Enhanced Bioavailability of Exemestane Via Proliposomes based Transdermal Delivery

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ABSTRACT: Exemestane, a novel steroidal aromatase inactivator used in the treatment of advanced breast cancer has limited bioavailability (42%) due to poor solubility, extensive first-pass metabolism, and also the absorption is dependent on formulation type and food. The present study is aimed to evaluate the feasibility of proliposomes for transdermal delivery of exemestane. The prepared proliposomes were characterized for size, zeta potential, and entrapment efficiency. The size of the vesicles was found to be between 440 and 700 nm with high entrapment efficiency for the formulation containing greater amounts of phosphatidylcholine. Differential scanning calorimetry and Fourier transform infrared studies were performed to understand the phase transition behavior and mechanism for skin permeation, respectively. The drug release across cellophane membrane follows zero-order kinetics by diffusion. *Ex vivo* permeation enhancement assessed from flux, permeability coefficient, and enhancement ratio were significantly higher for proliposome gels compared with control. A significant improvement in the bioavailability (2.4-fold) was observed from optimized proliposome gel compared with control (oral suspension). The stability data reveal that the formulations are more stable when stored at 4°C. In conclusion, proliposomal gels offer potential and prove to be efficient carriers for improved and sustained transdermal delivery of exemestane. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3208–3222, 2011

Keywords: proliposomes; cholesterol; liposomes; exemestane; stratum corneum; permeability; flux; transdermal; pharmacokinetics; bioavailability

INTRODUCTION

Transdermal drug delivery serves as a surrogate for oral route of administration to improve the bioavailability of drugs. Apart from avoidance of first-pass metabolism, the enormous surface area of the skin, easy administration, and termination of action make the transdermal route of delivery more attractive.[1] However, the major challenge in transdermal delivery is facilitating drug permeation across the skin impeding the barrier function of stratum corneum (SC), which is the rate-limiting step for absorption.[2]

Drug delivery systems using colloidal vesicular carriers such as liposomes, niosomes, ethosomes, transferosomes, elastic liposomes, and so on proved to have distinct improvement in transdermal delivery.[3,4] Among them, the liposomes have gained much interest and often been considered to be potential candidates for drug delivery. In spite of many advantages, the success rate of liposomes is limited because of significant problems in the general application of liposomes for drug delivery such as aggregation, sedimentation, phospholipid hydrolysis, and oxidation.[5,6] The proliposome concept introduced by Payne et al.[7] has resolved many of the stability issues pertaining to the liposome dispersions. Proliposomes, a semisolid liquid crystal (gel) product, composed of drug and lipid portion (lecithin and cholesterol) with minimum quantities of ethanol and water.[4] The formulation upon application onto the surface of skin get hydrated with water from skin under occlusion and renovate liposomes, which favor the drug delivery via the SC.[8,9]

Several mechanisms could be explained for the ability of liposomes to modulate the diffusion across skin. The fusion of vesicles on the surface of the skin can lead to the establishment of large concentration

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gradients of the intercalated drug across the skin and hence enhanced skin permeation.[10] Furthermore, the vesicle intercalation into the intercellular lipid layers of the skin might result in fluidization and disorganization of the regular skin structure and thus obviate the barrier function of SC.[3] However, the size and composition of vesicles,[11] nature of drug, and biophysical factors[10] are the important factors to be taken into consideration, which contribute for efficient vesicle–skin interaction.

Exemestane is a potent third generation steroidal aromatase inactivator used in the treatment of advanced breast cancer in postmenopausal women. The bioavailability of exemestane is limited to only 42% due to poor solubility and extensive first-pass metabolism and further the absorption is highly variable, which is dependent on formulation type and food.[12] The current oral therapy with exemestane pose problems such as unpredictable dissolution and absorption, poor patient compliance with common adverse effects such as body weight change, fatigue, dizziness, hot flushes, arthralgia, and myalgias.[13] Earlier, attempts were made to improve the oral bioavailability of exemestane from proliposome beads,[14] cyclodextrin inclusion complexes,[15] and self emulsifying drug delivery systems (SMEDDS).[16] As the physicochemical characteristics of exemestane (log p 3.5, low-molecular weight 296.41 g/mol) are similar to the ideal requirements of a candidate for transdermal drug delivery and further the transdermal application of exemestane has not been investigated so far, we have made an endeavor to formulate proliposome-based transdermal gels to improve the bioavailability and to maintain sustained drug levels with minimal fluctuations in plasma. Moreover, the site-specific application is also feasible for proliposomal gels so as to obtain high local concentration of exemestane near the vicinity of cancerous breast tissue and thereby risk of systemic side effects can be reduced.[17] Furthermore, if any untoward reactions occur, the termination of drug action is also equally possible by simple removal of the formulation. Therefore, the present systematic study encompasses the formulation *in vitro* and *ex vivo* characterization of exemestane proliposome gels. Furthermore, the pharmacokinetic study was carried out in albino Wistar rats to assess the feasibility of these systems for efficient transdermal delivery.

MATERIALS AND METHODS

Materials

Exemestane was a kind gift sample from Dr. Reddy's laboratories, Hyderabad, India. Soy phosphatidylcholine [(PC), Phospholipon 90G] was generously donated by Lipoid, Ludwigshafen, Germany. Cholesterol was obtained from E. Merck (Mumbai, Maharashtra, India). Stearylamine (SA) was procured from Sigma (St. Louis, Missouri). Dialysis membrane [DM-70; molecular weight cutoff (MWCO) 12,000 Da] was purchased from Himedia (Mumbai, Maharashtra, India). All other chemicals used were of analytical grade and solvents were of high-performance liquid chromatography (HPLC) grade. Freshly collected double distilled water was used all throughout the experiments.

