varying volumes of APZ solution corresponding to 0.5-7.0 µg mL^{-1} were transferred into a series of 10 mL volumetric flasks. To each flask 1.5 mL of acetate buffer (pH 4), 2.5mL of 0.005M ferric chloride and 3.0 mL of 2,2' bi-pyridyl (0.02M) were added and kept in a water bath (75 ± 2 ^{0}C) for 25min, then immediately cooled to room temperature (27 ± 1^{0}C) using cold water and the solutions were made up to volume with distilled water. The absorbance of each solution was measured at 519 nm against the reagent blank. The calibration graph was constructed by plotting the absorbance versus concentration of the drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation

Method C

Aliquot of a solution containing 0.5-9.0 µg mL^{-1} of APZ were transferred into a series of 10mL standard flasks. To each flask 3.0mL of ferric chloride (0.005M) was added and kept in a water bath (75 ± 2 ^{0}C) for 30min. the flasks were then added each with 3.0 ml of potassium ferricyanide and 1.0 ml of 1N HCl, cooled to room temperature, diluted to mark with distilled water and mixed well. The absorbance of the resulting solution was measured at 796 nm for APZ against a reagent blank prepared similarly. The concentration of the unknown was read from the calibration graph or computed from the regression equation.

Procedure for pharmaceutical formulations

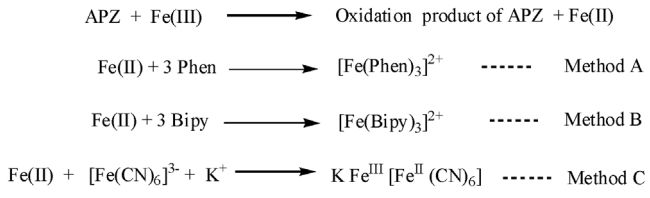
APZ containing ten tablets were weighed and ground into a fine powder. An amount of the powder equivalent to 25 mg of APZ was weighed into a 50-mL volumetric flask, 30 mL methanol added and shaken thoroughly for about 20 min, diluted to the volume with the same solvent, mixed, and filtered using Whatmann No. 41 filter paper into a 50-mL volumetric flask. The filtrate was further diluted with methanol to get concentration of 50 µg mL^{-1} of APZ for Method A, Method B and Method C. A suitable aliquot was then subjected to analysis.

Results and Discussion

It is known that 1, 10-phenanthroline and 2, 2-bipyridyl form highly stable, intensely red colored, water-soluble chelates with iron (II). The complex formation stabilizes the iron (II) oxidation state, as the formal redox potential of complexes (+1.06 V) is higher than that of the iron aqua-complex. These complexes were applied to the determination of reducing substances by measuring the amount of ferrous iron produced when the analyte solution is treated with an excess of ferric salt. Trace amounts of the reductants are conveniently measured by this way through spectrophotometric determination of ferrous iron using 1, 10- phenanthroline or 2, 2-bipyridyl.

The proposed methods involve the oxidation of APZ with ferric chloride and subsequent complexation of resulting Fe (II) with 1, 10-phenanthroline or 2, 2-bipyridyl to form a red-colored complex, $[Fe(phen)_3]^{2+}$, with an absorption maximum at 508 nm or

$[Fe(bipy)_3]^{2+}$ exhibiting an absorption maximum at 519 nm. The general reaction path of proposed methods is represented in scheme 1. The absorption spectra of colored complexes are shown in Figs 2 and 3.



Scheme-1

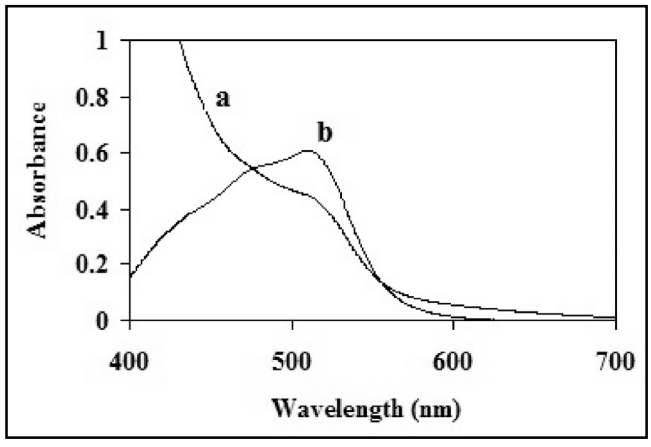


Fig.2: Absorption spectra. (b) Product of APZ (3 µg mL^{-1})-Fe (III)-Phen against reagent blank and (a) reagent blank against water

