Formulation and Evaluation of Proniosome Based Drug Delivery System of the Antifungal Drug Clotrimazole

S. Pankaj, T. Rini and P.M. Dandagi
Department of Pharmaceutics, KLEU’s College of Pharmacy, JNMC Campus, Belgaum, Karnataka, India.

ABSTRACT
In the present study proniosomal gel of clotrimazole was formulated by using lecithin, cholesterol as encapsulating agents, nonionic surfactants Span and Tween with different grades. Evaluation of proniosomal gels for pH, vesicle size analysis, encapsulation efficiency, drug diffusion profiles, ex-vivo skin permeation and ex-vivo drug deposition studies on guinea pig skin, irritation test on rabbit and stability studies was performed. The preliminary compatibility studies revealed that there were no interactions between clotrimazole and excipients which was evident from FTIR and DSC studies. The physical characteristics of proniosomal gels were found to be within the acceptable limits. The vesicle size was found to be in the range 5.25-15.23 μm. The proniosomes were spherical and homogenous in structure when observed under optical microscopy. The ex-vivo skin permeation and ex-vivo drug deposition studies showed the drug release from formulations F3, F4 and marketed formulation was 48.60%, 36.9% and 27.48 % respectively and the percent of clotrimazole deposited in skin after 24 h was found to be 35.7%, 43.6% and 15.17% for formulation F3, F4 and marketed formulation respectively. The release from the proniosomal gel was prolonged when compared to conventional formulation and showed a two fold increase in the drug deposition in the skin compared to conventional cream. No obvious erythema, edema or inflammation was observed on rabbits’ skin after one week of application of the selected formulation. Results of antifungal studies revealed that the developed proniosomal gel is more efficient when compared with the marketed formulation. The stability studies showed that proniosomal gels were stable at 5±3°C and 25±2°C. The above results indicated that the proniosomal gels of clotrimazole could be formulated for sustained release.

Keywords: Clotrimazole, Proniosomal gel, Lecithin, Cholesterol, Ex-vivo study.

Introduction
The purpose of topical and dermatological dosage forms is to conveniently deliver drug molecules across a localized area of the skin. To develop an ideal dosage form one must take into account the flux of the drug across skin, the retention of the dosage form on the skin’s surface, the reservoir capacity of the dosage form, and the patient’s acceptability of the formulation.

Proniosomal gels are semisolid liquid crystal products of nonionic surfactants easily prepared by dissolving the surfactant in a minimal amount of an acceptable solvent and small amount of aqueous phase. Proniosomal gels offer a great potential to reduce the side effects of drugs and increase the therapeutic effectiveness of transdermal drug delivery. This would be possible if proniosomes form niosomes upon hydration with water following topical application under occlusive conditions (Fang et al., 2001). Proniosomes minimizes problems associated with niosomes i.e. physical stability such as aggregation, fusion and leaking and provide additional convenience in transportation, storage and dosing (Hu et al., 1999). The Proniosomal gels are becoming more popular due to ease of application and better percutaneous absorption, than other semi solid preparations. Gels can resist the physiological stress caused by skin flexion, mucociliary movement, adapting to the shape of the applied area and for controlling drug release (Shamsheer et al., 2011).

The purpose of this study was to determine the factors influencing the encapsulation of clotrimazole in proniosomal gel and to optimize release parameters in order to achieve a suitable delivery system. Fungal infections on the skin can be treated by antifungal drugs. Clotrimazole is an imidazole derivative with a broad spectrum antymycotic activity. It acts by inhibiting biosynthesis of sterol ergosterol, an important component of fungal cell membranes. It is widely used for the treatment of local fungal infections such as ringworm, athlete’s foot and jock itch (Sweetman et al., 2007). Clotrimazole given orally is metabolized in liver to inactive compounds and excreted in the faeces and urine, after oral administration of clotrimazole lozenges caused nausea, vomiting, unpleasant mouth sensation and pruritus have been reported.

The objective of the study was to optimize and evaluate various clotrimazole proniosomal formulations. Proniosomes were prepared by using two types of nonionic surfactant including Span and Tween with different grades and two different quantity of the cholesterol was used. We also investigated the feasibility of proniosomal gel of clotrimazole for topical application. To enhance the
stability and increase the viscosity of the system, the proniosomes were mixed with carbopol gel.

