Preparation and In Vitro Evaluation of Miconazole Nitrate–Loaded Topical Liposomes

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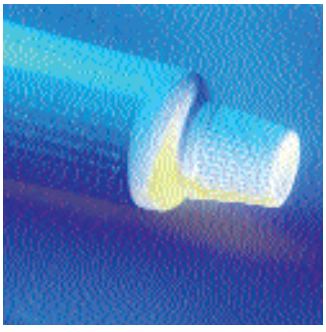

IMAGE 100

Miconazole nitrate is a widely used antifungal agent, but its use in topical formulations is not efficacious because deep-seated fungal infections are difficult to treat with conventional topical formulations. The entrapment of a drug in liposomes can facilitate localized delivery of the drug, and improved availability by means of a controlled-release pattern can advance the treatment of deep fungal infections. This study examined critical parameters such as lipid composition, type of lipid, and charge, and the prepared products were characterized for liposome-specific properties such as microscopic appearance, size, and degree of drug entrapment. Significant retention of miconazole in skin was observed with the use of liposomes.

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Miconazole nitrate (MCZ) is a broad-spectrum antifungal agent of the imidazole group (1). It acts by means of a combination of two mechanisms: ergosterol biosynthesis inhibition, which causes lysis of fungal cell membranes because of the changes in both membrane integrity and fluidity, and direct membrane damage of the fungal cells. The drug is primarily used as a topical treatment for cutaneous mycoses (2); poor dissolution and lack of absorption make it a poor candidate for oral administration. However, MCZ can be used as a systemic antifungal agent when amphotericin B or ketoconazole is either ineffective or contraindicated.

MCZ's poor skin-penetration capability presents a problem in the treatment of cutaneous diseases by topical application. For effective treatment, the drug must be delivered in sufficient concentration to the site of infection (3). Various approaches have been used to enhance the access of such poorly skin-partitioned drug molecules. For example, the use of complexation with cyclodextrins has been reported to improve oral and topical delivery of MCZ (4,5). Other approaches have used submicron emulsions of MCZ for improved topical delivery (6,7) and chewing gum containing MCZ for buccal delivery (8,9). Several reports have described the potential use of liposomes to topically deliver drugs into the deep layers of the skin.

The authors examined the use of closed lamellar vesicles whose unique structural and functional features facilitate the delivery of dermal drugs because they are biodegradable, nontoxic, amphiphilic in nature, penetration enhancing, and effective for modulating drug-release properties. These smectic mesophasic structures can be modified in terms of their size, shape, lamellae nature, and the type of composition used. The successful launch of the antifungal agent econazole (Pevaryl, CILAG, AG, Switzerland) on the market encouraged further exploration and exploitation of these molecules to make them more effective (10).

Various studies have assessed the individual characteristics of MCZ, including degree of entrapment, size profile, drug-release profile, and ability to carry the drug across the skin barrier. The analysis of MCZ in liposomes was determined using second-order derivative spectrophotometry (a simple spectrophotometric technique is not sensitive enough to produce accurate results for estimating a low amount of drug) (11,12).

Table I: Effect of lipid composition on encapsulation efficiency of miconazole in liposomes using PCS.

Batch No.	Composition Ratio by Weight (MCZ:PCS:CHOL)	Entrapment Efficiency (mg) Mean ± SD	Total Lipid (mg)
PLH 1	10:100:5	8.45 ± 0.05	115
PLH 2	10:100:10	8.79 ± 0.04	120
PLH 3	10:100:15	9.40 ± 0.10	125
PLH 4	10:100:20	9.76 ± 0.04	130
PLH 5	10:100:30	8.99 ± 0.04	140
PLH 6	10:100:50	8.88 ± 0.03	160
PLH 7	10:100:60	7.62 ± 0.02	170
PLH 8	10:100:70	8.32 ± 0.02	180
PLH 9	10:100:80	7.96 ± 0.04	190

Table II: Effect of lipid composition on encapsulation efficiency of miconazole in liposomes using PCU.