Preparation of Proliposomes

The preparation of proliposomes was carried out by using the method reported elsewhere with slight modification.[18] Accurately weighed amounts of lipid mixture (1 mM) comprising of PC and cholesterol at various ratios (1:0, 2:1, 1.5:1, 1:1, and 1:1.5, respectively) were taken in a clean and dry, wide mouthed glass vial. The drug was added to the lipid mixture followed by the addition of 400 mg of absolute ethanol. After ensuring the homogenous dispersion of the ingredients, the vials were tightly sealed in order to prevent the evaporation of the solvent and warmed in a thermostatic water bath at 55°C–60°C for about 5 min with intermittent shaking until the ingredients were dissolved. To the resultant transparent solutions, about 160 μL double-distilled water maintained at the same temperature was added stream wise while warming in the water bath till a clear or translucent solution was obtained, which upon cooling formed a yellowish translucent liquid or yellowish translucent gel or a white creamy proliposomal gel. The obtained gels were stored in the same closed glass vials for overnight in dark for characterization. The positively charged vesicles were prepared by adding 100 μM of SA (10 mol % of total lipid) to the proliposome formulation comprising of PC and cholesterol in 2:1 ratio and processed as described above. The composition of different proliposomal formulations is represented in Table 1.

Formation and Morphological Evaluation

The formation and morphology of the liposomes were evaluated by optical microscopy. For the morphological evaluation, the proliposomal gels were hydrated with 7 mL of phosphate-buffered saline (pH 7.4), mixed gently, and final volume was adjusted to 10 mL with the same vehicle. The liposomes formed after hydration was observed at a magnification of 450× through an optical microscope (Coslabs micro, Ambala, Haryana, India).

For transmission electron microscopy (TEM) studies, a drop of the final liposome dispersion formed after hydration was placed onto a carbon-coated copper grid, forming a thin liquid film. The film on the grid was negatively stained by adding immediately a drop of sodium phosphotungstate solution (2%, w/v);

Table 1. Composition and Appearance of Exemestane Loaded Proliposomal Gels

Formulation Code	Molar Ratio (PC: Chol)	PC (mg)	Chol (mg)	SA (mg)	Ethanol (mg)	Water (μL)	Appearance
PL	1:0	750	–	–	400	160	Yellowish liquid
PL	2:1	495	127	–	400	160	Yellowish translucent gel
PL-2	1.5:1	450	154	–	400	160	White creamy gel
PL-3	1:1	375	193	–	400	160	White creamy gel
PL-4	1:1.5	300	231	–	400	160	White creamy gel
PL-SA	2:1	495	127	26	400	160	Yellowish translucent gel

PC, phosphatidylcholine; Chol, cholesterol; SA, stearylamine.
Total 1 mM lipid mixture was used in all the preparations.
All formulations contained 10 mg exemestane.

excess staining solution was removed with a filter paper and followed by a through-air drying. The resultant films were then viewed on a transmission electron microscope (JEOL-200 CX; Jeol, Tokyo, Japan) and photographed.

Vesicle Size, Surface Charge, and Entrapment Efficiency

The proliposomal gels were hydrated with phosphate-buffered saline (pH 7.4) and subjected to bath sonication (Sonica, Milano, Italy) for 3 min and the resultant dispersion was used for the determination of size, zeta potential (ZP), and entrapment efficiency measurements.

The mean size of liposomes was determined by photon correlation spectroscopy using Zetasizer Nano ZS90 (Malvern Instruments, Malvern, Worcestershire, UK). Each sample was suitably diluted and size analysis was performed at 25°C with an angle of detection of 90°C. Size and polydispersity index (PI) of liposomes were obtained from the instrument. Surface charge (zeta potential) of the liposomes was also measured by using Zetasizer Nano ZS90 (Malvern Instruments). For the determination of entrapment efficiency, ultrafiltration was carried out using Centrisart (Sartorius AG, Gottingen, Germany) at 3500 rpm for 15 min, which consist of filter membrane (MWCO 20 kDa) at the base of the sample recovery chamber.[19] The amount of exemestane in the aqueous phase was quantified by HPLC. Entrapment efficiency was calculated from the difference between the initial amount of exemestane added and that present in the unentrapped form, and was expressed as a percentage of the total amount of exemestane added.

Rheological Studies

The rheological behavior of proliposomal formulations was studied by using a controlled stress rheometer with the cone (24 mm) and plate geometry (Brookfield Programmable DVIII+ Digital Rheometer, Massachusetts). Before carrying out the measurement, the sample was allowed to equilibrate for 5 min and the torque sweep was in the range of 10%–110%. The measurements were performed in triplicate at ambient temperature and the rheological properties were calculated using Rheocalc 32 software (Brookfield Engineering Laboratories Inc., Massachusetts, USA). The rheological behavior of thixotropic fluid system is analyzed by Ostwade power equation:

$$\eta = KS^{-n} \tag{1}$$

The logarithmic transformation of the equation yields:

$$\ln \eta = \ln K - n \ln S \tag{2}$$

where η is the apparent viscosity, K is the constant, S is the shear rate, and n is the thixotropic degree obtained from the slope of the curve when ln η is plotted against ln S.[20]

