DEVELOPMENT AND PHARMACOLOGICAL EVALUATIONS OF ECONAZOLE NITRATE MICROSPERES ENRICHED GEL

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ABSTRACT

The objective of the current investigation is to reduce dosing frequency and improve patient compliance by designing and systematically evaluating sustained release microspheres enriched gel of econazole nitrate. An antifungal agent, Econazole nitrate, is delivered through the microparticulate system using ethyl cellulose as the controlled release polymer. Microspheres were developed by the emulsion solvent diffusion-evaporation technique by using dichloromethane solvent system. The resulting microspheres were evaluated for particle size, morphology, production yield, viscosity, gel strength, mucoadhesive force, spreadability, *in vitro* drug release behavior and *in vivo* studies. The formulated microspheres were discrete, spherical with relatively smooth surface, and with good spherical shape. The particle size ranged from 6.37 to 26.03 µm and the microspheres enriched gel was found to be in the range 29694 to 53896 cps. The MLG formulations exhibited maximum gel strength was found to be (300 sec.), highest value of mucoadhesion force was found to be (34.55 dynes/cm^2), and remarkable spreadability was found to be in range range (40.02-53.03 gm.cm/sec). The microspheres were incorporated in various concentrations of gel formulations and the drug release from these formulations was up to 9 hours.

Keywords: *In vivo studies,* SEM, Microspheres enriched gel, HPLC

INTRODUCTION

Imidazole compounds are useful in the treatment of fungal keratitis, the available imidazoles are clotrimazole, miconazole, ketoconazole and econazole [1]. Econazole nitrate (EN) which is: 1-[2,4-dichloro-β -(pchlorobenzyloxy) phenethyl]-imidazole nitrate [2,3]. It is broad spectrum antifungal drug against yeast, mold, dermatophytes and it is quite active against some bacteria of the actinomycets, and highly active against some gram positive cocci and bacilli [4]. Candidiasis is the fungal infection that can manipulate the private parts. Genital infection is extremely more recurrent in women than men, nevertheless when it does appear in males; thrush affects the head of the penis and the foreskin [5]. Emerging modified release systems for highly water-soluble drugs have constantly been a task to the pharmaceutical technologists [6–8]. Microspheres are unique of the multi-particulate dosage forms that have been formulated to modify or retard the drug release rate of the highly water soluble drugs in pharmaceutical formulations. They characterize a polymeric matrix system including the drug in a state of uniform distribution throughout the matrix [9–11].

Cellulose based polymers such as ethylcellulose (EC) find extensive application in the preparation of matrix-type microspheres of water-soluble drugs to control the dissolution rate of drugs from the dosage forms [12–14]. Numerous formulation and process parameters, such as type of organic solvent, drug/polymer ratio, emulsion stirring rate, and phase ratio of the emulsion system, can influence the physicochemical properties of matrix-type microspheres, especially the drug release rate, to a greater or lesser extent. In recent years, several attempts to modify the drug release rate have been investigated, such as the use of additives like various surfactants [10, 15]. One of the new popular approaches is the addition of a plasticizer having a higher affinity towards the dissolution media, which may enhance drug release by acting as a pore former in the matrix structure of the microparticles [16–19]. The recent work was envisaged to reduce dosing frequency on adherence to topical therapies by designing econazole nitrate microspheres enriched gel.

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MATERIALS AND METHODS

Materials:

Econazole nitrate (SM Pharmaceuticals Sdn. Bhd. Sungai Petani, Malaysia), which is a water soluble drug, was chosen as a model drug. Polyvinyl alcohol

(Esses, United Kingdom) was used as surfactant. Ethyl cellulose (Esses, United Kingdom) was used as a matrix-forming agent and xanthan gum (Esses, United Kingdom) was used as a gelling agent in this study. All other chemicals were of analytical or reagent grade and used without further purification.