Optimum reaction conditions

The optimum reaction conditions for the quantitative estimation of APZ were established via a number of preliminary experiments

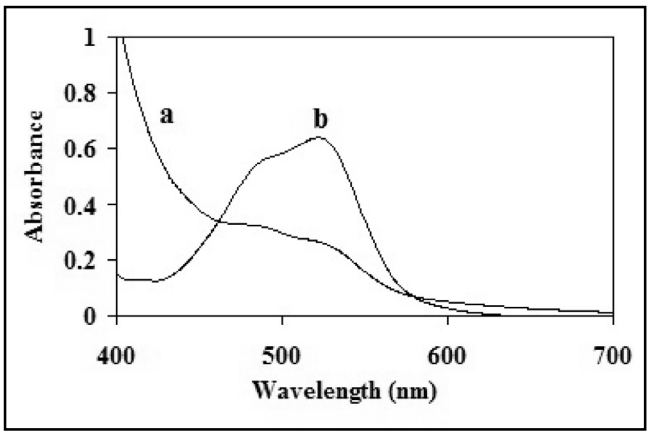


Fig.3: Absorption spectra. (b) Product of APZ (4 µg mL^{-1}) -Fe (III)-Bipy against reagent blank and (a) reagent blank against water

Effect of pH

The effect of pH for the quantitative determination of APZ with the proposed oxidation-complexation reaction over the pH range of 3-6 was examined using acetate buffer. At pH<3, the intensity of the absorption of the Fe(II)-phen or Fe(II)-bipy complex decreases most probably due to difficult oxidation of protonated forms of APZ by Fe(III).

An increase in pH >3.0 caused increased absorbance for both methods. So, pH 5.0 and 4.0 were chosen as the optimum pH in Method A and Method B, respectively. The optimum volume of buffer solution required was 1.0 ml in Method A and 1.5 mL in Method B in the total volume of 10 mL.

Effect of reagent concentrations

The effects of reaction variables (methods A and B) such as concentration of Fe (III) and 1, 10-phenanthroline or 2, 22 - bipyridyl have been investigated to develop maximum color by adding to 5.0 µg mL^{-1} of APZ. The effect of iron (III) chloride concentration on color development was studied. These results indicate that maximum absorbance was observed for 2.0 mL of 0.005M of ferric chloride for Method A and 2.5 mL of 0.005M of ferric chloride for method B. Larger volumes of Iron (III) chloride had no effect on the sensitivity of the reaction. Similar observations were made when varying volumes of 0.02M of phenonthroline or bipyridyl solution were added to fixed amounts of APZ (5.0 µg mL^{-1}) and diluted to 10 mL after full color development. It is found that the absorbance reaches its maximum when the amount of reagent is 3.0 mL.

Effects of temperature and heating time

The formation of colored complex was slow at room temperature and required longer time for completion. Hence efforts were made to accelerate by carrying out the reaction at higher temperatures. It was observed that the maximum absorbance was observed after heating the reaction mixture

on a water bath at 75°C for about 20 min and 25 min with Fe^{2+}-phen and Fe^{2+}-bipy colored complexes, respectively. Further heating caused no appreciable change in the color. The color of the complexes formed remained stable at room temperature for more than 12 h.

Method C

Absorption spectra

Fig. 6 shows the absorption spectra of the reaction product of APZ with iron (III) ferricyanide and the reagent blank. The greenish blue product from the studied drug exhibits an absorption maximum at 796 nm. The optimum conditions were established by varying parameters, such as iron (III), ferricyanide, reaction time and acid concentrations.