Number of Vesicles per Cubic Millimeter

The abundant formation of vesicles is one of the important parameter to optimize the composition. Proliposomal gel was hydrated with phosphate-buffered saline (pH 7.4) and the number of liposomes formed per cubic mm was counted by optical microscope using a hemocytometer.[21] The liposomes in 80 small squares were counted and calculated by using the following formula:

$$\text{Total no. of liposomes per cubic mm} = \frac{\text{Total number of liposomes counted} \times \text{Dilution factor} \times 4000}{\text{Total number of squares counted}}$$

Differential Scanning Calorimetry

The thermotropic properties and phase transition behavior of pure drug, PC, cholesterol, and proliposome gel (PL-1) was studied by using differential scanning calorimeter (Mettler DSC 821e, Mettler–Toledo, Switzerland). Average sample weight of 5 ± 2mg were heated in hermitically sealed aluminum pan over a temperature range of 20°C–300°C under a constant nitrogen gas flow of 30 mL/min at a heating rate of 10°C/min.

FTIR Analysis of Rat Skin

The prepared rat abdominal skin was treated with PL-1 equivalent to 5 mg of drug for 12 h. The treated skin samples were washed with water and blotted dry. The FTIR spectrum of the rat abdominal skin

was recorded in the range of 4000 to 400 cm^{-1} using FTIR multiscope spectrophotometer (Shimadzu Co., Tokyo, Japan). The FTIR spectrum of the control rat skin was also recorded.[22]

In Vitro Release Study

To understand the release behavior and stability of liposomes and further to have an insight on the barrier properties of the SC, *in vitro* release studies through an artificial cellophane membrane were conducted using fabricated vertical Franz diffusion cells with an effective diffusional surface area of 4.153 cm^2 and 14 mL of receptor cell volume. Prior to the study, the dialysis membrane was soaked and the proliposome formulation or control (drug suspended in the same solvent of formulations) equivalent to 5 mg of exemestane was placed in the donor compartment. The ethanolic phosphate-buffered saline (pH 7.4; 30%, v/v) filled in the receptor compartment was maintained at 37 ± 2°C under constant stirring up to 24 h. To prevent the evaporation of the diffusion medium, the donor chamber and the sampling port were covered by Parafilm. The samples were withdrawn periodically and replaced with an equal volume to maintain constant receptor phase volume. At the end of the study, the samples were suitably diluted and the amount of drug was quantified by HPLC. The obtained data were fitted into mathematical equations (zero order, first order, and Highuchi models)[23] in order to describe the kinetics and mechanism of drug release from the proliposomal formulations.

Ex Vivo Permeation Study

Male albino Wistar rats (180–200 g) were maintained under controlled condition of temperature and the rats had free access to water and food until they were sacrificed for skin harvesting. The study was conducted with the prior approval of Institutional Animal Ethical Committee, St. Peter's Institute of Pharmaceutical Sciences. The rats were sacrificed with excess ether inhalation. The abdominal skin was exposed and hair was removed with hair clipper taking extreme precautions not to damage the skin. The rat abdominal skin was isolated, excised, and the adhering subcutaneous fat, tissue, and capillaries were removed with a pair of scissors. The heat separation technique[24] was adopted to prepare the epidermis, which involves soaking of the entire abdominal skin in water at 60°C for 45 s. The epidermis was washed with water, wrapped in aluminium foil, and stored at −20°C till further use (used within 2 weeks of preparation).[25]

The permeation of exemestane from proliposome formulations was carried out using fabricated vertical Franz diffusion cells with an effective diffusion area of 4.153 cm^2. The skin was brought to the room temperature and sandwiched between two halves of the diffusion cell, with the SC side faced toward the donor compartment. The skin was allowed to equilibrate and the donor compartment was challenged with proliposome formulation and control (drug suspended in the same solvent of formulations) equivalent to 5 mg of exemestane. A 14 mL aliquot of 30% (v/v) ethanolic phosphate-buffered saline [(pH 7.4); containing 0.003% (w/v) of sodium azide to retard microbial growth] was used as receptor medium in order to maintain sink condition. The donor chamber and the sampling port were covered by Parafilm and the receptor compartment was maintained at 37 ± 1°C under constant stirring up to 24 h. At predetermined time intervals, an aliquot of 200 μL was withdrawn and replenished with an equal volume of fresh diffusion buffer. The samples were suitably diluted and assayed for exemestane by HPLC. The experiment was conducted in triplicate for all the formulations.

Determination of Drug Deposited into the Skin

The extent of drug deposited in the epidermal layers (DCS) was determined.[26] After 24 h of permeation study, the skin was removed from the diffusion cell and washed briefly in methanol for 15 s to remove the adhering formulation. The skin was allowed to dry at room temperature for 10 min, chopped into pieces, homogenized in diffusion medium (10 mL), and sonicated for 30 min using bath sonicator (Sonica, Milano, Italy) to leach out the drug. The samples were centrifuged and the supernatant layer was passed through 0.45 μm membrane filter and assayed for exemestane by HPLC.

Permeation Data Analysis

The cumulative amount of drug permeated (Q) was plotted against time. The steady-state flux (J_{ss}) was obtained from the slope of linear portion of the cumulative amount permeated per unit area versus time plot. The permeability coefficient (K_p) was calculated by dividing J_{ss} with initial concentration of exemestane in donor compartment. The enhancement ratio (ER) was calculated by using the following equation: ER = Transdermal flux from proliposomal gel/transdermal flux from control.