METHOD

Preparation of Microsponges:

Formulation of drug-loaded microspheres was carried out by the emulsion solvent diffusion-evaporation method [20]. The drug and polymer were used in different ratios with formulation batches MS1 to MS8, these ratios were shown in Table 1. An accurately weighed quantity of econazole nitrate (100 mg) and matrix-forming agent ethyl cellulose was dissolved at room temperature in dichloromethane. This solution was slowly introduced into 150 ml of 0.1 % PVA aqueous solution at room temperature and dispersed to form emulsion at stirring rates of 2000 rpm using a mechanical stirrer. Agitation offered by stirrer breaks the poured polymer solution to form an oil-in-water (O/W) type emulsion. This emulsion was formerly stirred for about 2 hours at room temperature. After stirring, the solidified microspheres were recovered by filtration, washed with distilled water. Recovered microspheres filtered and were dried at 40°C for 6h to remove solvents.

Preparation of microsphere enriched gel (MEG):

Accurate weighed amount of xanthan gum was soaked in distilled water for overnight and resultant was dispersed slowly in appropriate amount of microsphere with the help of overhead stirrer. The appropriate gellants was selected on the basis of compatibility with microsphere structure, texture and comfort of spreadability.

Viscosity Studies:

The rheological studies were conducted by using brookfield programmable DVII+ Model pro II type (USA). The viscosity of MEG was determined at 0.3 rpm and means of two readings were used to estimate the viscosity [21].

Particle size studies:

The mean particle size and size distribution of the econazole microspheres were determined by Olympus 70G camera scope 9 DN-117M, USA. About 0.5 mg of microspheres were dispersed in purified water in the sample dispersion unit and then analyzed. Each determination was carried out in triplicate [22].

Scanning electron microscopy:

The morphology of microspheres was observed by Phenoworld scanning electron microscopy (Netherlands). Prepared microspheres were coated with gold and studied under vacuum at room temperature [22]. The microspheres were viewed at an accelerating voltage of 4.8kV.

Determination of loading efficiency:

To determine the loading efficiency, accurately weighted portions from each batch of econazole microspheres were dissolved in methanol, and this clear solution was analyzed for loading efficiency. The solution was then filtered and determined by RP HPLC method. RP HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. The LC solution version 1.25 was used for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 1.0 ml/min using a mobile that is phase constituted of acetonitrile, 0.5 mm ammonium acetate (pH 3.0) (60:40, v/v), and detection was made at 260 nm. The mobile phase was prepared daily, filtered through a 0.45µm membrane filter (Millipore) and sonicated before use. A Thermo C18 column (25cm × 4.6mm i.d., 5µ) was used for the separation. The drug content was calculated from the calibration curve and expressed as loading efficiency [23].

Determination of mucoadhesive force:

The mucoadhesive force of all the optimized batches was determined as follows, a section of the chicken mucosa fixed with mucosal side out onto each glass vial using rubber band. The vial with chicken mucosa was connected to the balance in inverted position while first vial was placed on a height adjustable pan. Microspheres enriched gel was added onto the mucosa of first vial. Then the height of second vial was so adjusted that the mucosal surfaces of both vials come in intimate contact. Two minutes time of contact was given. Then weight was kept rising in the pan until vials get detached. Mucoadhesive force was the minimum weight required to detach two vials. The chicken mucosa was changed for each measurement [24].

Detachment stress (dynes/cm^2) = mg/A

Where m is the weight added to the balance in grams; g is the acceleration due to gravity taken as 980 cm/s^2; and A is the area of tissue exposed.

Measurement of Gel Strength:

A sample of 50 gm of microspheres enriched gel was placed in a 100 ml graduated. The apparatus for measuring gel strength (weighing 27 gm) was allowed

to penetrate in gel. The gel strength, which means the viscosity of the gels was determined by the time (seconds), the apparatus took to sink 5cm down through the prepared gel [24].

Spreadability:

For the determination of spreadability, excess of sample was applied in between two glass slides and was compressed to uniform thickness by placing 1000g weight for 5 min. weight (50 g) was added to the pan. The time in which the upper glass slide moves over to the lower plate was taken as measure of spreadability [24].

