Ethosomes: A novel delivery system for antifungal drugs in the treatment of topical fungal diseases

M.K. Bhalaria, Sachin Naik & A.N. Misra*
Pharmacy Department, Faculty of Technology and Engineering, The Maharaja Sayajirao University of Baroda, Vadodara 390 001, India

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Aim of this work was to prepare and characterize fluconazole (FLZ) encapsulated ethosomes, incorporate it in suitable dermatological base, and assess its comparative clinical efficacy in the treatment of Candidiasis patients against liposomal gel, marketed product and hydroethanolic solution of the drug. Drug encapsulated ethosomes and liposomes were prepared and optimized by “Hot” method technique and lipid film hydration technique. Vesicular carriers were characterized for % entrapment efficiency, particle size and shape, in vitro drug diffusion study, mean % reduction in dimension of Candidiasis lesion and stability study by using suitable analytical technique. Vesicle size and drug entrapment efficiency of the optimized ethosomes and liposomes were found to be 144±6.8 nm and 82.68% and 216±9.2 nm and 68.22% respectively. Microscopic examinations suggest ethosomes to be multilamellar spherical vesicles with a smooth surface. The differential scanning calorimetry results suggest high fluidity of the ethosomes than liposomes. In vitro drug diffusion studies demonstrated that % drug diffused from ethosomes was nearly twice than liposomes and three times higher than the hydroethanolic solution across rat skin. From the clinical evaluation, the developed novel delivery system demonstrated enhanced antifungal activity compared to liposomal formulation, marketed formulation and hydroethanolic solution of the drug.

Keywords: Delivery system, Ethosomes, Fluconazole, Hydroethanolic solution, Liposome, Phospholipids

Fluconazole (FLZ) is a broad-spectrum, antifungal agent used for dermal infection caused by various species of pathogenic dermatophytes. It is used for the treatment of Candidiasis.

Candidiasis is an infection caused by Candida, a yeast-like fungus. Candidiasis usually affects the skin and mucous membranes (soft, moist areas around body openings, like the mouth and anus). Children of any age may develop Candida paronychia, an infection of the skin around the nails. Older girls and women may develop Candida vulvovaginitis, an infection of the vagina and the area around the vaginal opening. A number of anti-fungal creams are now in use, and employed for a variety of dermatological and other mycotic infections. However many types of such fungal infections have proven to be persistent and defeat any attempts to control or cure them. In addition, local reactions including irritation and burning sensation may occur in patients treated topically.

In dermal and transdermal delivery, the skin is used as a portal of entry for drugs, for localized and systemic treatment. Because of the barrier properties of the outer layer of the skin, in many cases, permeation-enhancing agents are needed to achieve therapeutic levels of drug. Classic liposomal systems were found to be effective at forming drug reservoir in the upper layers of the skin, for local skin therapy. Ethosomal carriers, phospholipids vesicular system containing relatively high concentrations of alcohol, were effective at enhancing dermal and transdermal delivery of both lipophilic and hydrophilic molecules.

Ethosomal systems are vesicular systems composed mainly of phospholipids, ethanol, propylene glycol and water. Unlike classic liposomes, that are known mainly to deliver drugs to the outer layers of skin, ethosomes were shown to enhance permeation through the stratum corneum barrier. Ethosomes are shown to entrap drug molecule with various physicochemical characteristics i.e. of hydrophilic, lipophilic, or amphiphilic.

Hence, an attempt has been made to formulate a supplement dermal therapy of FLZ. The ethosomal encapsulation of FLZ was found to
increase the skin residence time leading to a faster healing of external lesions and to a reduction of side effects and duration of therapy\textsuperscript{22,23}. FLZ ethosomes were prepared by “Hot” technique with required modifications after optimizing process and formulation variables\textsuperscript{24,25}. The present investigation focuses on comparative \textit{in vitro} skin permeation study and the clinical evaluation of new ethosomal gel formulation on Candidiasis patients\textsuperscript{26,27}.

