ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.7, No.2, pp 814-820, 2014-2015

ICONN 2015 [4th -6th Feb 2015]
International Conference on Nanoscience and Nanotechnology-2015
SRM University, Chennai, India

In vitro and *In vivo* behavior of a Carbamothioic acid Liposomal Gel for the Treatment of topical Fungal Diseases

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Abstract: The study describes the *in vitro* and *in vivo* evaluation of liposomal reservoir system bearing the anti-fungal, tolnaftate, for better-permeable, controlled and localized delivery via topical route. The differently charged tolnaftate loaded liposomes were developed by dried thin film hydration technique and incorporated to carbopol gel. The developed gels were characterized for particle size, poly-dispersity index, drug content, spreadability, microbial assay and *in vitro* permeation. Antifungal efficacy of developed gels was assessed by the permeation studies in albino wistar rats. The mean particle size of neutral, negative and positive liposomes was found to be 119 nm, 143 nm and 284 nm respectively. Among the three differently charged liposomes neutral charged has exhibited higher entrapment efficiency about 88.14%. The complete sign of cure have been shown by the animals on the 13th day of the drug therapy. Small sized vesicle of developed liposomal gel formulation attributed higher permeation and faster cure rate than the available commercial product. So, this study ensures that liposomal carriers have a high potential in topical drug delivery and can overcome the permeability and efficacy problems.
Keywords: Tolnaftate, liposomal gel, permeability, Topical antifungal, *Candida albicans.*

Introduction

Topical drug delivery is a pleasing route for local and systemic treatment. The delivery of drug through the topical is most effective treatment for the skin diseases[1]. Topical drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist. When the drug is applied to the topically, mainly hydrophobic drugs, only a minute quantity enters in to the systemic circulation remaining stays as such on the skin.

Tolnaftate is a synthetic thiocarbamate used as an anti-fungal agent. It inhibits the squalene epoxidase[2]. Squalene epoxidase is an important enzyme in the biosynthetic pathway of ergosterol (a key component of the fungal membrane). Tolnaftate was found to be only active by topical application and inactive by the oral and intra peritoneal routes of administration[3,4]. Tolnaftate is available in the market in different topical dosage forms like cream, powder, spray and liquid aerosol. But each have its own disadvantages like aerosols cause a mild temporary stinging, in case of creams and gels they are having poor penetration hence require long time of therapy for curing and decreases the patient compliance.

One of the approaches to increase penetrability of the drug thereby without compromising the efficacy is through colloidal drug delivery system. Liposomes are the microscopic spheres made up of an aqueous core surrounded by the lipid shell[6,7,8]. Liposomes are (1) pharmaceutically acceptable & stable (2) superior carriers (3) ability to encapsulate hydrophilic and lipophilic drugs (4) has higher protection against the degradation (5) enhanced bio-availability of drug[9,10]. They can entrap hydrophilic and lipophilic drugs and exhibit higher accumulation at the site of action and deliver the drugs in sustained and controlled rates[8]. An important aspect of liposomes is the protection that they afford as an encapsulating agent against potentially damaging conditions in external environments. Liposomes are also an important system in their own right in medical, cosmetic, and industrial applications[11]. They have affinity to pass keratin of horny layer of skin and can penetrate deeper into skin and hence give better absorption. Topical liposome formulations could be more effective and less toxic than conventional formulations[12]. Topical formulations are also smart route for local and systemic treatment[1]. Hence in this study an attempt has made to formulate tolnaftate liposomal gel for potent skin penetration thereby increasing its efficacy.

Experimental

Materials

Tolnaftate was obtained as gift sample from Mole craft life sciences, Hyderabad, India. Soya bean lecithin, cholesterol, stearyl amine, dicetyl phosphate were purchased from sigma Aldrich, Mumbai, India. Sodium hydroxide and PEG 400 was obtained from SD fine chemicals, Chennai, India. Chloroform and ethanol was obtained from Merck, Mumbai, India. carbopol 971 P was obtained from Lubrizol, Mumbai, India. The equipment used in this study is rotary vacuum evaporator (Rotavapor® R-215), UV-Visible spectrophotometer (Shimadzu 1700E), Equitron probe sonicator (42 KHz, 120 W).

