Chapter - IV
MATERIALS AND METHODS

DRUGS
Ketoconazole Sharon Bio-Medicine Ltd, Raigad.
Miconazole Medical Products of India, Mumbai.
(Miconazole nitrate)

MATERIALS
Phospholipids Ozone chemicals, Mumbai.
Distilled alcohol Changshu yangyuan chemical, China.
Phosphate buffer Ranchem laboratory chemicals Pvt. Ltd, Chennai
Tripton X-100 Ozone chemicals, Mumbai.
Propylene glycol Ozone chemicals, Mumbai.
All the reagents used were of analytical grade.

EQUIPMENTS
UV Spectrophotometer Szhimadzu, Japan.
Electronic balance Szhimadzu, Japan.
Trinocular microscope Nikon, Japan.
Mechanical stirrer Remi equipments Pvt Ltd, Mumbai.
Refrigerator LG, India.
Research centrifuge Remi equipments Pvt Ltd, Mumbai.
Magnetic stirrer Remi equipment Pvt Ltd, Mumbai.
METHODS

PREPARATION OF STANDARD GRAPH

Standard stock solution

The stock solutions (1mg/ml) of the drugs (Ketoconzole and Miconazole) were prepared in phosphate buffer saline (PBS) pH 7.4 and 10% methanol.

Scanning of drugs (KETO and MICO)

The standard stock solutions were scanned at wavelengths between 200 mm and 400 mm for the determination of $\lambda_{\text{max}}$. The $\lambda_{\text{max}}$ for KETO and MICO were found to be 260.2 nm and 222.4 nm respectively.\(^{28}\)

From the standard stock solution a series of dilutions were made using PBS pH 7.4 solution. The absorbances of these solutions were measured against a blank of PBS pH 7.4 solution in UV/Visible spectrophotometer at 260.2 and 222.4 nm for KETO and MICO respectively. Standard graphs were then plotted.

PREPARATION OF ETHOSOMES CONTAINING DRUGS (KETO and MICO)

The method mentioned by Touitou et al was adapted for the preparation of drug encapsulated ethosomes\(^{100}\).

The ethosomal system of KETO and MICO comprised of 2.0% drug, 2.0% phospholipids, 10% propylene glycol, 20-40 % ethanol and aqueous phase to 100 % w/w (Table No. 3).

Drugs (KETO and MICO) were dissolved in methanol to give a concentration of 2.0% w/w of drug solution. From the above stock solution 5ml (which is equivalent to 2.0 % w/w of KETO and MICO in the final formulation) were diluted with PBS pH 7.4. The phospholipids were dispersed in water by heating in a water bath at 40°C until a colloidal solution was obtained. In a
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separate vessel 2.0%w/w solution of drugs (KETO and MICO), ethanol and propylene glycol were mixed and heated to 40°C. Once both mixtures reached 40°C, the aqueous phase was added to the organic mixture with stirring at 700 rpm. After mixing, stirring was continued for another 5 minutes. Temperature was maintained at 40°C during the entire process. The final milky solution of ethosomes was left to cool at room temperature.11,12

Table No 3: Composition of different Ethosomal formulations of KETO and MICO.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Phospholipids (%w/w)</th>
<th>Ethanol (%w/w)</th>
<th>Propylene glycol (%w/w)</th>
<th>Drug (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KET₁</td>
<td>2.0</td>
<td>20</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>KET₂</td>
<td>2.0</td>
<td>30</td>
<td>10</td>
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<tr>
<td>KET₃</td>
<td>2.0</td>
<td>40</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>MET₁</td>
<td>2.0</td>
<td>20</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>MET₂</td>
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<tr>
<td>MET₃</td>
<td>2.0</td>
<td>40</td>
<td>10</td>
<td>2.0</td>
</tr>
</tbody>
</table>

FORMULATION OF CREAMS

Drugs (KETO and MICO) and ethosome enclosed drugs were triturated in water miscible cream base q.s to prepare creams. The final concentration of drugs in all formulations was maintained at 2.0% w/w. The creams were filled in to
20gm lacquer coated aluminium tubes and sealed securely. These were later used for evaluations.