**Materials and Methods**

Fluconazole (FLZ) was obtained as a Gift sample from Pfizer Pharmaceutical Pvt. Ltd., Baroda. Soyaphosphatidyl choline (SPC) was purchased from Sigma chemical co. USA. Ethanol and propylene glycol was purchased from HiMedia Laboratory Pvt. Ltd., Mumbai (India). All other reagents used were of analytical grade.

**Preparation of vesicular systems**

*Ethosomes preparation*—Ethosomes were prepared by “Hot” method with required modification reported by United State Patent –5,540,934 using Magnetic Stirrer, Probe Sonicator as well as High Pressure Homogenizer. Drug (FLZ): soyaphosphotidyl choline (SPC): ethanol ratios were altered and drug entrapment efficiency was studied. The ethosomal system prepared here comprised of 10 mg drug, 2-6\% (w/v) soyaphosphotidyl choline, 10-45 \% (w/v) ethanol, 20\% (w/v) propylene glycol and water up to 100\%. Drug was dissolved in the ethanol and propylene glycol mixture and added to the phospholipids dispersion of water at 40\°C on magnetic stirrer and mixing for 5 min. The preparation was sonicated at 4\°C for 3 cycles of 5 min each with rest of 5 min between each cycle using Probe sonicator. The formulation was homogenized at 15,000 psi pressure in 3 cycles using high-pressure homogenizer to get Liposome.

**FLZ vesicle characterization**

*Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM)*—Ethosome vesicles were visualized using TEM Philips Technai electron microscope (TEM, Eindhoven, The Netherlands) with an accelerated voltage of 20 to 200 KV. Sample was negatively stained with a 1\% aqueous solution of phosphotungstic acid (PTA). Ethosomal solution (10\(\mu\)l) was dried on a microscopic carbon-coated grid for staining. The excess solution was removed by blotting. After drying, the specimen was viewed under microscope at 80 and 200KV (at 200 KV magnification 750,000X).

For SEM, one drop of ethosomal system was mounted on a stub covered with clean glass. The drop was spread out on the glass homogeneously. A polaron E5100sputter coat the samples with gold, and the samples were examined under a Philips XL30 ESEM TMP + EDAX scanning electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 20 KV(1000X – 5000X).

**Vesicle size**—The size of all the batches of ethosomes and liposomes were measured by Particle size analyzer (Malvern mastersizer) Malvern Instrument Ltd., Model S., Ver.2.15, Malvern, UK.

*Determinition of vesicle entrapment capacity*—Fluconazole was estimated in ethosomes by ultra centrifugation method\textsuperscript{14,24} and in liposomes by protamine aggregation method followed by ultra centrifugation.

**Method for ethosomes**\textsuperscript{14,24,25}—The total volume of the ethosomal suspension was measured and 2ml of this formulation was transferred to 10 ml centrifuge tube. The suspension was diluted with distilled water up to 5ml and centrifuged at 2000 rpm for 20 min to separate out undissolved drug in the formulation. Ethosomes were separated by ultra centrifugation at 20,000 rpm for 30 min. Supernatant and sediment were recovered and their volume was measured. Sediment was diluted with distilled water up to 5ml. The unentrapped and entrapped drug contents were analyzed by estimating drug in supernatant and ethosomes (sediment) by spectroscopic method.

**Method for liposomes**\textsuperscript{17,13}—The liposomal (2ml) suspension was transferred to 10 ml centrifuge tube. This suspension was diluted with distilled water up to
5 ml and centrifuged at 2000 rpm for 20 min. By this we can separate out undissolved drug in the formulation. Suitable volume of the protamine solution was added to the resulting supernatant and retained for 10 min. Liposomes were aggregated in presence of protamine and then separated by ultra centrifugation at 15,000 rpm for 20 min. Supernatant and sediment were separated out. Volumes of the supernatant and sediment were measured. Sediment was diluted with distilled water up to 5 ml. The unentrapped and entrapped drug contents were analyzed by estimating distilled water and protamine and then separated by ultra centrifugation at 15,000 rpm for 20 min. Supernatant and sediment were separated out. Volumes of the supernatant and sediment were measured. Sediment was diluted with distilled water up to 5 ml. The unentrapped and entrapped drug contents were analyzed by estimating drug in supernatant and liposomes (sediment) by spectroscopic method.