Methods

Development of calibration curve for tolnaftate

A stock solution of 1mg/mL of standard drug was prepared, later dilutions were made with ethanol. From this stock solution 10, 20, 30, 40, 50 µg/mL dilutions were prepared using ethanol. The λ_{max} of the drug was determined by scanning the dilutions between 200 to 400 nm using a UV-Visible spectrophotometer [13].

Preparation of tolnaftate liposomes

The liposomes loaded with soya lecithin containing tolnaftate were prepared by dried thin film hydration technique using rotary vacuum evaporator. Accurately weighed drug and other chemicals was dissolved in 10 mL of chloroform and stirred in mechanical stirrer to form a homogenous mixture. By using rotary evaporator with vacuum of about 25 mm Hg the mixture was dried at 25 ^{0}C until all the chloroform gets evaporated to get a dried thin film on the surface of the vacuum flask. To get a liposomal suspension of multi lamellar vesicles (MLVs) 10 mL of phosphate buffer saline (PBS) pH 7.4 was added and rotated at 25 ^{0}C without vacuum. The layout of composition and ratios of lecithin, cholesterol and stabilizers used for different types of liposomes were shown in the (Table 1). The liposomal dispersion obtained after hydration was sonicated for 30 min by bath sonicator to get small and more uniform sized population of liposomes[14,15]. For the preparation of cabopol gel one gram of carbopol 971 P was dispersed into purified water under stirring. After that the specified quantity of benzyl alcohol was added to the above gel phase under stirring and the pH was adjusted to 6.8-7.0 using 2N NaOH. The previously prepared liposomes were added to carbopol gel under mixing to get a 1% tolnaftate liposomal gel. Finally the pH was adjusted to 6.8-7.0 using 2N NaOH and the final volume was make up[16].

Characterization of Liposomes

Percentage of entrapment efficiency

Liposomes were centrifuged and the supernatant was diluted with aliquot amount of ethanol and the concentration was determined by UV-Visible spectrophotometer. The amount of drug loaded was determined using the formula[17].

$$\%\ Entrapment\ efficiency = \frac{Amount\ of\ drug\ loaded}{label\ claim} \times 100$$

Table 1: Composition and ratios of lecithin, cholesterol and stabilizers for different types of liposomes

Ratio of ingredients	Types of liposome		
	Neutral	Positive	Negative
Lecithin: cholesterol: Stearylamine: dicetylphosphate	5:5:0:0	4.5:4.5:1:0	4.5:4.5:0:1
	6:4:0:0	5:4:1:0	5:4:0:1
	7:3:0:0	6:3:1:0	6:3:0:1
	8:2:0:0	7:2:1:0	7:2:0:1
	9:1:0:0	8:1:1:0	8:1:0:1
	4:6:0:0	4:5:1:0	4:5:0:1
	3:7:0:0	3:6:1:0	3:6:0:1
	2:8:0:0	2:7:1:0	2:7:0:1
	1:9:0:0	1:8:1:0	1:8:0:1

Characterization of Liposomal Gel

Determination of viscosity

Brookfield DVE viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) was used for the determination of viscosity of the formulations. About 0.5 g of sample was taken for analysis without dilution the sample by using spindle no. 63 using different rpm at 25±0.5°C[18, 19].

Microbiological Assay

Tolnaftate is active against *Candida albicans* (*C. albicans*) which is the most common species for dermal fungal infections. Hence it was chosen as fungal inoculum model for both microbial and *in vivo* studies. Petri-dishes containing 20 mL medium (Sabouraud dextrose agar) were seeded with 100 µl of the fungal inoculums *C. albicans*. The plates were dried at room temperature for 15 min. Wells, each 2 cm in diameter, were cut out of the agar. 2 g of neutral, positive and negative gel formulations were placed into each well. Marketed cream (1%) equivalent to 2 g of formulation was used as reference. The fungal plates of *C. albicans* were incubated at 25 °C for 2 days. The zone of inhibition was observed after 48 h. The results were recorded by measuring the zones of growth inhibition surrounding the wells[20].