Batch No.	Composition Ratio by Weight (MCZ:PCU:CHOL)	Entrapment Efficiency (mg) Mean ± SD	Total Lipid (mg)
PLG 1	10:100:5	8.58 ± 0.10	115
PLG 2	10:100:10	7.55 ± 0.15	120
PLG 3	10:100:15	7.20 ± 0.17	125
PLG 4	10:125:5	8.77 ± 0.19	140
PLG 5	10:125:10	8.58 ± 0.12	145
PLG 6	10:125:15	8.56 ± 0.18	150
PLG 7	10:150:5	9.40 ± 0.21	165
PLG 8	10:150:10	8.91 ± 0.18	170
PLG 9	10:150:15	8.74 ± 0.15	175

Experiment

Materials. The materials used in the study were MCZ (Glaxo Ltd., Mumbai, India), Phospholipon 90H phosphatidyl choline saturated, (Nattermann Phospholipids, Cologne, Germany), phosphatidyl choline 97.3% content (PCS) (Nattermann), Phospholipon 90G (unsaturated phosphatidyl choline) (Nattermann), phosphatidyl choline 98.0% content (PCU), (Nattermann), Sephadex G-50 medium cholesterol (CHOL) (Sigma, St. Louis, MO), dicetyl phosphate (DCP) (Sigma), and butylated hydroxy toluene (BHT) (Sigma).

Preparation of liposomes. Multilamellar liposomes were prepared using the thin-film hydration method. Accurately weighed quantities of drug, PCS, PCU, CHOL, and DCP in the ratios shown in Table I and Table II were dissolved in chloroform in 250-mL round-bottom flasks. A constant amount of BHT, equivalent to 2% of total lipid volume, was added as an antioxidant to the organic phase in the flasks. The chloroform was evaporated at 45 °C under reduced pressure at 150 rpm using a Rotavapor film evaporator (Büchi RE 121, Flawil, Switzerland). After the chloroform was completly evaporated, the flasks were kept overnight under vacuum pressure to remove residual solvent. The thin film was hydrated using pH 6.4 phosphate-buffered saline (PBS) for 2 h until vesiculation was complete.

Determination of drug entrapment in vesicles. Unentrapped, free drug was separated using the minicolumn centrifugation method (13). The 0.2-mL vesicular suspensions were first presaturated with empty vesicles and then placed in the column. Centrifugation was conducted at 2000 rpm for 3 min, and elutes containing drug-loaded vesicles were collected and observed under a light microscope for drug particles. The vesicular suspensions were analyzed using a spectrophotometer (Lambda 15, PerkinElmer, Shelton, CT) and the second-order derivative technique.

Analysis by second-order spectrophotometry. For this analysis, 0.2-mL liposomal elutes were added to 10 mL of methanol and vortexed for 10 min for the extraction. The solutions thus obtained were centrifuged at 3000 rpm for 15 min to settle undissolved lipid in methanol. The supernatants were decanted and analyzed at $^2D_{276.8}$ using the spectrophotometer.

Instrument. A PerkinElmer Lambda 15 recording double-beam UV–vis spectrophotometer was used. The scan speed was 120 nm/min. The auto mode of slit width ($\Delta\lambda \approx 3$) in the instrument smoothes the spectra without removing substantial peaks. The other parameters were slit 2 nm, response 0.5 s, and peak threshold 0.2 D2.

Microscopy. All the batches were viewed under an optical microscope to determine the shape and lamellarity of vesicles. The liposomes' vesicle sizes were determined by light scattering on the basis of laser diffraction using the Malvern Mastersizer (model S, version 2.15, Worcestershire, Malvern, UK).

Drug-leakage study from vesicles. The optimized liposomal batches (PLH 4, PLG 7) were sealed in 30-mL vials after purging with nitrogen and stored at various temperatures (4–8, 25 ± 2, 37, and 45 °C) for a period of two months. Samples from each batch at each temperature were withdrawn at defined time intervals, and the residual amount of drug in the vesicles (i.e., entrapment) was determined as described in the drug-entrapment section (see Figure 1).