S= ML/T

Where,

M = weight tide to upper slide (g)

L = length moved on the glass slide (cm)

T = time taken (sec)

Diffusion studies:

The *in vitro* release of MEG formulations were studied using cellophane membrane using modified apparatus. The dissolution medium used was phosphate buffer, freshly prepared (pH 7.4). Cellophane membrane previously soaked overnight in the dissolution medium, was tied to one end of a specifically designed glass cylinder (open at both ends). Equivalent to 1% w/w of econazole nitrate microspheres enriched gel was accurately placed into this assembly. The cylinder was attached to stand and suspended in 200 ml of dissolution medium maintained at 37±1°C, the membrane just touching the receptor medium surface. Aliquots, each of 5 ml volume were withdrawn periodically at predetermined time interval of 0.15, 0.30, 1.0, 2.0, 3.0, 4.0, 5.0 up to 9 hours and replaced by an equal volume of the receptor medium. The samples were appropriately diluted and measured by using RP HPLC method.

In vivo evaluation of therapeutic efficacy:

Animals: Adult wistar rats (280 ± 10 g) of either gender were obtained from SCS College of pharmacy, India. The animals were housed in large, spacious polyacrylic cages at an ambient room temperature with 12-h light/12-h dark cycle. Rats had free access to water and rodent pellets diet. The study was approved by the Institute Animal Ethics Committee of the SCS college of pharmacy, India and all the animal experiments were carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines.

Acute toxicity testing: The female rats were used for the acute toxicity testing. Hair present in the dorsal surface of the animal (2 X 2 cm) was removed by applying hair remover and cleaned with alcohol. The screening are was marked (1 X 1 cm) and 0.5 g of a microspheres enriched gel was applied to the surface of an animal's skin. During the observation period (14 days), signs such as erythema and edema were assessed [25].

Evaluation of therapeutic efficacy: The male rats were used for the experiment. The rats were divided into the four groups *viz.,* normal control (group I), *Candida glabrata* control (group II), standard treatment group (group III) and microspheres enriched gel treatment group (group IV). Group II to IV animals were changed with intravenous methylprednisolone (5mg/kg) for 3 days for induction and maintenance of cell-mediated immunosuppression (Organisms from stock isolates were stored in nutrient agar at 27°C, streaked onto nutrient broth, and incubated at 37°C for 24 h and included culture was used for further experiment).

Candida glabrata culture was diluted with PBS and swabbed in smooth muscle of rat pennies and allowed to grow for 3 days until the growth of *Candida* was observed on ischiocavernosus smooth muscle. The colony growth was confirmed by counting colony-forming-unit. The animals which as CFU value of more than 3 CFU/ml ware included in the study. The animals were treated for week period and visually observed its physical changes. The swab culture was collected on initial day, 4th and 7th day of the experiment for microscopical evaluation. End of the experiment the animals were sacrificed and ischiocavernosus smooth muscle was collected from all the experimental animals and preserved in 10% formalin.

Microscopical evaluation: The colony was collected in sterile cotton swab and transferred into 0.5 ml sterile phosphate buffer saline (PBS). The mixture was diluted 10 fold and inculcated in nutrient agar media, incubated for 48 h at 37°C. The yeast count was expressed as $\log_{10}$ of CFU/ml of PBS.

Histopathologic analysis: The liver and pancreas were dehydrated with alcohol for 12 h each and cleaned with xylene for 15-20 min. The tissue blocks were prepared and the blocks were cut using microtome to get sections of thickness 5 µm. The sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied and allowed for drying. Finally, Serial cross sections of the tissues were obtained and stained with hematoxylin and eosin stain for fungal visualization [26].

Statistical analysis:

All the data were expressed as mean ± SEM. Statistical significance between the groups were tested using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test post-hoc test. A P less than 0.5 were considered significant.

