DESIGN AND EVALUATION OF ULTRADEFORMABLE SOFT ELASTIC NANO VESICLE ETHOSOMES FOR DERMAL DELIVERY

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Abstract
Clotrimazole is an antifungal drug for treatment of cutaneous candidiasis infections. However, its oral administration is associated with number of drawbacks. The goal of the current investigation is to evaluate the transdermal potential of novel vesicular carrier, ethosomes, bearing Clotrimazole an antifungal having limited transdermal permeation. Clotrimazole loaded ethosomes were prepared, optimized and characterized for vesicular shape and surface morphology, vesicular size, size distribution, entrapment efficiency, and stability. The ethosomal formulation (E63 having 3% phospholipids content and 35% ethanol showing the greatest entrapment (58.75%). Stability study was performed for 120 days. Furthermore, ethosomal delivery system could be considered for the treatment of number of dermal infections with better efficiency.

Keywords: Enhanced drug delivery; ethosomes; candidiasis; TEM; vesicle

1. Introduction
Infections caused by fungi constitute a major public health problem in many parts of the world, both in developed and developing countries. Microbial skin infections are very widespread in population of various ages and it can be classified by wound depth and pathogen species. In most cases of dermal and subdermal, primary and secondary skin bacterial infections, the disease treatment by simple topical drug application is not sufficient, a deeper penetration of antifungal drug of choice during therapy is felt very much essential. This is due to lack of ability of drug molecules as well as its conventional topical formulations to get self-permeated to deeper sections of the skin viz. dermis and epidermis. Clotrimazole is a 1-(2-chlorophenyl) (diphenyl methyl)1-H-imidazole. It has a very broad spectrum of activity and inhibits nearly all clinically relevant dermatophytes, yeasts, and molds. There are several advantages offered by transdermal route, but only few drug candidates are administered via this route due to formidable barrier nature of stratum corneum. To overcome the constraint, a great number of vesicular approaches are under investigation and the major among them are elastic liposomes and ethosomes. Ethosomes, the high ethanol containing vesicles are able to penetrate the deeper layers of the skin and hence appear to be vesicles of choice for transdermal drug delivery. Ethosomes present interesting features correlated with ability to permeate through the human skin due to their high malleability. The physicochemical characteristics of ethosome allow this vesicular carrier to transport wide variety of active substances more efficiently across the skin barrier with the goal of developing a potential effective treatment for deep dermal and intracellular bacterial infections.

2. Materials and Methods
Clotrimazole was purchased from Sigma Lab. PONTA SAHIB. Lipoid S PC-3, containing not less than 98% PC was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Ethanol

2.1 Preparation Of Clotrimazole Loaded Ethosomes: The ethosomes was composed of 1.0% w/v of lipid S PC-3, containing not less than 98% PC, 25% v/v of ethanol, drug (clotrimazole, 0.77% w/w) and probe (Rhodamine red (RR), 0.03% w/w). Lipoid S PC-3 was dissolved along with drug and probe (RR) in ethanol. Triple distilled water was
added slowly in a fine stream with constant mixing at 700 rpm with a mechanical stirrer (Remi equipment, Mumbai, India) in a house built closed container. Mixing was continued for additional 5 min. The system was maintained at 30 ± 1°C during the preparation and then left to cool at room temperature for 30 min.

2.2 Entrapment Efficiency: Ethosomal preparations containing Clotrimazol was estimated by ultra centrifugation technique for measuring its entrapment efficiency, for that the total volume of the ethosomal preparation was measured and 2 ml of the formulation was transferred to 10 ml centrifuge tube. The preparation was diluted with distilled water up to 5 ml and centrifuged at 2000 rpm for 20 minute to separate out undisolved drug in the formulation. Supernatant and sediment were recovered and their volume was measured. Sediment was dilute with the distilled water up to 5 ml. The entrapment and unentrapped drug content were analyzed by estimating the drug in supernatant and ethosomes (Sediment) by spectroscopic method. The method was repeated at least three times.

2.3 Size and Shape Analysis: Microscopic analysis was performed to determine the average size of ethosomes and liposome. A sample of ethosomes was suitably diluted with distilled water in order to observe individual vesicle and a drop of diluted suspension was put on a glass slide covered with cover slip and examined under microscope (magnification 15 × 45 X ). The diameters of 150 vesicles were determined randomly using calibrated eyepiece micrometer with stage micrometer.