Solubility study. The solubility of MCZ was determined using a thermostatic water-shaker bath at 37 °C for 24 h. The excess of drug was added to 10 mL of phosphate buffer saline with a pH 6.4 value. An increasing concentration of the surfactant Tween 20 was added to stoppered 25-mL volumetric flasks. After the samples were shaken for 24 h, they were filtered using 0.45-μm membrane filters and analyzed after appropriate dilution using the spectrophotometer at 272 nm. These studies were conducted to determine the sink conditions for in vitro performance evaluation of the described systems.

In vitro drug release study. Drug release studies included dialysis-membrane and skin-permeation trials on the hairless abdominal skin of LACA mice using modified Franz diffusion cells. Various formulations of liposomal (i.e., PLH 4 and PLG 7) and conventional creams with MCZ, each equivalent to 5 mg of drug, were applied to the membrane and skin facing the donor chamber. The receptor phase was 150 mL PBS with a pH 6.4 value and 2% w/v Tween 20. An aliquot of 5 mL of sample was withdrawn from each batch and replaced with the same amount of buffer to maintain the receptor phase at 150 mL. The samples were quantitiated by UV spectrophotometer at 272 nm.

Skin-retention studies. After conducting the permeation study, the authors carefully removed the skin mounted on the Franz diffusion cells. The remaining formulation adhering to the skin was scraped with a spatula and then wiped with tissue paper.

Table III: Effect of DCP on entrapment efficiency of liposomal batches (PLH 4 and PLG 7).

	MCZ:PCS: CHOL:DCP	Entrapment Efficiency (mg)/Total Lipid (mg)
PLH 4	10:100:20:5	8.76 ± 0.04/130
	10:100:20:10	9.76 ± 0.06/130
	10:100:20:15	9.70 ± 0.09/130
	10:100:20:20	9.80 ± 0.121/130
PLG 7	10:150:5:5	8.80 ± 0.12/ 165
	10:150:5:10	9.40 ± 0.21/165
	10:150:5:15	9.50 ± 0.08/165
	10:150:5:20	9.45 ± 0.16/165

The cleaned skin piece was mashed, and 10 mL of methanol was added to the meshed mass and mechanically shaken in a water shaker bath at 37 ± 1 °C for 24 h for the complete extraction of the drug. The filtrate was removed and analyzed using derivative spectrophotometry.

Results and discussion

Entrapment efficiency. **Influence of process parameters.** Vacuum pressure, hydrating medium, hydration time, flask rotation speed, and method of size reduction were optimized to prepare lipid vesicles of MCZ. PBS at pH 6.4 was the best hydrating medium, with good stability and entrapment of the drug. Adjusting the evaporator's rotation to 150 rpm increased the surface area for evaporation and was sufficient to form thin, uniform, and completely dried film. The size of liposome prepared from PCS was found to be 6 ± 2.5 μm, and the size of liposomes prepared from PCU was found to be 4 ± 1.5 μm.

Analytical method. Analysis was completed using derivative spectrometry, which is a technique for the enhancement of sensitivity and specificity in qualitative and quantitative analysis of various compounds, including pharmaceuticals. The fine structural features of this derivative method have been sharpened and emphasized to provide an improved resolution of overlapping and have been potentiated to give greater sensitivity (14,15). Amplitude at $^1D_{284}$ and $^2D_{276.8}$ has been reported specifically for MCZ (16).