Pharmacokinetic Study

The study was conducted with the prior approval of Institutional Animal Ethical Committee, St. Peter's Institute of Pharmaceutical Sciences. Male albino Wistar rats (180–200 g) were selected for the study and had free access to food and water. Before dosing, the animals were kept for overnight fasting. The rats were divided into two groups containing six in each. The rats used for the application of proliposomal gel were prepared by trimming the hair on the abdominal area maximally with a pair of scissors, followed by careful shaving with electric shaver and cleaned

with water. Control group received an oral suspension of exemestane [2.0 mg/mL in 0.5% (w/v) of sodium carboxymethyl cellulose] and the test group received the optimized PL-1 at a dose of 5 mg/kg body weight. The proliposomal gel was applied uniformly onto the shaved area covering a surface area of 3.14 cm^2 and was covered with an impermeable backing membrane and further fixed with the help of an adhesive membrane. The treated animals were placed in separate cages and maintained under laboratory conditions throughout the study. At predetermined time intervals, blood samples (500 μL) were collected from retro-orbital plexus into heparinized microcentrifuge tubes. The plasma was separated by centrifugation at 6,708 g for 10 min in a microcentrifuge (REMI Equipments, Mumbai, Maharashtra, India) and stored at −20°C until analysis. Before analysis, the plasma samples were allowed to thaw, deproteinized with acetonitrile, centrifuged, and the drug content in the supernatant was quantified for exemestane by HPLC.

HPLC Analysis of Exemestane

The samples were assayed for exemestane by using an HPLC equipped with LC-10 AT solvent delivery unit, SPD-10 AVP UV-Spectrophotometric detector, Spinchrom software, Rheodyne injector fitted with 20 μL capacity (Shimadzu Co. Kyoto, Japan), and a Lichrospher C18 column (5 μm, 4.6 × 250 mm). Isocratic elution was carried out at a flow rate 1.0 mL/min. The mobile phase consisted of 80:20% (v/v) methanol and water, respectively, and the detection wavelength was set at 250 nm. The injection volume was 20 μL and the sensitivity was set at 0.005 absorbance units full scale (AUFS).

Pharmacokinetic Parameters

The peak concentration (C_{max}) and its time (T_{max}) were obtained directly from the plasma concentration versus time profile. The area under the curve (AUC_{0-t}) was calculated by using trapezoidal rule method. The $AUC_{t-\alpha}$ was determined by dividing the plasma concentration at last time point with elimination rate constant (K). The relative bioavailability (F) was estimated by dividing the $AUC_{0-\alpha}$ of proliposome gel with control oral suspension. Furthermore, the *ex vivo* and *in vivo* correlation was performed by comparing the cumulative amount permeated (μg) versus AUC obtained *in vivo* from proliposome gel.

Statistical Analysis

Statistical analysis of the data obtained was performed using one-way analysis of variance, followed by Neuman–Keuls post test, and the Student's t test with GraphPad Prism software (version 4.00; GraphPad Software, San Diego, California). The level of statistical significance was chosen as less than $p < 0.05$.

Skin Irritation Test

The skin irritancy potential of the proliposome formulations was evaluated in albino rats. The hair was removed on the back of the animal and the formulations were applied, and the animals were examined for any signs of skin irritation and erythema for a period of 1 week.

Stability Studies

The formulations stored in glass vials covered with aluminium foil were kept at room temperature and in refrigerator (4°C) for a period of 30 days. At definite time intervals (10, 20, and 30 days), samples were withdrawn and hydrated with phosphate-buffered saline (pH 7.4) and observed for any sign of drug crystallization under optical microscope. Furthermore, the samples were also evaluated for particle size and percent retention of exemestane.

RESULTS AND DISCUSSION

Preparation and Characterization of Proliposomes

The formation of liposomes was spontaneous with the addition of water as evident from Figures 1a–1d. Initially, upon contact with water, swelling of phospholipids resulted in tubular structures and with gentle agitation has been deformed into multilamellar vesicles acquiring spherical shape (Fig. 2a). Furthermore, the TEM image confirms the shape and the type of the liposomes formed from proliposomes (Fig. 2b). The physical appearance of the formulations has been converted from translucent gel state to creamy white gel upon increasing the concentration of cholesterol in the formulation (Table 1).

Exemestane could be loaded into proliposomes by a simple method, which involves the principle of coacervation-phase separation.[4] The formulation prepared with PC alone (PL-0) formed a flowable translucent yellowish liquid and the consistency was less, which is a prime factor for the application of the product onto the skin. Furthermore, the entrapment was also less with poor stability. Keeping the stability and industrial utility in mind, several techniques were used earlier in order to improve the stability of the vesicular formulations, which is a primary concern in the production scale-up. Among them, cholesterol, a common additive, can be used as a structural lipid to improve the stability and entrapment efficiency of vesicular formulations[27] and it is well known fact that the addition of cholesterol forms a compact bilayer, which acts as a barrier and prevents the leakage of drug from the bilayer and stabilizes the formulation. Lecithin and cholesterol present in proliposomal gel alter the structure of intercellular lipid barrier in the SC, gets fluidized, and becomes more permeable favoring the permeation of drugs.[28,29] The concentration