**Differential scanning calorimetry (DSC) measurements**—Transition temperature (Tm) of the vesicular lipid systems was determined by using the Mettler DSC 60 computerized with Mettler Toledo Star software system (Mettler, Switzerland). Sample weigh (about 20 mg) and the phospholipids concentration was the same for all samples tested. The transition temperature was measured in duplicate in aluminum crucibles at a heating rate 10°C/min. within a temperature range from 20°C-300°C.

**Preparations of gels**—Gel base (2% w/w) was prepared by dispersing 20 g of hydroxypropylmethyl cellulose K4M (HPMC K4M) in 980 ml of distilled water, containing 0.001% w/v of phenyl mercuric nitrate, using a magnetic stirrer. The ethosomal suspension was centrifuged at 2000 rpm for 20 min and the pellets obtained were incorporated into the prepared gel base to get 1% (w/w) FLZ in the gel base.

In the clinical study, two gel formulations were made as follows—Plain FLZ gel was prepared by triturating FLZ with HPMC K4M gel base. Ethosomal FLZ gel was prepared by incorporating ethosomes of FLZ into HPMC K4M gel base by trituration. The final FLZ concentration was 1% w/w in all the gels prepared. The gels were filled into 20 g lacquer coated aluminium tubes and sealed securely.

**Stability studies**—The prepared ethosomes (batches FLZ 4, FLZ 6 and FLZ 7), and their ethosomal gels (FLZ4G, FLZ6G and FLZ7G) of Fluconazole were subjected to stability studies for 3 months period. The ethosomes and ethosomal gel were stored at refrigeration temperature (2°C-8°C) and room temperature (25°C±2°C) for 3 months period. The compositions of different ethosomal formulations prepared in this investigation are recorded in Table 1.
The ethosomal formulation (FLZ 4) prepared with 30% (w/v) ethanol and 3% (w/v) phospholipids show an average vesicle size of 188±7.2nm. With phospholipids concentration range of 2-5% w/v (FLZ 2, FLZ 3, FLZ 4, FLZ 10, and FLZ 11), the size of the vesicles increased with increasing phospholipids concentration, the largest particles in preparation containing 5% w/v phospholipids 197±7.8 (FLZ 11) and smallest in preparation containing 2% w/v phospholipids 139±6.2 (FLZ 2) were observed during study (Table 2 and Fig 1). The liposomes, made from the same phospholipids by film forming method and not containing ethanol, show an average size of 212±9.7 nm.

The entrapment efficiency of FLZ ethosomes and liposomes was calculated as percent total drug entrapped within the vesicles. The entrapment efficiency of FLZ ethosomes and liposomes (FLZ 4 and LIP) was found to be 72.21 and 62.23%, respectively. The relatively high entrapment of FLZ within ethosomes may be explained by multilamellarity of ethosomal vesicles and by the presence of ethanol. The data indicate that the entrapment efficiency depends on ethanol concentration. By increasing the ethanol concentration up to 30% (w/v) the entrapment efficiency also increases and with further increasing the ethanol concentration (>30%w/v), the vesicle membrane becomes more permeable and that may have led to decrease in the entrapment efficiency of ethosomal formulation (Table 1).

Ethosomes were found to be spherical vesicles when observed under Olympus light microscope.

Table 1—FLZ ethosomes composition and characterization
[Values are ± SE derived from three different experimental batches]