Methanol was used for the extraction of the drug rather than a chloroform and methanol mixture because methanol results in more conspicuous peaks during analysis compared with a chloroform and methanol mixture. The authors found that simple absorbance estimation made it difficult to determine the drug content in liposomal elute obtained from sephadex columns because the sensitivity of the simple spectrophotometric technique is very low ($E = 17$). Previous studies have shown that imidazole rings containing antifungal drugs such as MCZ have a relatively low absorption in the UV range and are difficult to analyze in low concentrations (17). Similarly, the authors found that the concentration obtained from the liposomal elute of the column was very low, and further minor interference from lipids made the estimation of MCZ in liposomes difficult as well as error prone. The authors had developed a method that is sensitive enough to analyze the drug in liposomal elute from a sephadex column. They found that the $^2D_{276.8}$ method is sensitive and specific for the determination of MCZ without interference from the lipids used ($E = 273$, $R^2 = 0.998$, $Y = 0.0273X$).

Influence of formulation component variables. Table I and Table II summarize the various lipid concentration and drug entrapment efficiencies of the liposomal systems. Table III shows the effect of DCP on entrapment efficiency. The inclusion of DCP as a negatively charged agent into the lipidic layers could impede the aggregation and fusion of vesicles so as to maintain their integrity and uniformity. In addition, the antioxidant BHT (2% of total lipids) was added to each formulation to minimize the oxidative degradation of phospholipids that leads to stability problems. Table I lists the liposomes prepared with PCS. An increase in CHOL concentration with the same MCZ and PCS concentration (PLH 1 to PLH 4) led to an increase in entrapment levels of MCZ from 8.45 mg/115 mg to 9.76 mg/130 mg of total lipids. The increase in entrapment efficiency was attributed to the ability of CHOL to cement the leaking space in the bilayer membranes, which in turn leads to an enhanced drug level in liposomes (18). A further increase in CHOL level from 30 to 80 mg (PLH 5 to PLH 9) with the phosphatidyl choline amount kept constant decreased the entrapment efficiency, although the total lipid level also was increased. This occurrence indicates that MCZ entrapment is enhanced up to a certain level of PCS and CHOL and substantiates the importance of the appropriate proportions of PCS and CHOL to maximize the entrapment affinity of drug toward bilayer-forming phospholipids.

Table II lists the liposomes prepared with PCU. The authors found that increasing the PCU level increased the entrapment efficiency (PLG 1, PLG 4, and PLG 7). At the lower level of PCU at 100 mg (PLG 1), the entrapment efficiency was less. As the amount of PCU was increased to 150 mg (PLG 7) and CHOL was kept at a constant level of 5 mg, the entrapment efficiency increased to 9.4 mg per 165 mg of total lipids from 8.58 mg per 115 mg of total lipids, respectively. Analogus to PCS liposomes, increasing the CHOL level while keeping the PCU level constant decreased the entrapment efficiency of MCZ. In contrast to PCS liposomes, the authors found that PCU-containing liposomes required a very low amount of CHOL (PCU:CHOL in a ratio of 150:5) to achieve maximum entrapment (PLG 7), and an increase in CHOL from 5 mg to 15 mg decreased the entrapment of MCZ to 8.74 ± 0.15 per 175 mg of total lipids (PLG 9). With PCS-containing liposomes, an increased amount of CHOL (PCS:CHOL ratio of 100:20) was required to achieve maximum entrapment.

The entrapment efficiency data clearly suggest that the ratio of PCU to CHOL is crucial because an enhanced CHOL level disturbs entrapment, which also depends on the type of phosphatidyl choline used and the physicochemical nature of the drug. To study the effect of DCP level on both kinds of liposomes, varying amounts of DCP were added, and the degree of entrapment was determined. The addition of DCP considerably improved the liposomal formulation. Enhancing the amount from 5 to 10 mg helped raise drug loading from 8.76 to 9.45 mg, but beyond that range no appreciable change in entrapment was noted (see Table III). Furthermore, the improvement in the liposome-specific features (entrapment, vesicle uniformity, and stability) may be attributed to DCP's ability to incorporate into lipid-layer domains imparting the polarity (negative charge). These properties facilitate the vesiculation process and pro-