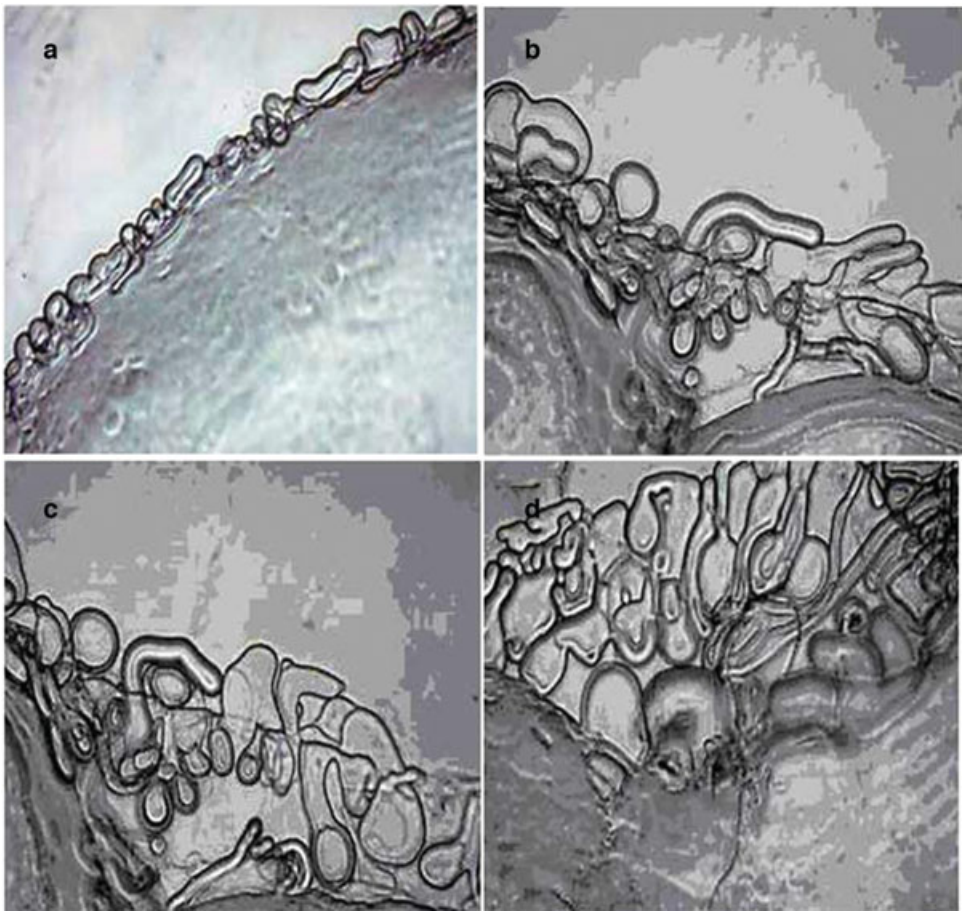

Figure 1. Microphotograph showing the formation of liposomes from proliposomes (PL-1) upon addition of phosphate-buffered saline (pH 7.4).

of cholesterol and lecithin are important as they tend to affect the morphology of the vesicles,[11] and an alteration in their composition leads to disruption of vesicles, which leads to leakage of free drug before fusion of the vesicles with the skin.[30] The effect of cholesterol was investigated by varying the composition of PC to cholesterol ratio keeping the total lipid constant at 1 mM.

Vesicle size and size distribution are important parameters for the topical administration of vesicular systems.[11] The size of all the formulations was within the range of 440–700 nm (Table 2). Small value of PI (<0.1) indicates a homogenous population, whereas a PI (>0.3) indicates a higher heterogeneity. The PI used as a measure of a unimodal size distribution was within the acceptable limits for all the proliposome

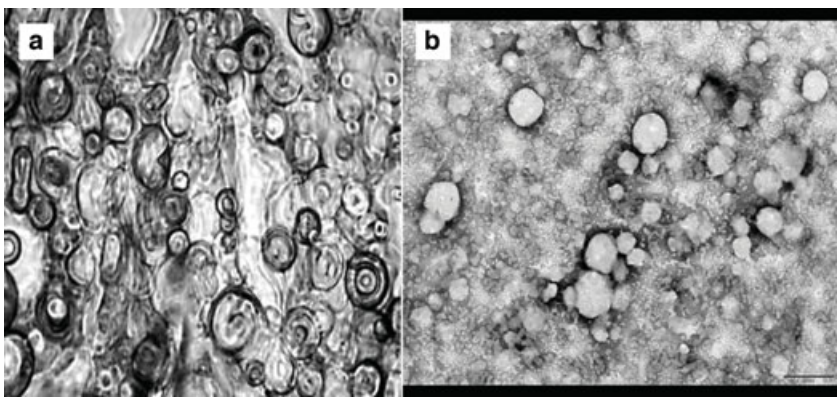

Figure 2. Optical (a) and transmission electron microphotographs (b) of liposomes formed from proliposome formulation (PL-1).

