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Ethosomes: A Novel Vesicular Carrier for Enhanced Dermal Delivery of Ciclopirox Olamine

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Abstract

Ciclopirox olamine 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 ciclopirox olamine an antifungal having limited transdermal permeation. Ciclopirox olamine loaded ethosomes were prepared, optimized and characterized for vesicular shape and surface morphology, vesicular size, size distribution, entrapment efficiency, vesicles skin interaction and stability. The ethosomal formulation (E9) having 3% phospholipids content and 45% ethanol showing the greatest entrapment (72.81±3.5%) and size range (152±11) was selected for further transdermal permeation studies. Stability study was performed for 120 days, which revealed low aggregation and growth in vesicular size (8.5±0.9%). Skin entrapment efficiency assessed by confocal laser scanning microscopy (CLSM) which revealed that enhanced permeation in the deeper layer of the skin (168 µm). The formulation retained its permeation power after storage. Vesicle skin interaction study also showed that there was no interaction between the formulation and rat skin. Further more ethosomal delivery system could be considered for the treatment of number of dermal infections with better efficiency.

Key words: Enhanced drug delivery, ethosomes, candidiasis, confocal laser scanning microscopy, vesicle

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Introduction

Infections caused by fungi constitute a major public health problem in many parts of the world, both in developed and developing countries [1]. Microbial skin infections are very wide spread 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

[2,3]. 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 [4].

Ciclopirox olamine is the ethanolamine salt of ciclopirox, which is a 6-cyclohexyl-1-hydroxy-4-methyl-2(1*H*)-pyridone. It has a very broad spectrum of activity and inhibits nearly all clinically relevant dermatophytes, yeasts, and molds, including the frequently azole-resistant *Candida* species *Candida glabrata*, *Candida krusei*, and *Candida guilliermondii*. It acts against a wide range of bacteria including many gram-positive and gram-negative species pathogenic for humans [5,6].

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 [7]. 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 [8,11]. With the goal of developing a potential effective treatment for deep dermal and intracellular bacterial infections, ciclopirox ethosomal delivery systems were designed and characterized [12].

Materials and Methods

Ciclopirox olamine was purchased from Sigma Lab. New Delhi. Lipoid S PC-3, containing not less than 98% PC was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Ethanol was purchased from Sigma Lab. New Delhi. All other chemicals were of analytical grade.

Preparation of Ciclopirox olamine loaded vesicles

The ethosomes was prepared by Jain et al. and Touitou et al. [13]. The ethanolic vesicular system investigated here was composed of 2.0% w/w of Lipoid S PC-3 (PC), 35% w/w of ethanol, drug (Ciclopirox olamine, 0.77% w/w). Lipoid S PC-3 (PC) was dissolved along with drug 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 ± 1^0C during the preparation and then left to cool at room temperature for 30 min.

Vesicular shape and surface morphology

Vesicular shape of the ethosome preparations were assessed by using Transmission Electron Microscope (TEM) (Philips CM12 Electron Microscope, Eindhoven, Netherlands. Samples were dried on carbon-coated grid and negatively stained with aqueous solution of phosphotungstic acid. After drying the specimen was viewed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 100 kV. Scanning electron microscopy (SEM) was also conducted to characterize the surface morphology of the ethosomal vesicle. One drop of ethosomal system was placed on clear glass stub, air dried and coated with Polaron E 5100 Sputter coater (Polaron, UK) and visualized under Scanning Electron Microscope (Leo-435 VP, Cambridge, UK).

Size distribution and vesicular size

The size distribution of ethosomal preparation was measured in two sets of triplicates, in a multimodal mode, by Dynamic Light Scattering (DLS) technique using a computerized Malvern Autosizer 5002 inspection system (Malvern, UK). For vesicle size measurement, ethosomal preparation were mixed with the appropriate medium (PBS, pH 6.5) and the measurements were taken in triplicate.