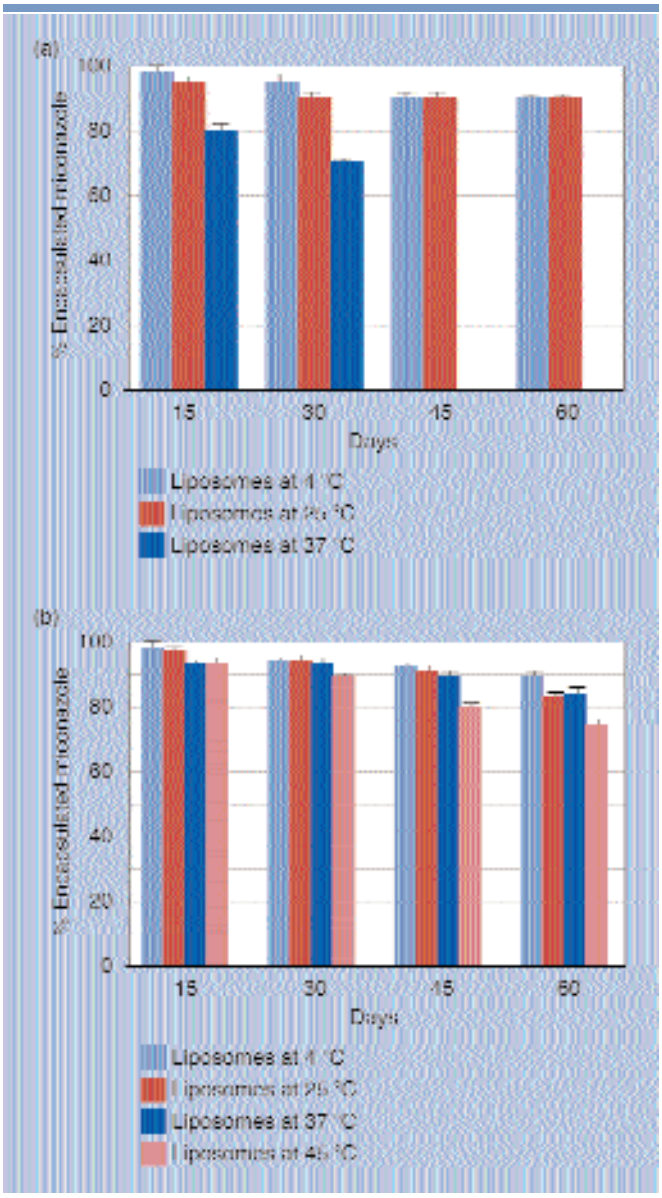

Figure 1: (a) The percentage of drug remaining in PLG 7 liposomes after subjecting them to temperatures of 4, 25, and 37 °C for 15, 30, 45, and 60 days. (b) The percentage of drug remaining in PLH 4 liposomes after subjecting them to temperatures of 4, 25, 37, and 45 °C for 15, 30, 45, and 60 days.

nounced uniformity in size distribution because the desired polar and nonpolar interactions are reached.

Drug-leakage studies. The authors conducted a two-month study of liposomal stability with respect to the liposomes' ability to retain an entrapped drug during a defined time period. The PLG 7 batch of liposomes was withdrawn from the study at 45 °C after 2 weeks and at 37 °C after 4 weeks because storage at these temperatures led to a substantial loss (≈ 90%) of drug from the liposomes by the end of a one-month period (see Figure 1a). Drug leakage at elevated temperatures may be a result of chemical degradation (oxidation and hydrolysis) of lipids in the bilayers, leading to defects in membrane packing. Thus, earlier reports of the low-temperature stability of liposomal products may be attributed to the deformation of gel-state lipid membranes that help hold MCZ molecules in place. Figure 1b shows that the drug-retention capacity of liposome PLH 4 is higher than that of PLG 7 in all conditions. The better stability of liposomes PLH 4 compared with PLG 7 may be attributed to the high phase-transition temperature of PCS (56 °C) relative to the low phase-transition temperature of PCU (28–30 °C), the greater affinity of PCS toward the drug molecule, and the liposomes' greater structural integrity. Both PCU and PCS have shown fairly high retention of drug inside the vesicles at 4 °C and 25 °C for as long as two months (≈ 90%).