Ex Vivo Permeation Studies

Permeation studies were performed for neutral, positive, negative liposomal gel formulations and marketed cream by using Franz diffusion cells having diffusion area of 1.813cm^2. Ethanol:PBS (pH 7.4) in 20:80 ratio was used as diffusion media by maintaining constant stirring with a magnetic stirrer at 300 rpm. The temperature of 32±0.5^{0}C was maintained with the help of water bath. 200mg of each formulation was applied separately on the dorsal surface of pork skin. Five Franz diffusion cells were being used simultaneously for five different formulation i.e. neutral, positive, negative, marketed cream and blank. One mL of sample was withdrawn from each in the time intervals of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 12 h respectively. The concentration of tolnaftate in the collected sample was determined by using UV-Spectrophotometer[21,22].

After carrying out the permeation study for 12 h, the percentage drug retained on the skin at end of 12 h was determined by washing the skin three to four times with the ethanol solution. The solution was filtered through 0.22 µm filter and was analysed for the entrapment efficiency using UV-Visible spectrophotometer. For the estimation of drug retention in the skin, the skin was cut into small pieces and was homogenized, which was then sonicated in the ultrasonicator for 10 min followed by vortex mixing for 15 min. The obtained sample was then centrifuged at 6000 rpm for 15 min. After centrifugation, supernatant was taken, filtered through 0.22 µm filter and was analyzed for entrapment efficiency[23].

In vivo Studies

Albino wistar rats (male) weighing 150±20 g were used for topical fungal cure rates studies. All animal experiments were approved by institutional animal ethical committee, J.S.S. College of Pharmacy, Ooty (Proposal no. JSSCP/IAEC/M.PHARM/PH CEUTICS/08/2012-2014). Animals were grouped in to 4 groups containing 3 animals in each group. Group I animals were treated neutral formulation, group II with positive formulation, group III with negative formulation and group IV with marketed cream. Negative control was maintained to confirm the cure is because of formulations but not a natural process.

The two months old male wistar rats were maintained by giving food and water at 21 ^{o}C. *C. albicans* was being used as a infectious agent for this *in vivo* study. The micromycetes were maintained on sabouraud dextrose Agar (SDA), which contains 40 g of glucose, 10 g of agar and 10 g of peptone in pure distilled water. The cultures were stored at 4^{0}C and sub cultured once in a month. For the purpose of inoculation the dorsal surface of the each rat, the areas of 5 cm^2 were cleaned and depilated. The infectious inoculums were prepared from a 6 day old culture of *C. albicans.* The inoculum was applied on the animal's dorsal surface immediately after the depilation and left for 3 days for the development of infection. The treatment was started on the 5th day of post inoculation and continued until complete recovery from the infection was achieved. Animals were treated once a day and the infected areas were scored visually for inflammation and scaling, as well as the presence of the pathogens by cultivating skin scales from infected loci in SDA plates containing 100 units/mL of penicillin and streptomycin, each day[23,24].

Results and Discussion

Preformulation Studies

Standard calibration curve of tolnaftate in UV spectrophotometer

The UV absorbance of tolnaftate standard solutions was in the range of 10-50μg/mL of drug in ethanol showed linearity at 257 nm. The linearity was plotted for absorbance (Abs) against concentration (μg/mL) with R^2 value of 0.996 and with the slope equation y =0.018x-0.003.

Preparation of tolnaftate liposomal gel

The liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug and carrier (soya lecithin) with different stabilizers like stearylamine and dicetyl phosphate and all other parameters like temperature, vacuum and stirring speed were kept constant. The optimized batch of liposomes were mixed into the carbopol 971 P gel with an electrical mixer and finally the pH was adjusted to 6.8-7.0 using 2N NaOH and final volume was make up.