**Material and Methods**

**Chemical and Drugs**

Clotrimazole was obtained as a gift sample from Saika Pharmaceuticals (Rohtak, India). Span-40, Span-60, Tween-60, Tween-80, were purchased from Ranbaxy Fine Chemical Limited, (New Delhi). Cholesterol was purchased from Loba Chemie Pvt. (Mumbai, India) and Lecithin purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Chloroform, Ethanol and Potassium dihydrogen phosphate were of analytical reagent grade and obtained from Research Lab (Mumbai, India). The protocol for the animal study was approved by Institutional Animal Ethical Committee.

**Methodology**

**Drug – excipients compatibility study**

The Drug – Excipient Compatibility Studies were performed in order to check any interaction between drug and excipients. The differential scanning calorimetry (DSC) of pure drug and its physical mixture with various excipients was obtained using DSC-60 Instrument and results are reported in Fig 1. FT-IR (IR Affinity, Shimadzu) spectrum of drug sample and its physical mixture with excipients was recorded by KBR pellets method at resolution of 4 cm⁻¹. The results are reported in Fig 2.

![Fig.1. DSC Thermogram of drug (a) and drug with excipient (b).](image-url)

**Method for Preparation**

Proniosomes were prepared by the method reported by Perrett et al. Weighed amount of surfactant mixture, surfactants: alcohol (1:1) and drug were weighed in a clean and dry, wide mouthed small glass tube. After mixing all the ingredients, the open end of the glass tube was covered with a lid to prevent loss of solvent and then warmed on a water bath at 60–70°C for about 5 min, until the surfactants were dissolved completely. The aqueous phase was then added and warmed on a water bath till clear solution was formed. The mixture was allowed to cool to room temperature until the dispersion was converted to proniosomal gel. Proniosomal gel was then mixed with 1% Carbopol gel in 1:1 ratio. The gel obtained was preserved in dark until characterization was done (Perrett et al., 1991).

In order to optimize and evaluate various clotrimazole proniosomal formulations, different grades of non-ionic surfactants: Span (sorbitan esters) i.e. Span 40, Span 60 and Tween (polyoxyethylene sorbitan esters) i.e. Tween 60 and Tween 80 were used in addition to different quantities of cholesterol as summarized in Table 1.

**TABLE 1**

Composition of proniosomal gel formulations.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Drug (mg)</th>
<th>Span 40 (mg)</th>
<th>Span 60 (mg)</th>
<th>Tween 60 (mg)</th>
<th>Tween 60 (mg)</th>
<th>Lecithin (mg)</th>
<th>Cholesterol (mg)</th>
<th>Ethanol (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>100</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>200</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>100</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>200</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>100</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>200</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F7</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>100</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F8</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td>500</td>
<td>200</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>80</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>400</td>
<td>1500</td>
</tr>
</tbody>
</table>
Evaluation of Formulations

Organoleptic properties

Proniosomal gels were characterized for appearance, color, homogeneity by visual inspection and pH was determined using a digital pH meter.

Determination of percentage entrapment efficiency

To 0.5 g of proniosomal gel weighed in a glass tube, 10 ml of the aqueous phase (phosphate buffer pH 7.4) were added; the aqueous suspension was then sonicated. Niosomes containing clotrimazole were separated from unentrapped drug by centrifugation at 9000 rpm for 45 min at 4°C. The supernatant was recovered and assayed spectrophotometrically using Shimadzu UV spectrophotometer (Japan) at 261 nm. The encapsulation efficiency was calculated by the following equation (Mokhtar et al., 2008).

\[
\% \text{Encapsulation efficiency} = \frac{(\text{Total drug} - \text{(unencapsulated drug / total drug)}) \times 100}{\text{Total drug}}
\]

Vesicle size and morphology analysis

Particle size of different batches of proniosomal gel was determined by optical microscopy at 100X magnification. Hydration of proniosomal gel (100 mg) is done by adding aqueous phase (phosphate buffer pH 7.4) in a small glass vial with occasional shaking for 10 mins. The dispersion is observed under optical microscope. The size of 50 vesicles was measured using stage micrometer (Jain et al., 1998).

Zeta potential determination

Zeta potential for the formulations was measured by using Zetasizer instrument (Mokhtar et al., 2008).