CHARACTERIZATION OF ETHOSOMES

SIZE AND SHAPE ANALYSIS

The average size of ethosomes was determined by microscopy. A sample of ethosomes was suitably diluted with distilled water in order to observe individual vesicle and a drop of diluted suspension was examined under a microscope (magnification 15 x 45 X) using calibrated eyepiece micrometer with stage micrometer. The diameters of 150 vesicles were determined randomly. The average diameter was calculated using the formula:

\[
\frac{\sum nd}{\sum n} = \text{Average diameter (d_{ave})}
\]

\(n = \text{number of vesicles}
\)
\(d = \text{diameter of the vesicles}
\)

Images of the vesicles were transferred to computer (IBM, China) through a video camera (JVC, Japan). Shapes of vesicles were analyzed automatically using special software developed by Leica imaging systems, UK.

ENTRAPMENT EFFICENCY

The entrapment efficiency of drugs (KETO and MICO) into ethosomal vesicles was determined by ultracentrifugation. 10 ml of ethosomal formulation were mixed with 1 ml of 1% triton X-100 solution. Each sample was vortexed for 2 cycles of 5 minutes with 2 minutes rest between the cycles. 1.5ml of each vortexed sample and fresh untreated ethosomal formulations were taken into different centrifugal tubes. These samples were centrifuged at 20,000 rpm for 3 hours. The supernatant layer was separated, diluted with water suitably and drug concentration was determined at 260.2 and 222.4nm respectively in both vortexed and unvortexed samples.
The entrapment efficiency was calculated as follows

\[
\text{Entrapment Efficiency} = \frac{T-C}{T} \times 100
\]

‘T’ is total amount of drug detected from supernatant layer of vortexed sample.
‘C’ is the amount of drug unentrapped and detected from supernatant layer of unvortexed sample.

**STABILITY STUDIES**

Stability study was carried out for ethosomal formulations at two different temperatures i.e. refrigeration temperature (4 ± 2° C) and at room temperature (30 ± 2° C) for 8 weeks. The formulations subjected for stability study were stored in borosilicate container to avoid any sort of interaction between the ethosomal preparation and glass of container, which may affect the observations. The ethosomal formulations were analyzed for any physical changes such as color and appearance. Other studies carried out were changes in vesicular size and shape, entrapment efficiency and drug content\[16,20,64\].

**VESICULAR SHAPE AND SIZE DURING STABILITY STUDIES**

Samples of ethosomal formulations were focused under the microscope for an interval of two weeks to know any change in shape and size as reported earlier under the heading of characterization\[16\].

**DRUG ENTRAPMENT AND DRUG CONTENT DURING STABILITY STUDIES**

A study was carried out for a period of eight weeks to determine the total drug in ethosomal formulations and to find out drug entrapped into vesicular system. Samples were withdrawn at an interval of 2 weeks and study was performed as mentioned earlier in the thesis under the drug entrapment efficiency by ultracentrifugation.
**IN-VITRO SKIN PERMEATION STUDIES**

**PREPARATION OF RAT SKIN**

Rats 6 to 8 weeks old weighing 120 to 150 gms were humanely killed by inhaling chloroform. The hair of test animals were carefully trimmed short to less than 2mm with a pair of scissors and abdominal skin was separated from the underlying connective tissue with scalpel. The excised skin was placed on aluminium foil and dermal side of the skin was gently teased off for any adhering fat and subcutaneous tissue. Cleaned skin was used for in-vitro skin permeation study\(^\text{10}\).

**IN-VITRO SKIN PERMEATION STUDY**

The *in-vitro* skin permeation of drugs (KETO and MICO) from ethosomal formulation was studied using Franz glass diffusion cell specially designed in laboratory according to the literatures with modifications\(^\text{10}\). The effective permeation area of the diffusion cell and receptor cell volume were 3.14 cm\(^2\) and 20ml respectively. The temperature was maintained at 37 ± 0.5°C. The receptor compartment containing 20 ml of PBS pH7.4 and 10% methanol was constantly stirred by magnetic stirrer at 100 rpm. Prepared rat skin was mounted between the donor and the receptor compartments. 1 ml ethosomal formulation was applied to the epidermal surface of skin and the content of diffusion cell was kept under constant stirring. 1 ml samples were withdrawn from the receptor compartment of diffusion cell at predetermined time intervals and analyzed by spectrophotometric method at 260.2 and 222.4nm respectively for KETO and MICO after suitable dilution. An equal volume of fresh phosphate buffer was replaced into the receptor compartment after each sampling.