Entrapment efficiency

Ethosomal preparations were separated from the free (unentrapped) drug by a Sephadex G-50 minicolumn centrifugation technique [14,15]. The method was repeated at least three times with a fresh syringe packed with gel each time until the fraction collected was free from unentrapped drug. The vesicles were lysed by Triton X-100 (0.5% w/w) and entrapped drug were estimated using HPLC.

Confocal laser scanning microscopy (CLSM)

CLSM was used to investigate depth and mechanism of skin penetration of ciclopirox olamine loaded ethosome preparation, as reported previously. Briefly, unentrapped probe was removed from probe-loaded vesicles by minicolumn ultracentrifugation thereafter formulation was applied non-occlusively for 8 h to the dorsal skin of 5–6week old nude albino rat. The rat was then sacrificed by heart puncture, dorsal skin was excised, washed, placed on aluminium foil and adhering fat and/or subcutaneous tissue was removed. The skin was then sectioned into the pieces of 1 mm size and evaluated for depth of probe penetration for various formulations. The full skin thickness was optically scanned at different increments through the z-axis of a confocal laser scanning microscope (LSM 510 with an attached universal Zeiss epifluorescence microscope). All investigations were performed as per the protocol approved by the Institutional Animals Ethical Committee (Reg. No. 949/a/06/CPCSEA) [16].

Vesicle skin interaction studies

To observe the ultrastructural changes in the skin upon exposure to various ethosomal formulations the vesicles were applied on the skin of rats (Male Sprague Dawley, 5–6 week old, 80–100 g). Preparations were applied topically to the skin for 6 h, animals were sacrificed, skin were excised and stored in formalin solution (10%) in phosphate buffer saline (pH 7.4) followed by dehydration with alcohol. It was then treated with anti-media and embedded in paraffin for fixing. Controls skin section was prepared by similar procedure with out application of any preparation. Sections of 5 μm thickness were cut from each piece and stained with heamotoxyline and eosin and histological changes in stratum corneum, epidermis and dermis were examined under optical microscope (Leica, DMLB, Heerbrugg, Switzerland) [7].

Storage-physical stability of ethosomes

The ability of ethosomal preparations to retain the drug (i.e., drug-retentive behavior) was checked by keeping the preparations at different temperatures, i.e., 25 ± 2 (room temperature, RT), 37 ± 2 and 45 ± 2^{0}C for different periods of time (1, 20, 40, 60, 80 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 and morphology of the vesicles using DLS and TEM.

HPLC assay of ciclopirox olamine

The quantifications of ciclopirox olamine was done by HPLC using methanol/acetonitrile/pH 5.4 buffer solution (9.0/6.8/85 v/v) solvent system. Flow rate was kept at 0.6 ml min−1, by LC 10-AT vp pump (Shimadzu, Japan). 20 µl of injection volume was eluted in LUNA 54, C18, 4.6×150 mm, column (Phenomonex, USA) at room temperature. The column eluant was monitored at 302 nm using SPD-M10A vp diode array UV detector (Shimadzu, Japan), ciclopirox olamine peaks were separated with a retention time of 9.8 min.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of the mean and statistical analysis was carried out employing the Student's t test using the software PRISM (Graph Pad). A value of Pb0.05 was considered statistically significant.

Results and Discussion

Ciclopirox olamine 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). Surface morphology and three-dimensional nature of ethosomes were performed by further analysis of the preparation by Scanning Electron Microscopy (SEM), which confirmed the vesicular characteristics possessed by this novel carrier (Fig. 1b).