In vitro diffusion studies

In vitro diffusion studies of proniosomal gel was carried out using a dialysis bag (Hi-media dialysis membrane, 8000-10,000 MW cutoff) as donor compartment. Proniosomal gel equivalent to 10 mg drug was taken in the dialysis bag and placed in a beaker containing 100 ml of PBS, which acted as the receptor compartment. Previously, the dialysis membrane was soaked in warm water for 10 mins and both ends were sealed with closure clips after adding the proniosomal preparation. The beaker was placed over a magnetic stirrer (100 rpm) and maintained at 37±1°C. At predetermined time intervals during 24 h, aliquots (1ml) were withdrawn and replaced with fresh buffer. The sink condition was maintained throughout the experiment. Samples withdrawn were suitably diluted and analyzed spectrophotometrically at 261 nm (Aggarwal et al., 2005).

Ex vivo permeation studies

Permeation of drug through excised guinea pig skin from the selected proniosomal gel preparations was assessed. The abdominal hair of guinea pig was removed carefully. After the animal was sacrificed, the abdominal skin was excised. The whole skin was equilibrated in phosphate buffer solution (pH 7.4) for 1 h before the experiment. This membrane was mounted on a vertical Franz diffusion cell with the dermis facing the receptor compartment. The donor side was charged with 1 mg equivalent drug formulation. The membrane surface area available for diffusion was 2.54 cm². The receptor compartment was filled with 10 ml of phosphate buffer. Temperature was maintained at 37±0.5°C to simulate human body temperature. The receptor compartment was constantly stirred at 100 rpm. Samples from the receptor fluid (1 ml) were withdrawn at various time intervals up to 24 h and replaced immediately by fresh buffer solution. The samples were then assayed spectrophotometrically at 261 nm (Escribano et al, 2003).

Drug deposition study

At the end of the permeation experiments (after 24 h), the skin surface was washed with methanol. The skin was then cut into small pieces. The tissue was further homogenized with methanol: distilled water (1:1) and left for 6 h at room temperature. After manual shaking for 5 minutes and centrifuging the mixture for 5 minutes at 5000 rpm, the clotrimazole content was analyzed by spectrophotometric method after appropriate dilutions at 261 nm. (Kakkar et al., 2011).

Evaluation of optimised formulation

Formulation F4 was optimised on the basis of ex vivo permeation and drug deposition studies and evaluated for following parameters:

Antifungal study

Antifungal studies were carried out to ascertain the biological activity of the optimized formulation (F4) and compared with marketed formulation against Candida albicans (36082). A layer of nutrient agar (20 mL) was seeded with 0.2 mL of test micro-organism and allowed to solidify in the petri plate. Cups were made with the help of sterile borer at 4 mm diameter on the solidified agar layer. Fifty milligrams of optimized formulation (F4) were taken and suspended in normal saline. The 18 h and 24 h release sample solutions were poured into the cups for microbial assay. Marketed formulation was kept in another petri plate for comparison. After keeping petri plates at room temperature for 4 h, the plates were incubated at 37°C for 24 h. The diameter of zone of inhibition (ZOI) thus obtained was measured. Readings were taken in triplicates (Anantnarayan, 1997).

Skin Irritancy test

The test was performed using 2 healthy male albino rabbits (1.5±0.5 kg). On to the first rabbit, a single dose of 0.5 g of the selected medicated formulations (5 mg drug) was applied to the left side of the shaved back of the rabbit and to the right shaved side standard irritant (formalin solution) was applied. The shaved back of the second rabbit was kept as control. The development of erythema was monitored daily for 6 days by comparing the effect of prepared formulation on skin with that of the standard irritant and control. Extents of development of erythema were indicated on the basis of the following: (Van-Abbé et al., 1975).
0: No erythema development.
2: barely visible few blood vessels and light erythema development.
4: main blood vessels visible and slight erythema development.
6: main blood vessels more obvious and slight erythema development.

Irritation potential was calculated using the following equation.

\[
\text{Resultant Index} = \frac{A \times B}{\text{Number of observation days}}
\]

Where A and B represent erythema value and corresponding day, respectively. The study was approved by the Institutional Ethics Committee.

**Short-term stability study**

Stability of selected proniosomal gel (F4) formulation were carried out at 5°C ± 3°C and 25±2°C and 40 ± 2°C. The samples were withdrawn after 15, 30, 60, 90 days and tested for particle size, drug retained and in vitro diffusion study (Azarbayjani et al., 2009 and WHO. 2006).

**Results and Discussion**

**Drug – excipients compatibility study**

The IR spectra and DSC studies of pure drug, phospholipid (lecithin), cholesterol and physical mixture of excipients with drug were studied. Figs 2 indicate no interaction between clotrimazole and excipients (phospholipid, cholesterol) when compared with IR spectra of pure drug as all functional group frequencies were present. Also the results of DSC studies as shown in Fig 1 indicate no appreciable change in the melting endotherms of the physical mixture (drug + phospholipid) compared to pure drug. Pure clotrimazole showed a sharp endotherm at 149°C corresponding to its melting point. Phospholipid showed a sharp endotherm at 52.89°C corresponding to its transition temperature.