In vitro drug-release studies. Diffusion medium. Although physiological saline was potentially the most appropriate receptor phase, the drug was poorly soluble in water. Thus, an appropriate medium was required that could provide sufficient solubility for the drug to maintain the required sink condition during permeation studies. The authors used varying concentrations of Tween 20 to solubilize the drug. They found that increasing the surfactant concentration improved the solubility. A medium with 2% w/v Tween 20 provided a 0.239-mg/mL solubility, which was sufficient for the permeation study. Five milligrams of MCZ was placed in the donor compartment, and 150 mL of receptor medium was used. This permeation medium had an approximate sevenfold capacity to solubilize the added amount of drug.

Diffusion study. Figure 2 shows the release profile of various formulations and their release fluxes. The release-behavior studies of MCZ-loaded liposomes showed retarded transfer of drug molecules across the vesicular systems composed of PCS and PCU when compared with that of the plain drug solution. The flux values obtained with various formulations were 109.36 μg/cm^2/h (MCZ), 76.43 μg/cm^2/h (PLH 4), 70.4 μg/cm^2/h (PLG 7), 50.56 μg/cm^2/h (cream A), 33.6 μg/cm^2/h (cream B), and 32.53 μg/cm^2/h (cream C) for solution, PCS liposomes, PCU liposomes, commercial cream A, commercial cream B, and conventional cream, respectively. Analysis of these results showed that the liposomal systems composed of PCS and PCU had formed a release barrier through their multilamellar layers. PCU lipids had a greater release-retarding effect than did PCS lipids. Liposomes composed of saturated lipids normally show improved results because of the low leakage property of bilayers with an improved packing of lipid molecules. One could conclude that the change in the behavior of the PCU vesicles with a high lipid content strengthened the lipid barrier effect. However, the conventional and commercial creams showed decreased values of release flux because of the drug being dispersed in an insoluble form in cream C. The remaining formulation design factors of commercial creams did not affect the release flux.

Skin-permeation and skin-retention studies. Figures 3 and 4 show the various results obtained in the skin-permeation, permeation-flux, and skin-retention studies. The PCS and PCU liposomal systems had permeation flux values of 54.43 μg/cm^2/h (PLH 4) and 51.4 μg/cm^2/h (PLG 7). The nonliposomal systems permeated the drug at a flux value of 81.66 μg/cm^2/h (MCZ), 29.5 μg/cm^2/h (cream A), 41.23 μg/cm^2/h (cream B), and 28.73 μg/cm^2/h (cream C). In the skin-retention studies of the liposomal systems, the amounts of MCZ retained were 0.84 mg (PLH 4) and 0.72 mg (PLG 7) for liposomes composed of PCS