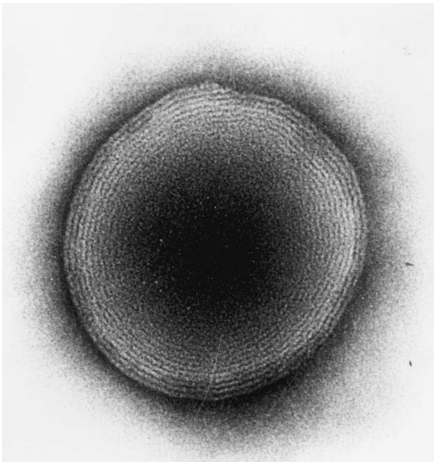


Fig 1a. Visualization of Ethosomal vesicles Tem vesicles(magnification 315 000)

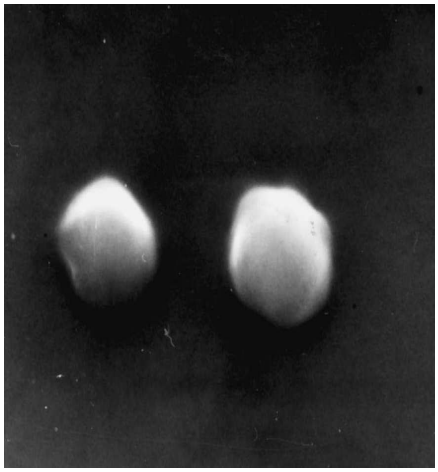


Fig 1b. Sem of Ethosomal (magnification(100 000)

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 ciclopirox olamine loaded ethosomal formulations, E9 (45% 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 (72.8±3.5%) and optimum size (152±11 nm) thus showing the greatest opportunity to the ciclopirox olamine loaded ethosomal preparation to attain a better skin

penetration, by providing a safe homing to the ciclopirox olamine and optimized vesicular size which has been reported to affect the skin permeation parameters [17]. An optimum polydispersity index (0.099±0.012) of ciclopirox olamine loaded ethosomal formulation could better justify the homogeneous nature of the prepared ethosomal formulation (Table 1).

Table 1. Composition and characterization of ethosomal formulations

Formulation	Composition		Vesicle size [a]	PI [b]	%Entrapment Efficiency
	Ethanol (%v/v)	Phospholipids (%w/v)			
E1	25	1	143±8.5	0.049±0.003	35.2±1.7
E2	25	2	199±9.2	0.052±0.004	41.1±2.5
E3	25	3	262±11.7	0.054±0.007	46.22±2.9
E4	35	1	133±8.0	0.046±0.005	43.1±1.8
E5	35	2	169±12.3	0.064±0.007	49.22±2.9
E6	35	3	194±13.1	0.083±0.008	59.75±3.0
E7	45	1	120±7.3	0.076±0.007	54.3±3.2
E8	45	2	141±10.2	0.096± 0.01	63.8±3.1
E9	45	3	152±11.0	0.099± 0.012	72.81±3.5

Values represent mean±SD (n=3); [a] Vesicle size just after preparation; [b] polydispersity index

To measure the extent of penetration and transdermal potency of the stored system, confocal laser scanning microscopic studies should be performed, from the study it showed that an increase in the depth penetration of the ciclopirox olamine loaded ethosomal formulation (up to 168 μm) shown in fig. 2 This prominently efficient delivery of ciclopirox olamine loaded ethosomal vesicle suggests their enhanced penetration and consequent fusion with the lipid membrane in the depths of the skin supporting the hypothesis of Touitou et al., 2000 [18]. The above mentioned parameters were similar for ciclopirox olamine loaded ethosomal formulation which stored for 120 days (depth of penetration=168 μm) which suggesting a stable nature of ethosomal preparation along with no change in transdermal potency of the stored system.

Before the proposal of a ciclopirox olamine as a potential carrier for transdermal drug delivery system, an important characteristic to be evaluated is its *in vivo* skin tolerability/irritancy studies. As, skin non-irritancy of ciclopirox olamine loaded ethosomal formulation is well justified by Kanninkkanan et al. [19] after applying the preparation. It was observed for the erythema scores upon exposure of hairless rabbit skin to various formulations of ethosomes, it was revealed that ciclopirox olamine loaded ethosomes showed no significant erythema, demonstrating that ethanol present in the ethosomal formulation is not able to act as a skin erythema inducing agent, even though present in high concentration.