Characterisation of Liposomes

Mean particle size and polydispersity index of the liposomes were determined by Malvern Zetasizer. The mean particle diameter of neutral liposomes, negatively charged vesicles and positively charged was found to be around 119 nm, 143 nm and 284 nm respectively. These results can be attributed to the inclusion of a charge inducer (Stearylamine, Dicetyl phosphate) in liposomes, which increased the spacing between the adjacent bilayers, resulting in the formation of liposomes larger in size compared with the neutral ones (Table 2). The percentages of entrapment efficiency in neutral and charged liposomes were found to be 88.14%, 82.8% and 84.23% respectively. The percentage drug loading was optimum in neutral liposomes when compared with charged liposomes.

Table 2: Particle size and Poly dispersity index (PDI) of tolnaftate liposomes

Type of liposome	Particle Size (nm)	PDI
Neutral	119	1.000
Positive	284	0.640
Negative	143	0.163

Characterization of Liposomal Gel

Microbial assay

The images for zone of inhibition are shown in the (Figure 1). The zone of inhibition of neutral, positive, negative and marketed cream were found to be 0.64±0.17 mm, 0.59±0.23 mm, 0.45±0.18 mm and 0.12±0.05 mm respectively which conclude that the neutral showed maximum inhibitory effect on fungal strain *C. albicans* followed by positive, negative formulations and commercialized cream. The results indicated that neutral, positive, negative formulations were having higher release behavior and higher penetration capacity compare to marketed cream. It was also found that neutral, positive, negative have high zone of inhibition than

the marketed cream this may be due to the highly lipophilic nature of the drug in the vesicles of the 3 gels compared to drug present in marketed cream as such, as a result high uptake of lipophilic compounds by fungal strains lead to higher zone of inhibition. This microbial assay has also exposed the fact that lower viscosity gel exhibit higher release rates in addition to low vesicle sizes which has higher penetration and together leads to high zones of fungal inhibition. Hence, it was further resolved that decrease in the vesicle size and viscosity of the formulation, the zone of inhibition produced was increased.

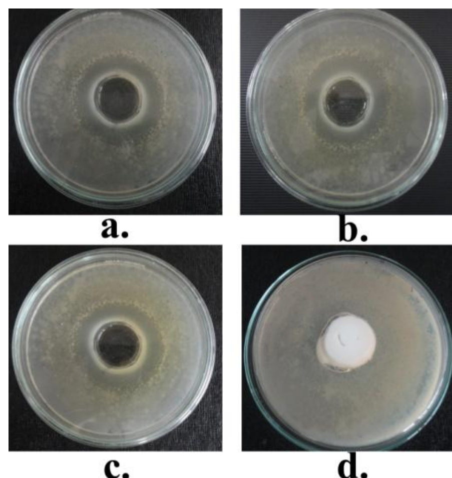

Figure 1: Images for zone of inhibition: a. Negative, Neutral, Positive and Marketed Cream

Ex Vivo Permeation Studies

The permeation profile was obtained by applying neutral, positive and negative formulations and marketed cream (each containing 2 mg of Tolnaftate) on the pork dorsal ear skin in the Franz diffusion cell. The results for permeation studies i.e. % drug permeated (receptor chamber), % retained in the skin, % retained on the skin (donor chamber) were obtained and listed in the table 3 (Table 3). It was observed that concentration of tolnaftate steadily increases in the receptor medium with increase in time, where the permeation profile generally followed Fick's diffusion law. The % cumulative amount of tolnaftate permeated from neutral, negative, positive formulations and marketed cream at the end of 12th h after application was found to be 58.2±0.6, 51.19±0.81, 44.84±0.9 and 19.78±1.12 respectively. For effective dermal drug delivery there is a necessity to provides larger area for permeation of drug in to skin and high drug concentration on the affected area which results in a larger concentration gradient, which has been efficiently attained with smaller vesicle size of neutral, negative, positive formulation. The marketed cream which was used as a control had retained large amount of the drug on the skin (56.32±0.3). Neutral liposomal gel has show the maximum retention of drug in the skin i.e (29.33±0.5), followed by negative (25.36±0.2), positive (20.56±0.43) which is due to less viscosity and low vesicle size of the neutral liposomal gel followed by negative and positive liposomal gel.