Table 2. Physicochemical Characterization of Exemestane Proliposomes

Formulation code	Size (nm)	Zeta Potential (mV)	PI	Entrapment efficiency (%)	n	No. of vesicles per mm^3 $\times$ 10^4
PL-0	412 ± 18	−2.75 ± 1.2	0.243	83.4 ± 2.3	0.764	3.58
PL-1	453 ± 23	−11.12 ± 1.4*	0.229	97.2 ± 1.9**	0.922	3.92
PL-2	487 ± 39*	−7.95 ± 2.7*	0.284	96.1 ± 2.2**	0.748	3.75
PL-3	567 ± 26**	−8.12 ± 2.3*	0.242	95.1 ± 3.1**	0.635	3.66
PL-4	634 ± 41**	−7.11 ± 3.5*	0.291	94.2 ± 2.6**	0.589	3.59
PL-SA	665 ± 22**	15.5 ± 2.5**	0.238	96.4 ± 3.8**	0.895	3.82

*$p < 0.05$ against PL-0 formulation.
**$p < 0.01$ against PL-0 formulation.
PI, polydispersibility index; n, thixotropic degree.

formulations (Table 2). We could establish a linear relationship between the size of the vesicles and concentration of cholesterol. As the concentration of cholesterol increased, the size was also increased. The zeta potential of the proliposome formulation devoid of cholesterol (PL-0) was −2.75 ± 1.2 mV and the incorporation of cholesterol (PL-1) led to a significant increase in ZP to −11.12 ± 1.4 mV ($p < 0.05$). However, we could not notice a significant change in ZP with further increase in cholesterol concentration. In case of PL–SA formulation, the size of the liposomes (665 ± 22 nm) increased significantly and also the surface charge has been inversed to positive charge (+15.5 ± 2.5 mV) with the inclusion of SA (at a concentration of 10 mol % of total lipid) in PL-1 formulation ($p < 0.01$) (Table 2). The entrapment efficiency of the proliposome formulation without cholesterol (PL-0) was found to be 83.4 ± 2.3%, whereas the same has been increased significantly (94%–97%) in proliposome formulations (PL-1 to PL-4) containing varying ratios of PC to cholesterol ($p < 0.01$). This could be due to the increased bilayer hydrophobicity and reduced permeability of the bilayer, leading to the effective intercalation of hydrophobic drug within the hydrophobic core of the bilayer with an enhanced drug pay load.[31] Interestingly, the entrapment values declined with higher cholesterol content in the formulation (PL-4). The higher amounts of cholesterol may compete with the drug for the packing space available in the bilayer during the liposome formation and also perturb the linear regular structure of bilayer, resulting in limited accommodation of drug molecules.[32] Our observations are also in coincidence with the reports. The hydrophilic amphiphiles (dicetyl phosphate and SA) result in vesicle enlargement due to the increase in vesicle surface energy.[33] Accordingly, the increase in size with optimum entrapment efficiency for PL–SA formulation can be attributed to the swelling of the lipid bilayer due to the magnitude of surface charge.[34]

The rheological behavior is an important parameter to be evaluated for the formulations meant for percutaneous application, and the viscosity of proliposome formulations was decreased with an increase in shear rate. We could notice an increase in viscosity of proliposome formulations containing higher amounts of cholesterol. It is apparent from the rheological data that the thixotropic degree (n value) was also seems to be dependent on cholesterol content; lower the cholesterol, higher the thixotropic degree (Table 2). The number of vesicles formed from the hydration of proliposome formulation is an important parameter to be considered for the optimization of the variables and we could observe an abundant formation of vesicles in case of all formulations (Table 2). However, with PL-1 formulation, we could notice more number of vesicles per cubic mm (3.92 × 10^4) compared with other formulations.

DSC and FTIR Analysis

The exemestane and cholesterol has shown sharp endothermic peaks at 195.70°C and 148.42°C, respectively (Fig. 3a). The disappearance of the transition peak corresponding to the melting point of exemestane in proliposome formulation is obviously due to the transformation of crystalline form of the drug to molecular state in PC and cholesterol mixture. Furthermore, the absence of characteristic peak of cholesterol in proliposome indicates a change in the phase transition behavior of pure lipids and could be due to the formation of liquid crystalline state.

The peaks in the IR spectrum represent the molecular vibrations of lipids and proteins present in the skin. The bands at 2922 and 2852 cm^{-1} present in the functional group region of FTIR spectra can be attributed to the asymmetric -CH_2 and symmetric -CH_2 vibrations of long chain hydrocarbons of lipids[35] (Fig. 3b). Both the peak height and area are proportional to the amount of lipids present and any extraction of lipids from SC results in a decrease of peak height and area.[36] We could observe a reduction in the area of these peaks, suggesting the fluidization of the SC due to the fusion of the vesicle bilayer with skin membrane and/or due to the interference of PC and cholesterol with the intercellular lipids of the SC, thus altering the lipid composition.

In Vitro Release Study

To know the *in vitro* release behavior and the effect of composition on the stability of liposomes, artificial cellophane membrane was used. The percentage drug

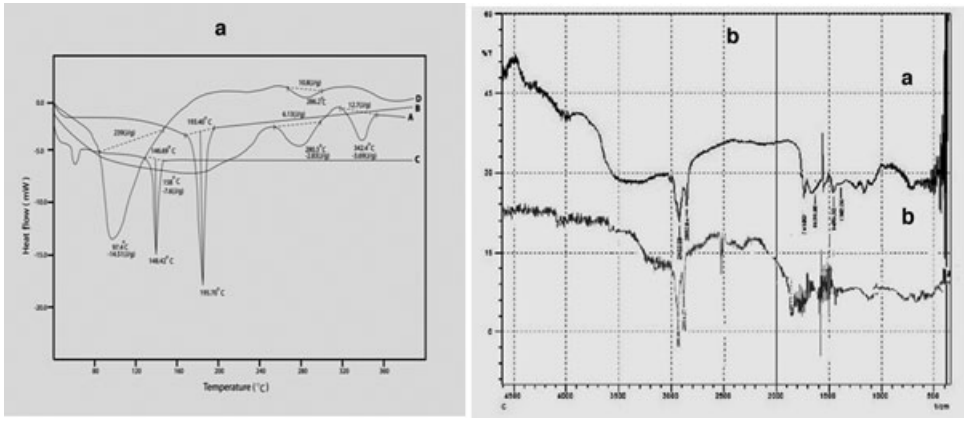

Figure 3. DSC thermograms (I) of (a) phosphatidylcholine, (b) exemestane, (c) cholesterol, and (d) proliposome formulation (PL-1); and (II) FTIR spectra of (a) control skin and (b) skin treated with proliposome formulation.