RESULT AND DISCUSSION

In this paper, different econazole nitrate loaded matrix-type ethyl cellulose microsphere formulations were prepared by the W/O emulsion- solvent evaporation technique. This technique works best for highly dosed freely water-soluble drugs like econazole nitrate to provide high drug loading and suitable particle size. Microspheres were prepared with drug and polymer at two different ratios (0.5-1.0 and 1-1.25 w/w) to investigate the particle size on the physicochemical characteristics of the prepared microspheres. When a polymer is incorporated into a drug material, it improves the matrix formation around the microspheres. According to this approach, the addition of a suitable amount of a polymer as a matrix forming agent to the microsphere formulations can modify the various physicochemical properties of the prepared formulations, especially the release rate of the incorporated drug.

The results of the encapsulation efficiency and particle size analysis given in Table 1. The loading efficiency of econazole nitrate into the microspheres were found in a wide range for all the formulations (55.41 to 61.24%) and were affected neither by the drug/ polymer ratios studied. All of these results indicated that the emulsion solvent diffusion-evaporation technique was a very suitable preparation method for encapsulation of hydrophilic drugs.

The statistics describing the mean particle size of the econazole microspheres showed that the mean particle diameter was affected by both the drug/polymer ratio for all the formulations as seen in Table 1, the mean particle diameter of the microspheres increased with increasing polymer ratio in the disperse phase based on the viscosity of the all the prepared formulations. This can be explained due to increasing the viscosity of the disperse phase by increasing the drug amount and reducing the polymer ratio; the diameter of the droplets in the emulsion system increases, which is mirrored in the increased mean diameter of the microspheres as shown in table 1.

The shape and surface characteristics of the optimized microsphere formulations are illustrated in Fig. 2. The in vitro release profiles of the econazole nitrate from microspheres enriched gel formulations based on xanthan gum ratio are given in figure 4. As the gelling agent concentration increased from 0.5 to 1.25, the drug release rate decreased based on the concentration of gelling agent. It is apparent from the drug release

SEM observations showed that all of the microspheres prepared were spherical in shape, and the morphological characteristics of the EC microsphere formulations were extensively affected by the polymer ratio investigated. As shown in Fig. 3.

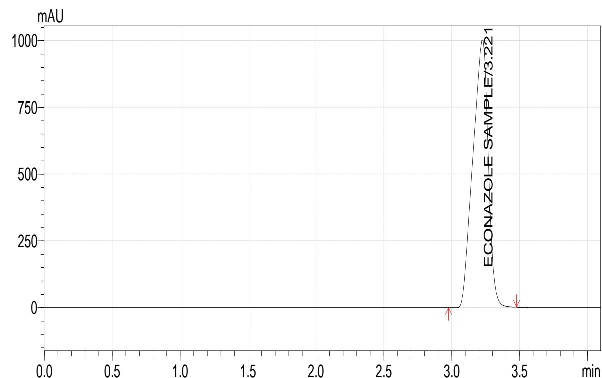

Figure-1: Typical chromatogram of econazole nitrate

Figure-2: Showing the SEM studies with 0.5 mg of polymer

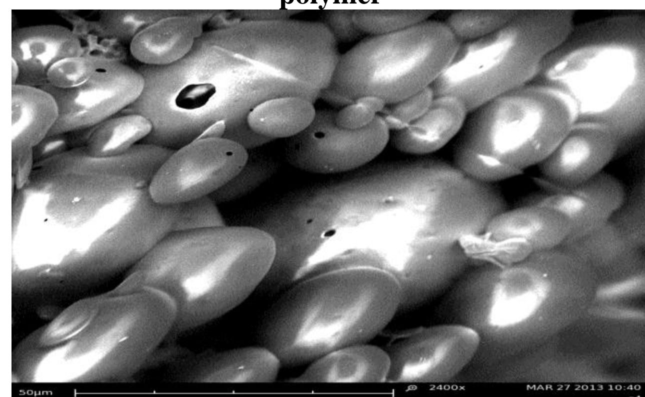

Figure-3: Showing the SEM studies with 1.25 mg of polymer

profiles that EC microspheres prepared with 1% of xanthan gum showed the fastest release behavior at each time interval. A further increase in the xanthan amount causes a reduction in the drug release.