2.4 In vitro permeation study through rat skin: The rate of the drug release from the carrier is an important parameter in the evaluation of the drug delivery. In this study a Franz diffusion cell was used for the diffusion studies. The study was carried out for 48 h at 37 ± 1°C. Rat skin was obtained from the abdominal of albino rats (aged 17–22 weeks, weight 250–300 g). All efforts were made to minimize animal suffering and to limit the number of animals used. Rats were killed by diethyl ether inhalation. After depilation and washing, abdominal skin was excised, thoroughly washed with pH 7.4 buffer solutions, dried and carefully cleaned and then preserved at 25°C. Before using, the skin was thawed, pre-hydrated for 1 h with the pH 7.4 buffer solution and then mounted in the diffusion chamber of the Franz cell with the horny layer facing the donor compartment and the dermal side toward the receptor fluid, which was stirred with a magnetic bar at 50 rpm. Sample of 1 ml each was withdrawn at the regular time interval for 48 hours (1, 2, 4, 6, 12, 24, 48 hr), refilling with fresh medium was carried out at the same time. Samples were suitably diluted and analyzed for drug content by a using UV spectrophotometry at 262 nm and mean cumulative of the drug released across the rat skin was calculated. Experiments were performed in triplicate. Rat skin was removed at the end of the diffusion study. The amount of the drug deposited in the skin and the amount of unabsorbed drug were calculated.

2.5. Storage-physical stability of ethosomes: The stability chamber was used to determine the ability of ethosomal preparation to retain the drug (i.e., drug-retentive behavior) was checked by keeping the preparations at different temperatures, i.e., refrigerator (4±10°C), room temperature (RT) 25 ± 20°C, 37 ± 2 and 45 ± 20°C at relative humidity (RH) 60% for different periods of time (1, 20, 40, 60, 80, 100 and 120 days). The ethosomal preparations were kept in sealed vials (10 ml capacity) after flushing with nitrogen. The stability of ethosomes was also determined quantitatively by monitoring size of the vesicles using photo correlation spectroscopy and by checking the % of drug leakage for different period of times (1, 20, 40, 60, 80, 100 and 120 days).

2.6. Preparation of clotrimazole loaded liposomes: Liposomes containing 2% lipid S PC-3 (PC) and clotrimazole were prepared by the classic mechanical-dispersion method. Initially, a methanolic solution of lipid S PC-3 (PC) was first completely dried in a rotary evaporator (Rotavapor-R, Buchi, Germany) at 55°C until a thin lipid film on the wall of a round-bottomed flask was obtained. The resulting lipid film was then hydrated with solution of clotrimazole (prepared by methanol: water) which was mixed at 700 rpm.
with a mechanical stirrer (Remi equipment, Mumbai, India) at room temperature.

2.7. Determination of liposome entrapment efficiency (EE): Liposomes encapsulation efficiency was determined by using ultra centrifugation technique, for that the liposomal (2ml) preparation was transferred to the 10 ml centrifuge tube. The preparation was diluted with distilled water up to 5 ml and centrifuge at 2000 rpm for 20 minute. 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 minute. Supernatant and sediment were separated out. Volume of the supernatant and sediment were measured. Sediment was diluted with distilled water up to 5 ml. The unentrapped and entrapped drug content were analyzed by estimating the drug supernatant and liposomes (sediment) by spectroscopic method. The method was repeated at least three times.

2.8. Determination of liposomal particle size: The average diameter and size distribution of liposome suspensions were determined by photo correlation spectroscopy using a 90 plus particle size analyzer (Brookhaven instrument, New York, USA) at a fixed angle of 90° and at 25°C. Liposome suspensions were suitability diluted with phosphate buffer (pH 7.4) and filtered through a 1 μm polycarbonate membrane to minimize interference particulate matter before sizing. Each measurement was done in triplicate.

2.9. In vitro permeation study through rat skin: Franz diffusion cells were used in the permeation studies of clotrimazole through rat skin. The study was carried out for 48 h at 37 ± 1°C. Rat skin was obtained from the abdominal skin of albino rats (aged 17–22 weeks, weight 250–300 g). All efforts were made to minimize animal suffering and to limit the number of animals used. Rats were killed by diethyl ether inhalation. After depilation and washing, abdominal skin was excised, thoroughly washed with the pH 7.4 buffer solutions, dried and carefully cleaned and then preserved at 25°C. Before using, the skin was thawed, pre-hydrated for 1 h with the pH 7.4 buffer solution and then mounted in the diffusion chamber of the Franz cell with the horny layer facing the donor compartment and the dermal side toward the receptor fluid, which was stirred with a magnetic bar at 50 rpm. Sample of 1 ml each was withdrawn at the regular time interval for 48 hours (1, 2, 4, 6, 12, 24, 48 hr), refilling with fresh medium was carried out at the same time. Samples were suitably diluted and analyzed for drug content by a using UV spectrophotometry at 262 nm and mean cumulative of the drug released across the rat skin was calculated. Experiments were performed in triplicate. Rat skin was removed at the end of the diffusion study. The amount of the drug deposited in the skin and the amount of unabsorbed drug were calculated.