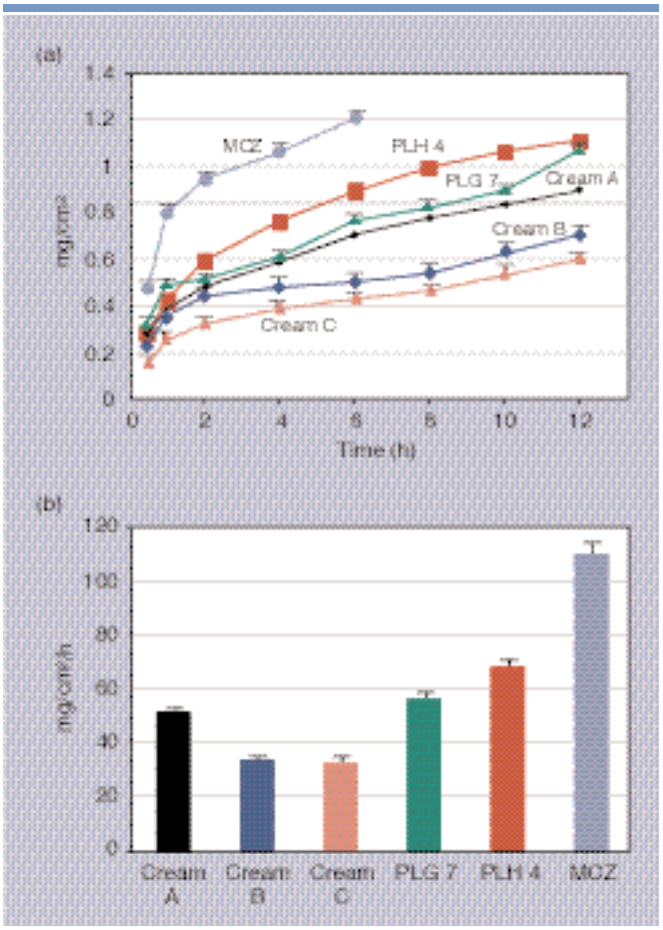

Figure 2: (a) Plot between the amount of MCZ released per unit area and the time for the optimized batches of PLH 4 and PLG 7 liposomes and drug in a cream base (cream A: commercial gel; cream B: commercial gel; cream C: prepared cream base). (b) A comparison of the rate of flux of miconazole released from liposomal and cream formulations (cream A: commercial gel; cream B: commercial gel; cream C: prepared cream base).

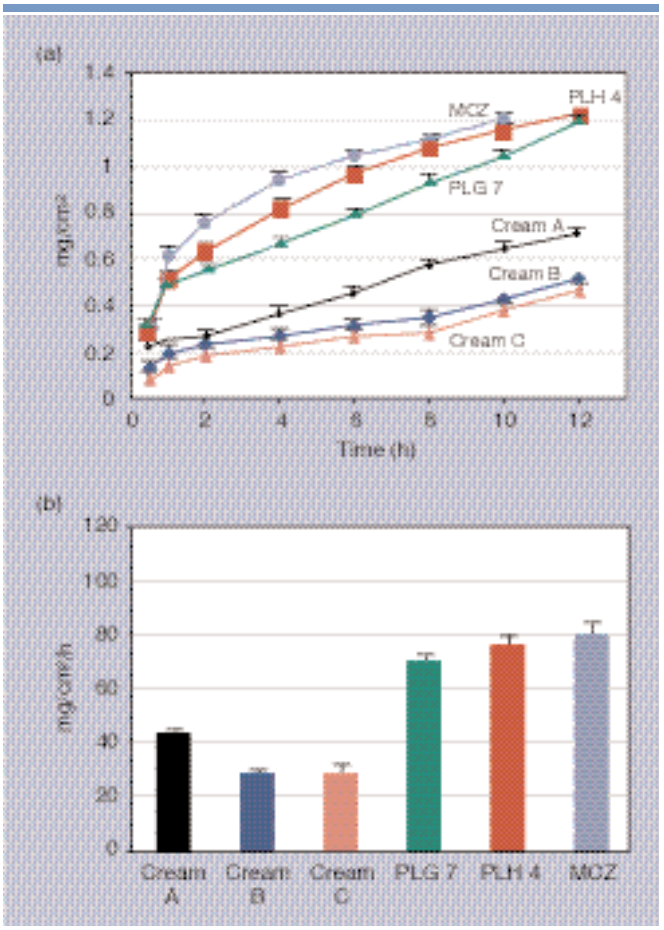

Figure 3: (a) Plot between the amount of MCZ permeated per unit area and the time for the optimized batches of PLH 4 and PLG 7 liposomes and drug in cream base (cream A: commercial gel; cream B: commercial gel; cream C: prepared cream base). (b) A comparison of the rate of flux of miconazole permeated from the liposomal and cream formulations (cream A: commercial gel; cream B: commercial gel; cream C: prepared cream base).

and PCU, and in the nonliposomal studies the skin retention values were 0.09 mg (MCZ), 0.15 (cream A), 0.12 mg (cream B), and 0.25 mg (cream C).