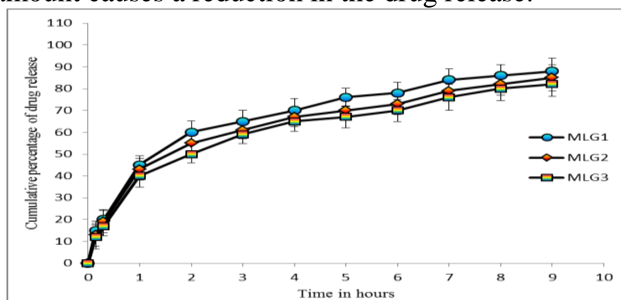

Figure-4: Showing the Drug release of microspheres enriched gel

All the MEG formulations were behaving as shear thinning systems as shown in figure 5. The formulation MEG3 having the maximum concentration of xanthan gum (2%) showed maximum viscosity in gels forms and hence was not suitable for application.

The values of spreadability denote that the gel is easily spreadable by small amount of force. The spreadability of formulation MEG1 AND MEG2 was found to be 41.04 and 40.02 gm.cm/sec respectively; this indicates all the optimized concentration of gel to spread on skin easily.

Gel strength is important because strong gels will support a much higher pressure than weak gels before they are washed out of the targeted site. The formulations exhibited moderate gel strength as seen in Table. 2, which may be due to concentration of xanthan gum 1 to 2%.

The mucoadhesive force is an important physic-chemical parameter for topical application. The formulations showed maximum mucoadhesive force, these may be due to increase in concentration of gelling agent in the formulations as seen in Table 2.

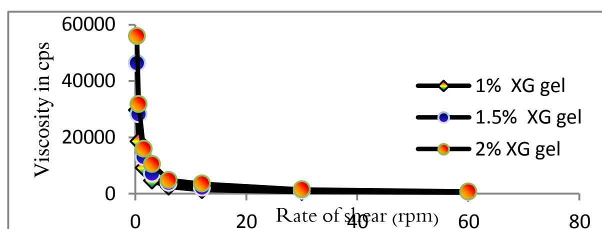

Figure- 5: Showing the viscosity behavior of gels with various rpm

The therapeutic efficacy of MEG was compared with *Candida glabrata* control by quantitative microbiological analysis and histopathological evaluations. MEG and standard marketed formulation treated animals showed significant reduction of CFU count on 4th day of the treatment onwards. The efficacy of the MEG is comparable with standard marketed formulation as seen in (Figure. 6 and 7).

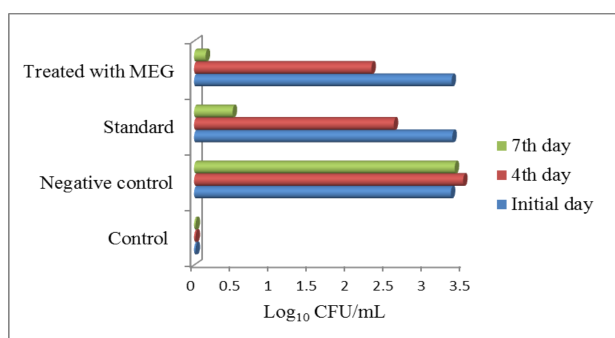

Figure-6: Quantitative microbiological analysis of the *Candida glabrata* in rat penal smooth muscle surface.

CONCLUSION

Morphological studies of microspheres showed that were affected by drug and polymer ratio used in the formulation. The results obtained also showed that an increase in the concentration of gelling agent in a reduction in the release rate of drug from the microspheres. The release data showed that the higher viscosity of medium and the lowest release rates were obtained from gel containing econazole microspheres. The *in vivo* animal studies conducted on adult wistar rats against *Candida glabrata* in penile candidiasis revealed that the formulation made with econazole nitrate microspheres enriched gel were eradicating completely the fungal burden in comparison to the standard.