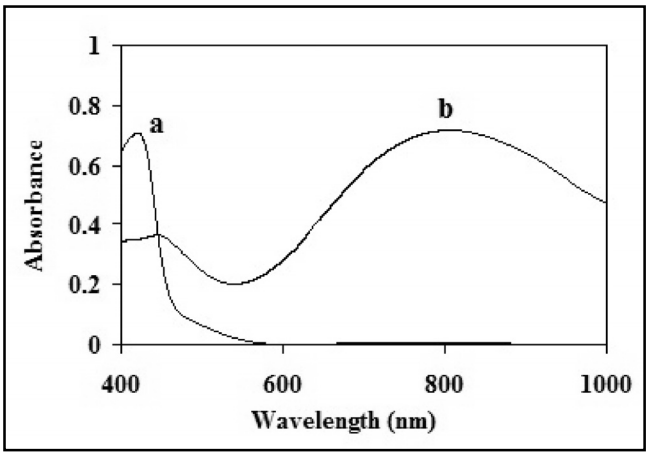


Fig.4: Absorption spectra. (b) Product of APZ (4 µg mL^{-1}) -Fe (III)-K_3 [Fe (CN)$_6$] against reagent blank and (a) reagent blank against water

Optimum iron (III) and ferricyanide concentrations

When a study on the effect of iron (III) chloride concentration on the color development was performed, it was observed that the absorbance increased with increase in the volume of 0.005M iron (III) solution and reaching a maximum upon the addition of 3.0 mL of the 0.005M iron (III) solution to 5.0 µg mL^{-1} of APZ. The influence of the amount of potassium ferricyanide on absorbance was studied. It is found that the absorbance reaches its maximum when the amount of potassium ferricyanide is 3.0 mL.

Effects of temperature and heating time

The color reaction occurred at room temperature, though at high temperature the color developed more rapidly. The absorbance of complex was measured different time intervals at 75± 2^0C in water bath. It was observed that the absorbance was maximal when heating time was equal to 30 min. The temperature of 75 ± 2^0C and a reaction time of 30 minutes were selected for reproducible results. Further heating caused no appreciable change in the color. The absorbance of the complexes remained constant at room temperature for more than 12 h.

Effect of nature of acid and its concentration

The reaction product, Prussian green, was found to flocculate within 20–30 min of color development. To delay the flocculation, addition of acid after full color development and before diluting to the mark was found necessary. Hydrochloric acid was found to give more stable color and reproducible results compared to sulphuric acid. A 1.0 mL volume of 1N HCl in a total volume of 10 mL was found to be adequate.

Validation of proposed methods

Linearity

Under optimum conditions, a linear relation was obtained between absorbance and concentration of APZ in the range 0.5-7.0 µg/mL in Method A and Method B and 0.5-9.0 µg/mL in Method C. The regression analysis of the plot using the method of least squares was made to evaluate the intercept (a), slope (b), regression coefficient (r^2) and standard deviations of slope and intercept (Table 2). In all cases, Beer's law plots were linear with good correlation coefficients as shown Table 1. The moderately high sensitivity of the method was indicated by the fairly high value of molar absorptivity and low values of sandell sensitivity. The limits of detection (LOD) and limits of quantitation (LOQ) [15] were determined using the formula: LOD or LOQ= k SDa/b, where k=3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope.

Table 1
Analytical and regression parameters of proposed methods

Parameter	Method A	Method B	Method C
λ_{max} (nm)	508	519	796
Beers law limit (µg/mL)	0.5-7.0	0.5-7.0	0.5-9
Molar absorptivity (l mol^{-1} cm^{-1})	$8.8751x10^4$	$7.2128x10^4$	$7.7406x10^4$
Sandell's sensitivity (µg/cm^2)	0.005	0.0062	0.0057
Regression equation (Y = a + bC)			
Slope (b)	0.1979	0.1609	0.1726
Intercept (a)	0.0163	0.0063	0.0133
Correlation coefficient (r^2)	0.9997	0.999	0.9991
Standard deviation of slope (S_b)	$1.4x10^{-3}$	$2.1\ x10^{-3}$	$1.79\ x10^{-3}$
Standard deviation of intercept (S_a)	$5.87\ x10^{-3}$	$8.3\ x10^{-3}$	$9.56\ x10^{-3}$
Detection limit LOD (µg/mL)	0.098	0.17	0.18
Quantification limit LOQ (µg/mL)	0.297	0.51	0.55

Precision and Accuracy

In order to determine the accuracy and precision of the proposed methods, solutions containing three different concentrations of APZ were prepared within linearity and analyzed in six determinations. The analytical results of precision and accuracy are show in Table .2. The precision of the proposed methods is fairly high, as indicated by the low values of SD and %RSD, respectively.