These results show the influence of vesicular systems and nonvesicular formulations on the penetration, retention, and permeation tendencies of the drug molecules in various conditions. The study data show that liposomal systems can make the drug molecules accessible within the skin layers. The high retention but reduced permeation of the drug in vesicular systems can be attributed to intrinsic liposome–skin interaction behavior. This conclusion compares favorably with an earlier study that showed that the drug associated with liposome bilayers within the liposomal compartments can be better routed into the skin (20). Other studies have shown that liposomal ambience may help modify the permeability characteristics of the stratum corneum, and the systems keep the drug molecules within the skin layers so that their skin-substantivity is high (21–27).

When the authors analyzed the effect of the liposomes' composition, they found that liposomes composed of PCS show higher retention than those composed of PCU; therefore, drug molecules in saturated phospholipid bilayers are better localized in skin. Retention and permeation of MCZ with the conventionally formulated cream was significantly low when compared with liposomal forms. The poor performance of the conventional cream may be ascribed to the dispersed state of the drug in an insoluble form, which impairs penetration of the outer skin barrier. The retention and permeation properties of commerical creams were shown to be lower than those of liposomal systems, and commercial cream A showed improved penetration when compared with the other two creams. The enhanced penetration of cream A may be attributed to its formulation design.

Conclusion

Entrapment of MCZ in liposomes was achieved after studying the effect of various process and formulation variables. The amount of drug loaded into these vesicles ranged from 7.2 mg per 125 mg to 9.76 mg per 130 mg of total lipid. These systems were found to have good size and stability characteristics and exhibited improved permeation properties. The in vitro permeation study indicated that MCZ in an exquisite amphiphillic

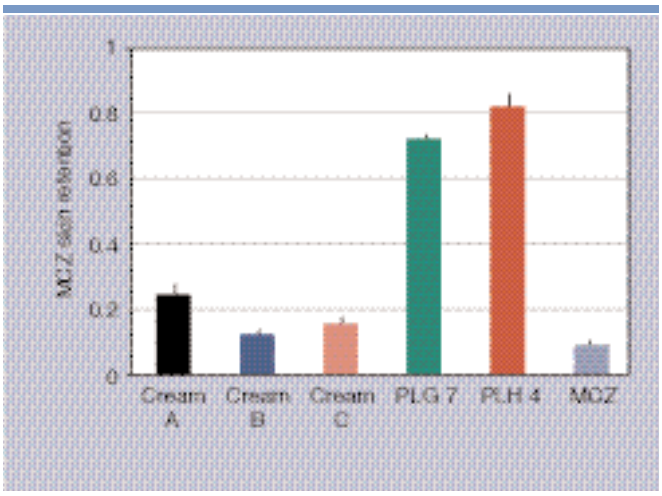

Figure 4: Skin retention of miconazole from PLH 4 and PLG 7 liposomal and cream formulations (cream A: commercial gel; cream B: commercial gel; cream C: prepared cream base).

atmosphere of a closed lamellar system forms depots in skin layers, revealed the merits of developed MCZ-loaded liposomes, and justified their potential for strengthening the efficacy and safety of a drug. The results of the skin-retention studies showed fairly high MCZ retention with liposomes when compared with commercial creams of MCZ. The higher permeation of drug across the skin and greater retention in the skin with liposomal systems clearly show that liposomes can penetrate and form depots in skin layers. The liposomal phospholipids (natural constituent of skin lipids) proved better for generating and retaining the required physicochemical state of skin for enhanced permeation and retention. This characteristic can be attributed to phospholipids' ability to vesiculate independently because they carry two bulky nonpolar lipid chains and a polar-head group, which helps them spontaneously form into closed bilayered systems and produce long-lasting effects.

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