3. Results and Discussion
Clotrimazole loaded ethosomes were prepared using varying concentration of Lipoid S PC-3 (PC) and ethanol, when examined by Transmission Electron Microscope (TEM) appeared as unilamellar vesicles with a predominant spherical shape (Fig. 1a), confirmed the vesicular characteristics possessed by this novel carrier (Fig. 1b).

Fig 1(a). Visualization of ethosomal vesicles.

Fig 2(b) Visualization of liposome vesicles.
The two basic parameters on the basis of which the formulations were optimized are vesicular size and entrapment efficiency on increasing the phospholipids concentration. It was observed that the vesicular size was increased, though with increase in ethanol concentration the vesicular size decreased (Table 1). This indicates that at higher ethanol the membrane thickness is reduced considerably probably due to the formation of phase with interpenetrating hydrocarbon chain. In terms of entrapment efficiency, among all the Clotrimazole loaded ethosomal formulations, E6 (35% v/v ethanol, 3.0% w/v Lipoid S PC-3 and drug) showed the greatest entrapment efficiency, thus justifying itself as the optimized formulation with greatest entrapment efficiency (58.75%) opportunity to the Clotrimazole loaded ethosomal preparation to attain a better skin. An optimum polydispersity index (0.073±0.008) of Clotrimazole loaded ethosomal formulation could better justify the homogeneous nature of the prepared ethosomal formulation (Table 1). All other chemicals were of analytical grade.

| Formulation | Drug (%w/w) | Ethanol (%v/v) | Phospholipids (%w/v) |
|-------------|-------------|----------------|----------------------|
| E1          | 0.77        | 25             | 1                    |
| E2          | 0.77        | 25             | 2                    |
| E3          | 0.77        | 25             | 3                    |
| E4          | 0.77        | 35             | 1                    |
| E5          | 0.77        | 35             | 2                    |
| E6          | 0.77        | 35             | 3                    |

**Table 2. Vesicular size of the ethosomal preparations**

| Formulations | Vesicle size | Polydispersity index (PI) |
|--------------|--------------|----------------------------|
| E1           | 148±8.5      | 0.041±0.003                |
| E2           | 192±9.2      | 0.048±0.004                |
| E3           | 261±11.7     | 0.054±0.007                |
| E4           | 130±8.0      | 0.043±0.005                |
| E5           | 158±12.3     | 0.057±0.007                |
| E6           | 192±13.1     | 0.073±0.008                |

Values represent mean±SD (n=3)

**Table 3. Entrapment efficiency of ethosomal formulations**

| Formulations | %Entrapment Efficiency |
|--------------|------------------------|
| E1           | 32.2                   |
| E2           | 41.1                   |
| E3           | 42.22                  |
| E4           | 40.1                   |
| E5           | 43.22                  |
| E6           | 58.75                  |

E6 formulation shows the better entrapment efficiency

**Table 4. Entrapment efficiency of liposomal formulation**

| Formulation | %Entrapment Efficiency |
|--------------|------------------------|
| Liposome     | 35.2±1.7               |

**Fig 3. Entrapment efficiency of ethosomal and liposomal formulations**

The stability study was performed on Clotrimazole loaded ethosomal and liposomal formulations, which was evaluated for substantial loss of drug at various temperatures (refrigeration, room temperature, 37±2 °C and 45±2 °C) which suggested the storage of ethosomal formulation at refrigerated temperature (4±2 °C), as at elevated temperatures greater drug loss was observed from the formulation (Fig. 3), that might be ascribed to the effect of temperature on the gelto-liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in the membrane packing.17
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Fig 4. Extent of Clotrimazole leakage from ethosomal formulation at different temperatures during storage

Values represents mean ± SD (n=3).

Fig 5. Extent of Clotrimazole leakage from liposomal formulation at different temperatures during storage

Values represents mean ± SD (n=3).

Vesicular size of ciclopiroxolamine loaded ethosomal formulations were measured over the period of 120 days and result showed in Table 2. Vesicular size measurements of ethosomes which stored at room temperature for various time periods showed only 9.2±0.9% size increase (in 120 days) which suggest a stabilizing effect of ethanol in the formulation, in terms of aggregation of vesicles by providing a net negative charge on the surface to the ethosomal formulations thus avoiding aggregation.