**DIFFERENTIAL SCANNING CALORIMETRY (DSC) STUDIES**

Samples of pure drugs, phospholipids and drug loaded ethosomes were subjected to DSC analysis. The analysis was performed with 5mg samples sealed in standard aluminium pans. Each sample was scanned between 0°C and 200°C. Thermograms were obtained at a scanning rate of 20°C/minute\(^\text{102}\).
IN-VITRO DRUG RELEASE STUDIES FROM CREAM BASED FORMULATIONS

In-vitro drug release studies were carried out using Franz glass diffusion cell specially designed in laboratory according to the literatures with modifications\textsuperscript{10}. The effective permeation area of the diffusion cell and receptor cell volume were 3.14 cm\(^2\) and 20ml respectively. The temperature was maintained at 37 ± 0.5°C. The receptor compartment containing 20 ml of PBS pH7.4 and 10% methanol was constantly stirred by magnetic stirrer at 100 rpm. Regenerated cellulose acetate membrane with thickness of 60-65µm and 0.45 µm pore size was mounted between the donor and the receptor compartment. A sample cream was placed evenly on surface of membrane in donor compartment. 1ml of receptor fluid was withdrawn from the receiving compartment at 0, 1, 2, 4, 6, 12, 24, 36, 48, 60 & 72 hours and replaced with 1ml of fresh solution. Samples were analyzed spectrophotometrically for drug content at 260.2 and 222.4nm respectively for KETO, MICO.

IN-VIVO STUDIES OF ETHOSOMAL FORMULATIONS

Four groups, each having three rabbits were taken for the in vivo studies. The animals (Male New Zealand rabbits) weighing 2.0±0.2kg were housed in separate cages maintained as per guidelines on care and use of laboratory animals given by CPCSEA, India. The experimental protocol for all the in vivo studies was approved by the institutional animal ethical committee (Registration no: APTUS/IAEC/234) which is an approved body under CPCSEA. The studies were carried out according to the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Before treatment, the hair was shorn from the back of rabbits with electric clippers (Super proclipper RC 2000) and four areas of the skin, approx. 3cm\(^2\), each in two sides were scarified by sandpaper type 60. These abraded portions of the skin were then infected with suspensions of
\( C.\text{albicans} \) containing \(1.0 \times 10^6\) infective blastocell. 48 hours after the challenge, an inflammatory state ensued in each treated area of animals. The prepared ethosomal creams were applied twice daily on 15 consecutive days beginning 48 hours after \( C.\text{albicans} \) inoculation. The activity of test groups were assessed in comparison with reference group treated with a drug loaded cream and a control group treated with the vehicles only. During treatment, evaluations of the lesions were taken daily with degree of inflammations and diameter of lesions. Evaluations of in vivo activity were made on days 6, 10 and 18. Yeasts were removed from scales, transferred to Sabouraud agar and incubated for evaluations. Animals considered to be cured when attempts to reisolate \( C.\text{albicans} \) after 4 days of incubation at 37\(^\circ\)C failed\(^43\).

**Preparation of fungal culture**

\( Candida\ \text{albicans} \) cultures on receipt were sub cultured in Sabouraud dextrose agar plates and further stored in slants as stock cultures. For the experiments, stock culture was prepared by inoculating each culture from slants to flask in sterile Sabouraud dextrose broth and incubated at 28\(^\circ\)C for 48 h. The stock culture was serially diluted by ten fold with sterile peptone water and 0.1ml from each dilution was spread over Sabouraud dextrose agar plates and incubated at 28\(^\circ\)C for 48 h. The number of colony forming units (cfu) was counted from plates of each dilution and there by the total cfu was calculated in the stock culture. For wound healing studies the stock cultures of \(1x10^6\) cfu per ml were used.