**Organoleptic properties**

The proniosomal gel was off-white in color, odorless and semisolid in nature. It was stable and did not show sedimentation. pH was found to be in the range of 5.7-6.2 as shown in Table 2.

**Entrapment efficiency**

Entrapment efficiency of proniosomes formulations ranged from 67.70% to 87.64%. The drug encapsulation efficiency of all nine formulations is shown in Table 2. The proniosomes formed exhibited good encapsulation efficiency. This could be explained on the basis that the highly lipophilic portion of the drug is expected to be housed almost completely within the lipid bilayer of the proniosomes. Most of the surfactants used to make nonionic surfactant vesicles have a low aqueous solubility. However, freely soluble nonionic surfactants such as Tweens can form micelles on hydration in the presence of cholesterol. The Tween formulations in the present study were also able to entrap clotrimazole efficiently. However, the encapsulation efficiency was relatively low as compared to those composed of Span. This is because the vesicles can be successfully formed by Tween only in the presence of cholesterol. Also among the two grades of Span i.e. span 40 and 60, Span 60 was found to show more efficient entrapment. As the cholesterol content of the formulation decreased, the encapsulation of drug also decreased.

**Vesicle size and shape**

The mean vesicle sizes of the clotrimazole proniosome formulations ranged from 4.49 µm to 15.23 µm (Table 2). The differences in vesicle size among the proniosomes prepared with Span were not significant. On the other hand, proniosomes prepared with Tweens were significantly larger than those prepared with Spans. The relationship observed between proniosome size and Span hydrophobicity has been attributed to the decrease in surface energy with increasing hydrophobicity, resulting in the smaller vesicles. This would also explain the large vesicle size of proniosomes prepared with Tweens which has a much lower hydrophobicity than Spans. Increasing the cholesterol content also contributed in increasing the hydrophobicity, with a subsequent slight reduction in vesicle size. The microscopic images of the proniosomes formulations are shown in Fig 3. Most of the vesicles are well identified, spherical and discreet with sharp boundaries having large internal aqueous space. Zeta potential of all formulations is shown in Table 1.

**TABLE 2**

Evaluation parameters of proniosomal gel formulations.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>pH*</th>
<th>Entrapment Efficiency (%)*</th>
<th>Particle Size (µm) **</th>
<th>Zeta Potential (mV) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>7.01</td>
<td>77.79</td>
<td>7.58±0.47</td>
<td>-28.71</td>
</tr>
<tr>
<td>F2</td>
<td>7.23</td>
<td>84.14</td>
<td>5.29±0.49</td>
<td>-25.10</td>
</tr>
<tr>
<td>F3</td>
<td>7.48</td>
<td>83.10</td>
<td>6.20±0.19</td>
<td>-25.29</td>
</tr>
<tr>
<td>F4</td>
<td>7.06</td>
<td>87.64</td>
<td>4.49±0.56</td>
<td>-26.29</td>
</tr>
<tr>
<td>F5</td>
<td>6.78</td>
<td>67.70</td>
<td>13.16±0.80</td>
<td>-28.10</td>
</tr>
<tr>
<td>F6</td>
<td>6.94</td>
<td>72.15</td>
<td>11.23±1.09</td>
<td>-29.55</td>
</tr>
<tr>
<td>F7</td>
<td>7.01</td>
<td>69.42</td>
<td>15.23±1.22</td>
<td>-26.21</td>
</tr>
<tr>
<td>F8</td>
<td>7.32</td>
<td>75.51</td>
<td>12.02±1.51</td>
<td>-24.21</td>
</tr>
<tr>
<td>F9</td>
<td>7.48</td>
<td>72.46</td>
<td>9.14±1.05</td>
<td>-26.21</td>
</tr>
</tbody>
</table>

*Values expressed are mean of triplicate, **Values expressed are Means S.D. (n=3)
In vitro drug permeation study

Fig 4 illustrates that a higher drug release was observed from proniosomes prepared with Span 60 that was 95.83% than from the other formulations. This could be due to the emulsification effect of the surfactant after the hydration of the noisome by the dissolution medium and formation of elution channels within the gel structure due to loss of lipid bilayers. Increasing the cholesterol content resulted in a more intact lipid bilayer as a barrier for drug release and decreased its leakage by improving the fluidity of the bilayer membrane and reducing its permeability, which led to lower drug elution from the vesicles. Drug release from proniosomes prepared with tween showed less drug release compare to formulation prepared with span it may be due to its hydrophilic nature. The burst release of the drug from proniosome formulation F9 was observed (which does not have lecithin), may result from the disrupted structure of the vesicles.