Table 3: Comparative data of % drug permeated, retained in skin and on skin

% Drug	Neutral	Negative	Positive	Marketed cream
Permeated	58.2±0.6	51.19±0.81	44.8±0.9	19.78±1.12
Retained in skin	29.33±0.5	25.36±0.2	20.56±0.43	10.66±1.6
Retained on skin	5.71±1.6	27.46±0.3	32.33±0.94	56.32±0.3

***Values are Mean±S.D (n=3)**

After permeation studies ANOVA by post tukey multiple comparison of all columns was used to calculate the significance (P<0.05) of permeation difference between each formulations. Based on the ANOVA data using post tukey multiple comparison of all columns at 12th h release of each formulation, it was conclude that the neutral, negative, positive, and marketed cream were extremely significant (P<0.05) in their permeation, possibly due to slower release and poor permeation of marked cream and lower vesicle size of neutral liposomal gel compare to negative and positive liposomal gel the killing of fungus is possible only if larger amount of drug retains into the skin layers and with higher permeability.

In vivo studies

First symptoms (small scaly redness) on the rats inoculated with *C. albicans* were observed on the 5th day of the experiment, while later (8th day), they were exhibiting in blood wounds, around 5 mm in diameter. The treatment was started on the 5th day of the experiment. The lesions and wounds started curing by the 8th day itself for neutral, positive, negative formulations. On the 13th day of the treatment with all above formulations, the rats were completely cured, there were no visually observed symptoms,in comparison animals treated with the commercial cream, were got cured after 16 days of treatment (Figure 2).

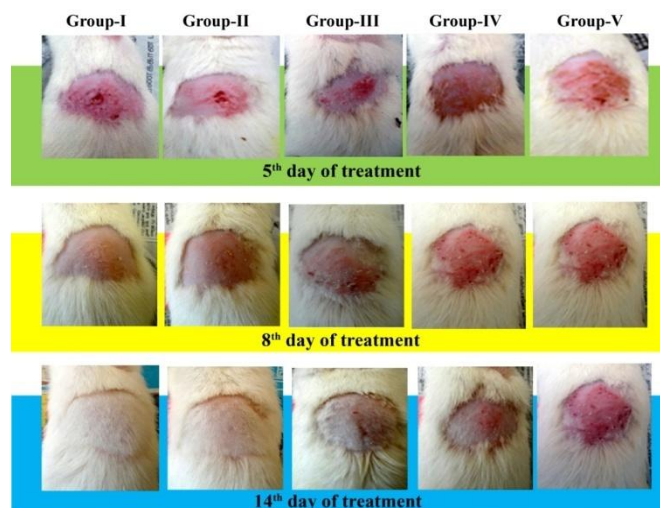

Figure 3: Images of animals during 5th, 8th and 14th day of treatment with neutral, positive, negative liposomal gel, marketed cream and negative control

To make conformation that fungus has completely cured on the final day (Day 14) small quantity of skin was scrapped from infected area and cultured on SDA plates and then incubated. Up on 4 days of incubation the plates were scored for fungal colonies and statistical data produced using one way ANOVA followed by post-tukey multiple comparison test. Based on ANOVA data it was concluded that neutral, positive and negative formulations are extremely significant ($P < 0.05$) than marketed cream and negative control, since the number of colony forming units (CFU) were more for marketed cream which is near similar to that of negative control. This is due to the fact that neutral, positive and negative liposomes have less vesicle size and high lipophilicity which allow high concentrations of drug to penetrate to the skin and functionally create a drug depot in the stratum corneum and epidermis. Hence neutral, positive and negative formulations possess good therapeutic and antifungal effect and could represent possible alternative for the treatment of patient infected by dermartomycoses compared to marketed cream.

Conclusion

This study further confirms that liposomal gels provided greater permeation followed by cure rates of poorly soluble drugs which are intended for topical use to overcome the permeability and efficacy problems.

Acknowledgements

The author would like to thank Mole craft life sciences, Hyderabad, India for kindly providing the gift sample of tolnaftate.

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