Table 5. Stability of ethosomes: Vesicle size (nm).

| Days (after preparation) | Vesicular size of Ethosomes (nm) |
|--------------------------|----------------------------------|
| 1                        | 142±2.9                          |
| 20                       | 145±3.1                          |
| 40                       | 148±3.0                          |
| 60                       | 150±3.5                          |
| 80                       | 153±4.0                          |
| 100                      | 155±3.2                          |
| 120                      | 156±4.2                          |

Table 6. Stability of liposome: Vesicle size (nm).

| Days (after preparation) | Vesicular size of Liposome (nm) |
|--------------------------|---------------------------------|
| 1                        | 211±19                          |
| 20                       | 214±25                          |
| 40                       | 219±18                          |
| 60                       | 224±22                          |
| 80                       | 231±26                          |
| 100                      | 233±37                          |
| 120                      | 237±27                          |

3.1 *In vitro* drug diffusion study of ethosomes and liposomes through rat skin

The rate of the drug release from the ethosomal carrier is an important parameter in the evaluation of the drug delivery for ethosomes. This study was performed by using Franz diffusion cell for the 48 hr. and % drug diffused was calculated by using UV spectrophotometer and results are shown in the Table for ethosomes of which Fig. was plotted between time and % of drug diffused to represent cumulative amount of drug permeated across the rat skin.

The drug release rate for liposomal carrier also be performed by same method for the 48 hr., and % drug diffused was calculated by using UV spectrophotometer and results shown in the Table. The % drug release through the diffusion study is shown on y axis and time plotted in x axis shown in the Fig.
Table 7. *In vitro* drug diffusion study of ethosomes and liposomes

| Time (hr) | % of Drug Diffused |
|-----------|-------------------|
|           | Ethosomes         | Liposomes        |
| 1         | 05.14±0.52        | 03.43±0.44       |
| 2         | 9.23±0.78         | 5.71±0.99        |
| 4         | 18.42±2.51        | 11.83±1.88       |
| 12        | 37.32±3.45        | 21.56±2.33       |
| 24        | 62.11±3.92        | 27.44±2.98       |
| 48        | 74.33±4.60        | 38.11±3.32       |

Fig. *In vitro* drug diffusion study through rat skin

Conclusion

Ethosomes have been studied as a possible vehicle for transdermal delivery of clotrimazole, an antifungal agent, from the study it was confirmed that ethosomal formulation of Clotrimazole showed a higher entrapment efficiency and better stability profile. The enhanced accumulation of Clotrimazole via ethosomal carrier within the skin might help to optimize targeting of this drug to the epidermal and dermal sites. Thus it concluded that ethosomes is a very promising carrier for transdermal delivery and creating a new opportunities for topical application of clotrimazole in the fungal infections.

Clotrimazole is an antifungal drug for treatment of antimycotic that acts against fungi by inhibiting ergosterol. However its oral administration is associated with number of drawbacks. The goal of the current investigation is to evaluate the transdermal potential of novel vesicular carrier. six formulations of Clotrimazole loaded ethosomes were prepared using varying concentration of lipoid S PC-3 (PC) and ethanol also liposomal formulation containing Clotrimazole was prepared by using lipoid S PC-3 (PC), methanol and clotrimazole. The two basic parameters on the basis of which the formulations were optimized are entrapment efficiency and vesicular size on increasing the phospholipids concentration.

In terms of entrapment efficiency, among all the Clotrimazole loaded ethosomal formulations, E6 (45% v/v ethanol, 3.0% w/v lipoid S PC-3 and drug) showed the greatest entrapment efficiency (shown in Table 6 & fig 10), thus justifying itself as the optimized formulation with greatest entrapment efficiency (58.75%) and optimum size (192±13.1 nm) thus showing the greatest opportunity to the Clotrimazole loaded ethosomal preparation to attain a better skin penetration, by providing a safe homing to the Clotrimazole and optimized vesicular size which has been reported to affect the skin permeation parameters.

Ethosomes was examined by transmission electron microscope (TEM) for its vesical shape which appeared as unilamellar vesicles with a predominant spherical shape. It was observed that the vesicular size of ethosomes was increased (148±8.5 to 192±13.1 nm), though with liposomes was the vesicular size (231±23) Diffusion study should be performed to observe the % drug diffused in the rat skin from the ethosome and liposome formulation. The cumulative amount of the drug permeated across the rat skin per unit area was plotted as a function of time and % drug diffused was calculated from the slope of linear portion. The % drug diffused from the ethosome and liposome of clotrimazole were found to be 74.33±4.60 and 38.11±3.32, which shown in Table and Fig . From the data we can predict that % drug diffuse from the ethosome was nearly twice than liposome formulation. When ethosomal carrier, which contain ethanol and soft small vesicle are applied to the skin a number of concomitant process may take place, involving the stratum corneum and pilosebaceous pathway.
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