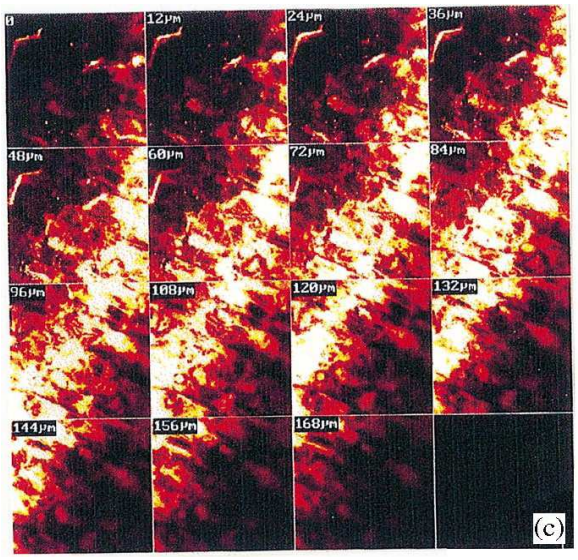
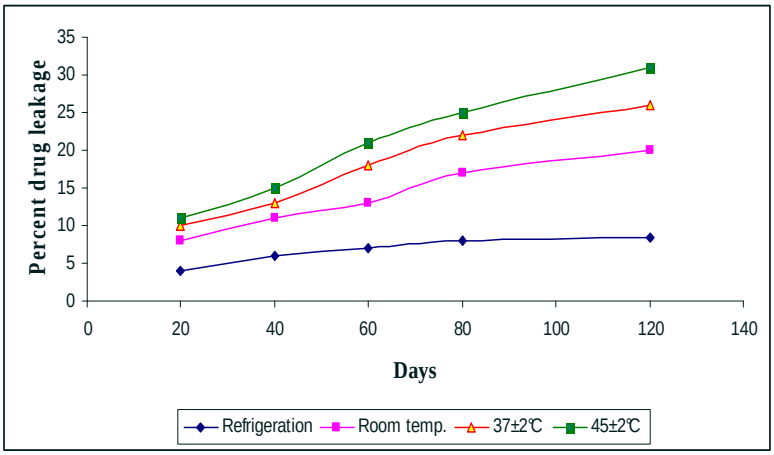


Fig. 2: Confocal laser scanning photomicrograph of penetration of ciclopirox olamine from ethosomes applied non-occlusively onto nude rat skin

The stability study was performed on ciclopirox olamine loaded ethosomal 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 gel-to-liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in the membrane packing [20]. Still a small amount of drug was loaded out from the ethosomal system at refrigerated temperature (8.5±0.9%), a phenomenon possibly occurring due to hydrosoluble nature of ciclopirox olamine.



Values represents mean ± SD (n=3).

Fig. 3: Extent of ciclopirox olamine leakage from ethosomal formulation at different temperatures during storage

Vesicular size of ciclopirox olamine 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 2. Stability of ethosomes: vesicle size (nm)

Days (after preparation)	Vesicular size (nm)
	Ethosomes
1	152±07
20	154±11
40	157±12
60	158±09
80	163±11
120	165±08

Ciclopirox olamine loaded ethosomal formulation showed greater skin penetration, thus justifying its use as a carrier for choice in dermal and transdermal delivery of this anti-fungal drug. Also, the ethosomal formulation is reported to be non-irritant with the skin, which establishing the potential transdermal drug delivery. The enhanced transdermal efficacy obtained from the ethosomal system could be justified on the basis of dual function performed by ethanol present in the ethosomal formulations i.e. fluidizing both the vesicular lipid bilayers and Lipoid S PC-3, thus providing a greater malleability to the vesicles and enhancing permeability of the skin [18,21].

Conclusion

Ethosomes have been studied as a possible vehicle for transdermal delivery of ciclopirox olamine, an antifungal agent, from the study it was confirmed that ethosomal formulation of ciclopirox olamine showed a higher entrapment efficiency and better stability profile. The enhanced accumulation of ciclopirox olamine 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 ciclopirox olamine in the fungal infections.

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