release from different formulations was represented in Figure 4a. The release of exemestane from control was nearly 65% within 12 h, which clearly suggests the permeability of the membrane and prevalence of the sink condition for the drug. A typical biphasic release pattern was observed with proliposome formulations with an initial rapid burst release (4 h), followed by sustained release for a period of 24 h (Fig. 4a). The high concentration gradient prevailing at the initial time points might have contributed for the rapid drug release and also may be due to the faster release of unentrapped drug from the proliposome formulations. The amount of drug release from the proliposome formulation containing lower amounts of cholesterol (PL-1 and PL-2) was less, which could be due to the more ordered and compact structure of the bilayer, thus retarding the drug release.[37] However, further increase in cholesterol concentration led to an increase in drug release, which could be due to the disordered or perturbation of the bilayer. The entrapment efficiency results also support the *in vitro* release data. The *in vitro* release data subjected to mathematical modeling reveal that the drug release from proliposome formulations is diffusion controlled, following zero-order kinetics (higher R^2 values) (Table 3).

Table 3. *In Vitro* Release Kinetics of Exemestane from Proliposomes Across Cellophane Membrane

	Zero Order		First Order		Higuchi
Formulation Code	K_0 (h^{-1})	R^2	K (h^{-1})	R^2	R^2
Control	0.286	0.982	0.151	0.821	0.858
PL-0	3.485	0.963	1.391	0.626	0.985
PL-1	3.253	0.823	1.241	0.525	0.977
PL-2	8.468	0.716	1.807	0.432	0.926
PL-3	8.608	0.685	1.499	0.351	0.897
PL-4	7.181	0.738	1.593	0.442	0.936
PL-SA	2.677	0.964	1.029	0.574	0.989

K_0, zero-order rate constant; K, first-order rate constant.

Ex Vivo Permeation Study

The *ex vivo* permeation study was conducted across the rat abdominal skin and the cumulative amount of drug permeated is represented in Figure 4b. After the hydration of proliposomal gel with the skin fluids, permeation of exemestane results due to the release of the drug from the liposomes formed.[38] As we could not notice lag time with all the formulations, it clearly suggests the formation of liposomes. Moreover, the drug was detected within 0.25 h, which explains the procedures of diffusion of water from the receptor fluid to the skin membrane, release of drug, and permeation across the skin occurred rapidly.[39] The cumulative amount of drug permeated was 26.42 μg within 24 h from control. A significant improvement in permeation was observed with all the proliposome formulations compared with control (Fig. 4b). From Figure 5, it is evident that the inclusion of cholesterol has improved the permeation of exemestane compared with proliposome gel without cholesterol (PL-0). However, the increase in concentration of cholesterol leads to a decrease in transdermal delivery.[40] In accordance, the permeation was very much less for the formulation containing high amounts of cholesterol (PL-3 and PL-4), which is obviously due to the increased hydrophobicity and an enhanced solubility of exemestane in the hydrophobic core of the bilayer, resulting in reduced partitioning promotion of the hydrophobic drug into the SC lipid barrier. The maximum flux obtained was 19.72 and 21.57 μg/cm^2/h from control and proliposome formulation without cholesterol (PL-0), respectively (Table 4). The maximum flux was significantly higher ($p < 0.001$) for the PL-1 formulation and decreased with an increase in cholesterol. However, the flux was higher at all time points for the proliposome formulations compared with control and PL-0,

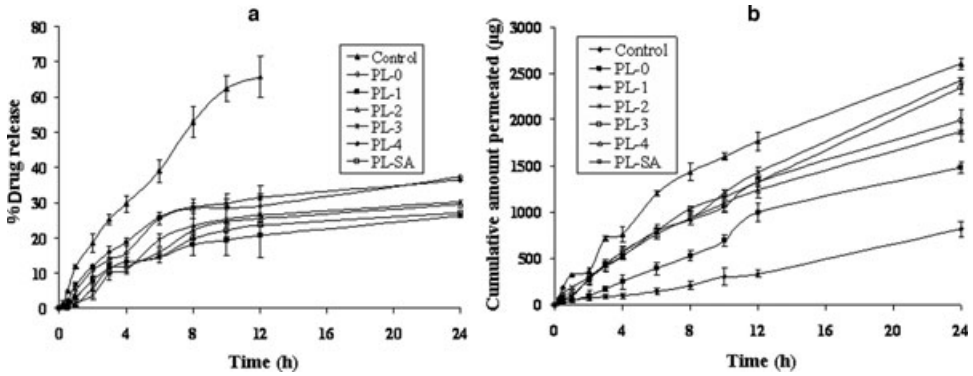

Figure 4. *In vitro* release and *ex vivo* permeation profiles of exemestane from various proliposomal formulations across (I) cellophane membrane and (II) excised rat skin (mean ±SD; $n = 3$).