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Drug (mg)</th>
<th>SPC (% w/v)</th>
<th>Ethanol (% w/v)</th>
<th>PG (% w/v)</th>
<th>Water (up to) (% w/v)</th>
<th>+/-</th>
<th>Particle-size (nm)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLZ1</td>
<td>10</td>
<td>1.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FLZ2</td>
<td>10</td>
<td>2.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>139±6.2</td>
<td>48.81</td>
</tr>
<tr>
<td>FLZ3</td>
<td>10</td>
<td>2.5</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>146±6.8</td>
<td>55.45</td>
</tr>
<tr>
<td>FLZ4</td>
<td>10</td>
<td>3.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>188±7.2</td>
<td>72.21</td>
</tr>
<tr>
<td>FLZ5</td>
<td>10</td>
<td>3.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>211±10.4</td>
<td>51.34</td>
</tr>
<tr>
<td>FLZ6</td>
<td>10</td>
<td>3.0</td>
<td>35</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>184±9.2</td>
<td>67.74</td>
</tr>
<tr>
<td>FLZ7</td>
<td>10</td>
<td>3.0</td>
<td>40</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>171±8.4</td>
<td>64.51</td>
</tr>
<tr>
<td>FLZ8</td>
<td>10</td>
<td>3.0</td>
<td>45</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>159±7.9</td>
<td>56.11</td>
</tr>
<tr>
<td>FLZ9</td>
<td>10</td>
<td>3.0</td>
<td>50</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>134±6.3</td>
<td>45.44</td>
</tr>
<tr>
<td>FLZ10</td>
<td>10</td>
<td>4.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>194±8.4</td>
<td>63.45</td>
</tr>
<tr>
<td>FLZ11</td>
<td>10</td>
<td>5.0</td>
<td>30</td>
<td>20</td>
<td>100</td>
<td>+</td>
<td>197±7.8</td>
<td>65.57</td>
</tr>
<tr>
<td>LIP</td>
<td>10</td>
<td>6.0</td>
<td>1.2 %w/v cholesterol</td>
<td>100</td>
<td>+</td>
<td>212±9.7</td>
<td>62.23</td>
<td></td>
</tr>
</tbody>
</table>

Table 2—Effect of phospholipids concentration on the size of ethosomal vesicles
[Values are ± SE derived from three different experimental batches]

<table>
<thead>
<tr>
<th>Phospholipid (%)</th>
<th>Particle size (nm)</th>
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</thead>
<tbody>
<tr>
<td>2.0</td>
<td>139±6.2</td>
</tr>
<tr>
<td>2.5</td>
<td>146±6.8</td>
</tr>
<tr>
<td>3.0</td>
<td>188±7.2</td>
</tr>
<tr>
<td>4.0</td>
<td>194±8.4</td>
</tr>
<tr>
<td>5.0</td>
<td>197±7.8</td>
</tr>
</tbody>
</table>

Fig. 1—Effect of phospholipids concentration on the size of ethosomal vesicles

However, to confirm multilamellarity of ethosomes vesicles at higher concentration of ethanol, the ethosomes were subjected to transmission electron microscopy after negative staining with phosphotungstic acid. Ethosomes are multilamellar vesicles with mean size of 50 nm to 200 nm (Fig. 2). Further investigation of the formulations by SEM, which allows for analysis of the 3-D surface
properties, confirms the ethosomes to be three dimensional with a smooth surface as seen in Fig. 3.

In order to ascertain the reason of better penetration of ethosomes within the skin efficiently, free energy measurement of bilayers of ethosomes using DSC was carried out. Thermogram of the DSC is expected to express the consumption of the energy in phase transfer and fluidity of bilayers. On comparison of thermograms, phase transition temperature of liposomes and ethosomes was found to differ by 7.79°C (Fig. 4). The results suggest that the ethosomes are comparatively more in fluid state than liposomes. The difference may be attributed to the presence of alcohol in ethosomes.

The percentage drug retained in ethosomes and ethosomal gel was conducted at refrigerated

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Fig. 2—Ultrastructural characterization of ethosomes loaded with fluconazole. Representative TEM shows FLZ ethosomal vesicles seem to be very malleable, as evident by imperfect round shape. This characteristic can be explained by the fluiding effect of ethanol on FLZ ethosomes (at 200 KV magnification 750,000X).

Fig. 3—Surface Morphology of ethosomes loaded with fluconazole. The three- dimensional nature of the phospholipids vesicles was confirmed by SEM. (at 20 KV magnification 5000X).