Table-1: Composition of econazole nitrate microspheres

Formulation Code	Drug/polymer ratio (w/w)	Loading efficiency (%)*	Production yield (%)	Mean particle size (μm)*
MS1	1.0:0.5	92.45±0.91	55.41	6.37±1.52
MS2	1.0:0.75	92.62±0.54	53.16	7.46±1.40
MS3	1.0:1.0	91.35±0.34	59.12	12.80±1.85
MS4	1.0:1.25	90.43±0.81	60.75	26.03±1.92
MS5	0.5:1.0	89.17±0.95	57.61	6.75±1.61
MS6	0.75:1.0	90.61±0.52	56.88	8.12±1.20
MS7	1.0:1.0	92.66±0.36	59.10	12.23±1.75
MS8	1.25:1.0	91.98±0.73	61.24	19.31±1.08

*mean ± SD, n=3

Table-2: Characteristics of econazole microspheres enriched gel

Formulation	Viscosity (cps)	Mucoadhesive force(dynes/cm^2)	Spreadability (gm.cm/sec.)	Gel strength (seconds)
MEG1 (1% XG)	29694	30.16±0.32	53.03±0.18	128
MEG2 (1.5% XG)	46290	32.83±0.45	41.04±0.13	290
MEG3 (2% XG)	53896	34.55±0.14	40.02±0.16	300

* XG- Xanthan Gum

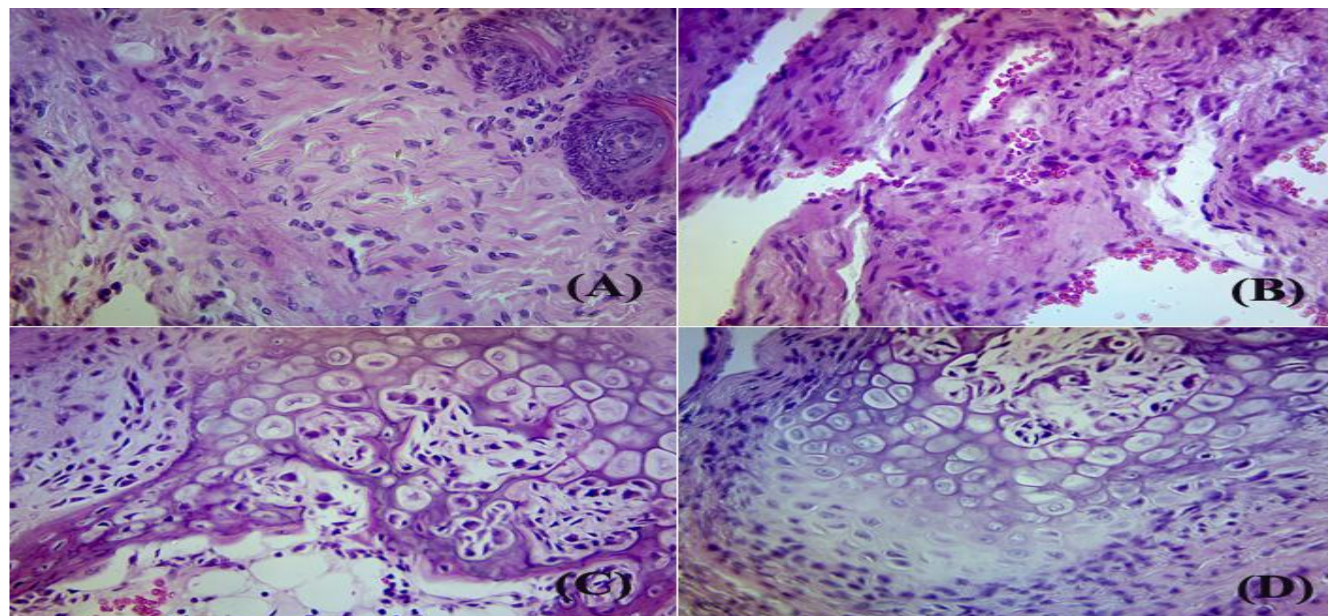

Figure-7: Histopathological analysis of the rat penal smooth muscle section from (A) control showed normal artícheture and (B) showed *Candia* microorganism infection in smooth muscle surface (C) and (D) showed reduction in growth of *Candia* due to antifungal effect of standard and MEG, H and E , 40X

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