Table 2
Evaluation of precision and accuracy of proposed methods

Proposed method	Concentrations(µg/mL)		RSD (%)	R.E (%)
	Taken	Found*±SD		
Method A	1	1.01±0.025	2.47	1.0
	3	3.03±0.06	1.98	1.0
	6	6.05±0.078	1.29	0.83
Method B	1	0.99±0.018	1.81	-1.0
	3	3.02±0.048	1.59	0.67
	6	6.03±0.078	1.60	0.5
Method C	1	1.01±0.018	1.78	1.0
	3	3.04±0.062	2.04	1.33
	7	6.98±0.078	1.12	-0.28

*Mean value of six determinations; SD. Standard deviation; RSD. Relative standard deviation ; RE. Relative error

Accuracy and recovery

The accuracy and reliability of the methods were ascertained through recovery experiments. To a fixed and known amount of drug in the tablet powder, pure APZ was added at two different levels, and the total content was found by the proposed methods. The recoveries of the pure drug added to the tablet powder were shown in (Table.3). The results reveal that the proposed methods are not liable to interference by tablet fillers, excipients and additives usually formulated with pharmaceutical preparations.

Analysis of pharmaceutical formulations

The results (Table 4) indicate that the methods developed compare well with the reference method [11] with respect to accuracy and precision.

Conclusions

The methods proposed are fairly simple and do not require any pretreatment of the drug and tedious extraction procedure. The methods have been demonstrated to be free from rigid experimental conditions. The procedures are based on well established and characterized redox and complex formation reactions and use cheaper and readily available chemicals. The methods have wider linear range with good accuracy, precision and stability of the colored species for $\leq$ 12 h. Thus, the developed methods are sensitive, selective, offer advantages of reagent availability, stability, less time consumption and free from interferences by common additives and excipients qualifying them for the analysis of aripiprazole in pharmaceutical formulations.

Acknowledgement

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Table 3
Results of recovery experiments by standard addition method

Proposed methods (%)Recovery* ± SD

Proposed methods	Formulation taken (µg/mL)	Pure drug added (µg/mL)	ARPIT-10	ARIP MT-10	ARPIZOL-10
Method A	3	1.5	99.6±1.83	102.9±1.77	99.22±1.36
	3	3	101.67±1.9	102.16±1.05	99.6±0.86
Method B	3	1.5	101.06±0.35	100.91±2.1	98.48±1.84
	3	3	100.67±1.42	99.84±0.77	100.17±0.57
Method C	4	2	100.81±2.6	98.91±1.4	99.2±1.82
	4	4	100.52±1.93	101.07±2.03	99.07±1.57

*Mean value of three determinations

Table 4
Application of proposed methods to the determination of APZ in pharmaceutical formulations

Pharmaceutical preparations #	Reference method (%Found* ± SD)	Proposed methods (% Found* ± SD)		
		Method A	Method B	Method C
ARPIT-10[a]	101.81±1.31	100.08±1.94 t=1.81 F=2.14	101.33±1.46 t=0.599 F=1.24	100.12±1.68 t=1.93 F=1.64
ARIP MT-10[b]	101.61±1.79	101.09±1.87 t=0.49 F=1.1	99.95±0.94 t=1.41 F=3.62	101.38±1.95 t=0.12 F=1.18
ARPIZOL-10[c]	99.73±1.18	98.79±1.75 t=0.732 F=2.2	100.3±0.65 t=1.09 F=3.29	100.14±1.5 t=0.52 F=1.61

Marketed by: a. Crescent Pharma, India; b. Torrent Pharma, India; c. Sun Pharma, India. All aripiprazole tablets containing 10 mg per tablet
*Mean value of six determinations. The theoretical values of t (2.57) and F (5.05) at confidence limit at 95% confidence level and five degrees of freedom (p=0.05)

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