Ex vivo drug permeation study

The proniosomal gel formulations (F3 and F4) and a marketed formulation Clotrimazole cream USP 1% were characterized for their drug permeation through guinea pig skin and the results are reported in Fig 5. The drug permeation was high for formulation containing 100 mg of cholesterol (F3) as compared with F4 formulation that contains 200 mg of cholesterol and marketed formulation. From the permeation profile it was seen that the drug release for F3, F4 and marketed formulation was 48.60%, 36.9% and 27.48 % respectively after 24 h study, results are shown in Fig 5. Increasing the cholesterol content resulted in a more intact lipid bilayer as a barrier for drug release and decreased its leakage by improving the fluidity of the bilayer membrane and reducing its permeability, which led to lower drug elution from the vesicles. Thus proniosomal gel prepared by using span-60 containing 100 mg of cholesterol exhibited better permeation and optimum entrapment efficiency.

Drug deposition study

The percent of clotrimazole deposited in skin after 24 h was found to be 35.7%, 43.6% and 15.17 respectively for formulation F3, F4 and marketed formulation as shown in Fig 6. The cholesterol concentration was higher in F4, so its drug deposition was higher i.e 43.60% and cholesterol concentration was lower in F3, so its drug deposition was also lower 35.73%. The data indicates that drug deposition depends on cholesterol content. As the concentration increases, increase in deposition of drug in the skin was seen. Due to small particle size, enhancer properties of lecithin and nonionic surfactant, the drug penetrates in
to the skin whereas due to lipoidal nature of the stratum corneum, penetrated drug concentrates into the skin and remains localized for longer period of time.

**Antifungal Study**

The microbiological assay was carried out for F4 proniosomal gel with marketed formulation. The diameter of ZOI obtained with marketed formulation and proniosomal gel is shown in Fig 7 and Fig 8. Marketed formulation shows a ZOI of 29.3 mm compared to ZOI of 35.1 mm obtained for proniosomal gel after 24 h. Results revealed that the developed proniosomal gel is more efficient when compared with the marketed formulation in antifungal action.

**Skin Irritancy Test**

The selected proniosomal gel formulation (F4) showed an irritation potential of 1.4976 shown in Table 3, thus proving to be non-irritant as it was mentioned by Van-Abbé et al., that a value between 0 and 9 in an irritancy test indicates that the applied formulation is generally non-irritant to human skin (Van-Abbé et al., 1975). Results as shown in Fig 8 indicate no obvious erythema, oedema or inflammation on rabbits’ skin after one week of application of the selected formulation.

**Stability Studies**

The residual drug content was determined at the end of three month. It was observed that the drug leakage from the vesicles was least at 5±3°C followed by 25±2°C and 40±2°C. This may be attributed to phase transition of surfactant and lipid causing vesicle leakage at higher temperature during storage. Hence, it is concluded from the stability studies that proniosomal gels were stable at 5±3°C and 25±2°C as shown in table 4.
Conclusions

The *in vitro* permeation of clotrimazole from proniosomal gel of various compositions and types of nonionic surfactants have been studied and evaluated. Clotrimazole was successfully entrapped within the lipid bilayers of the vesicles with high entrapment. Significant difference was observed between entrapment efficiency of proniosomes prepared with different cholesterol contents. Transdermal proniosomal gels showed sustained drug release properties. The results of the present study indicated that clotrimazole proniosomal gel containing lecithin, cholesterol and surfactants like span 40, 60 and tween 60, 80 sustained release of drug over a period of 24 h. The proniosomal gel prepared by using span-60 containing 200 mg of cholesterol (F4) exhibited better skin deposition and optimum entrapment efficiency. The proniosomal gel could be an effective alternative vehicle for delivering the drug through transdermal route to avoid side effects associate with oral route. Thus proniosomal gel system has shown potential for delivery of antifungal drug candidate clotrimazole.

References


Address correspondence to: S. Pankaj, Department of Pharmaceutics, KLEU’s College of Pharmacy, Belgaum- 590010, Karnataka, India.

E-mail: pankajshukla367@gmail.com Tel: +91 8962383758, 7411088463.