Fig. 4—Differential scanning calorimetric (DSC) measurements of (A) plain FLZ, (B) FLZ ethosomes and (C) FLZ liposome.
Temperature (2°-8°C), room temperature (25°±2°C) and samples were withdrawn at specific time intervals. The samples were analyzed for drug content by Spectrophotometry. At the refrigeration and room temperature, there was more than 85-95% drug retention within the vesicles of ethosomes after 3 months of storage. However, at the body temperature there was negligible amount of drug retained in the vesicles. Particle size analyzer (Malvern mastersizer) was used for determination of the vesicle size of ethosomes (batches FLZ 4, FLZ 6 and FLZ 7) for the period of three months. The mean vesicle size remained unchanged for three months at room temperature (Table 3).

**Table 3—Vesicle sizes of ethosomes and ethosomal gel on storage for 3 months**  
[Values are ± SE derived from three different experimental batches]

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vesicle diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLZ4</td>
</tr>
<tr>
<td>Refrigeration temperature</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>188±7.2</td>
</tr>
<tr>
<td>B</td>
<td>±±±±</td>
</tr>
<tr>
<td>Room temperature</td>
<td>183±9.1</td>
</tr>
<tr>
<td>A</td>
<td>9.1±7.2</td>
</tr>
<tr>
<td>B</td>
<td>±±±±</td>
</tr>
</tbody>
</table>
A- Immediately after preparation  
B- After 3 months storage

**Diffusion studies**

The cumulative amount of drug permeated across mice skin per unit area was plotted as a function of time and % drug diffused was calculated from the slope of linear portion. The percent drug diffused from ethosomes, liposomes and hydroethanolic solution of FLZ were found to be 89.57, 49.48 and 32.64%, respectively (Table 4 and Fig. 5). From the data we can predict that % drug diffused from ethosomes was nearly twice than liposomes and 3 times higher than the hydroethanolic solution. The amount of drug deposited in the skin and the amount of unabsorbed drug remaining on the surface of skin for ethosomes, liposomes and hydroethanolic solution of FLZ were found to be 4.4±0.26 and 6±0.41, 3.1±0.32 and 42±0.41 and 3.3±0.29 and 60±0.35% respectively. When ethosomal carriers, which contain ethanol and soft small vesicles are applied to the skin a number of concomitant processes may take place, involving the stratum corneum and pilosebaceous pathways.

Ethanol has long been known to have penetration enhancing properties. However, the permeation enhancement from ethosomes observed in the present study is much greater than would be expected from ethanol alone, suggesting that some kind of synergisting mechanism between ethanol, vesicles and skin lipids.

The data suggest that the value of % drug diffused depends on the ethanol concentration. As the concentration of ethanol increased, % drug diffused of FLZ increased up to 30% and further increase in the ethanol concentration significantly decreased the % drug diffused. The reason for this is the deteriorating effect of ethanol on the lipid bilayers at higher concentration of ethanol. The statistical significant
difference in % drug diffused between ethosomal formulation, liposomal formulation and hydroethanolic solution clearly indicates that the ethosomal system is much more permeable through skin10,11 than liposomal formulation and hydroethanolic solution of the drug.

Clinical studies
The clinical evaluation demonstrated that the mean percent reduction in dimension of skin lesions of Candidiasis patients was 50-75 % with FLZ ethosomal gel as compared to 35-60 % with FLZ liposomal gel, 25-30% with marketed FLZ cream and 15-20% with hydroethanolic solution of the drug (Table 5 and Fig. 6).

Comparing the data using ANOVA [Fvalue*-calculated 3.304 and Fvalue-tabulated-3.49, Fvalue > Fvalue*, therefore, the test was passed] revealed that ethosomal FLZ gel significantly improved the therapeutic response at all evaluation time points at both levels as compared to marketed FLZ cream, FLZ liposomal gel and hydroethanolic solution of FLZ (Table 6).

The lesions were also reduced in thickness, a parameter, not mentioned in the study. This may be due to higher skin deposition of FLZ from ethosomal gel.

Conclusion
Results of the fore sent investigation have conclusively demonstrated the role of ethosomal FLZ gel formulation in treatment of Candididiasis patients. It provides better remission from the disease and reduces the duration of therapy. However, these formulations can find a place in clinical use after clinical evaluation with a large number of patients with special focus to adverse symptoms.

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