which indicates the sustained delivery of exemestane from proliposome formulations. The permeation enhancement assessed in terms of permeation parameters (flux, permeability coefficient, and enhancement ratio) were calculated and shown in Table 4. The J_{ss} and K_p were very much higher for the proliposomal gel formulation containing PC to cholesterol in 2:1 ratio (PL-1). The ER well above 1 indicates improved permeation and in our findings, we could notice an ER greater than 1 for all proliposome gel formulations compared with control (Table 4). On the basis of permeation parameters, the proliposome formulations can be ranked in the following decreasing order PL-1 > PL-2 > PL-3 > PL-SA > PL-4 > PL-0 > Control.

The flux ($20.97 \pm 1.67\,\mu g/cm^2$ /h) obtained from PL–SA was less compared with PL-1 formulation ($p < 0.001$) (Table 4). As skin membrane carries negative charge, it is expected that the positive charge vesicles can have an improved interaction and permeation across the SC due to electrostatic attraction.[29] As speculated, we could not observe a dramatic increase in the permeation of exemestane from positive charge vesicles (PL-SA), which implies that for mere absorption of drugs across the skin, the skin–vesicle interaction is not only important, rather the exchange of material due to fusion of liposomes with skin is responsible for the favorable permeation of exemestane.[41] Interestingly, the amount of drug released across the cellophane membrane was significantly lower than the permeation across skin except PL-0 and PL-4 formulations ($p < 0.05$). This can be explained based on the fact that the free drug released from liposomes has to diffuse across the cellophane membrane and further we cannot expect the direct transfer of vesicles as the pore size of the membrane is 2 nm. Furthermore, the retardation of drug release from proliposome formulations can be attributed to the favorable partitioning of exemestane in hydrophobic regions of liposome bilayers. In contrary, the marked improvement in the permeation of exemestane via skin clearly suggests that the vesicle–skin interaction and transfer of intact vesicles through skin are important factors for improved transdermal delivery of exemestane.[3] The results are in consistent with the reports.[33] Overall, it is evident from the results that the permeation of exemestane has been greatly improved from proliposomal gel formulations compared with control. One of the reason is that proliposomes can act as a drug carrier system and intact liposome can enter the SC with the entrapped drug under the influence of *in vivo* transcutaneous

Table 4. Permeation Parameters of Exemestane from Different Proliposome Formulations Across Rat Skin (Mean ±SD; $n = 3$)

Formulation Code	Q_{24} (µg)	J_{ss} (µg/cm²/h)	J_{max} (µg/cm²/h)	K_p (cm/h) × 10^{-3}	ER
Control	818 ±71	7.99 ±0.96	19.72 ±1.56	1.60 ±0.86	1.00
PL-0	1479 ±66^{***a}	15.84 ±1.23^{***a}	21.57 ±2.23	4.08 ±0.75	2.02
PL-1	2607 ±56^{***abdefg}	26.97 ±1.35^{***abfg}	89.75 ±6.79	5.28±0.03	3.44
PL-2	2423 ±35^{***abfg}	24.46 ±1.30^{***ab**f*g}	60.62 ±5.44	3.00 ±1.29	3.12
PL-3	2340 ±49^{***abfg}	24.03 ±1.84^{***ab**f*g}	34.70±4.32	4.68 ±0.85	3.06
PL-4	1865 ±41^{***ab12}	19.68±1.12^{***a**b1b2}	35.19 ±3.89	3.79 ±1.14	2.51
PL-SA	1997 ±23^{***ab**f}	20.97 ±1.67^{***ab}	34.24 ±2.45	4.08 ±1.06	2.67

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
^{a}control; ^{b}PL-0; ^{c}PL-1; ^{d}PL-2; ^{e}PL-3; ^{f}PL-4; ^{g}PL-SA.
Q_{24}, amount permeated in 24 h; J_{ss}, steady-state flux; J_{max}, maximun flux; K_p, permeability coefficient; ER, enhancement ratio.

hydration gradient,[42] and hence higher amount of the drug deposited in the skin bilayer. The other reason is that vesicles composed of phospholipids can act as a permeation enhancer with subsequent modification of the intercellular lipids of SC and there upon modify the structural and permeability characteristics, raising its fluidity and thus impede the barrier function.[29,43,44] To summarize, several mechanisms such as penetration enhancement property, intact vesicle permeation into the skin, and increased vesicle–skin interaction in combination might have played a significant role in enhancing the permeation of exemestane from proliposomes. Such an enhancement seems to be dependent and dictated by the composition, vesicle characteristics, and physicochemical properties of the drug.[45]

Estimation of Drug Deposited in Skin

The extent of drug deposited in the skin (DCS) layers upon treatment of rat skin with all the proliposome formulations and control was shown in Figure 5. As the permeation was increased with all the proliposomal formulations, obviously the drug in the skin layers was also increased. This can be well explained based on the fact that the saturation of skin layers at the termination point of the experiment, that is, 24 h could have led to the higher DCS values. Moreover, the entry of intact liposomes into the skin bilayer might also have contributed for the higher deposition of drug in the skin layers.[45] Interestingly, we could notice a significant enhancement in the drug deposition in the skin layers with positively charge vesicles (PL–SA) compared with control ($p < 0.01$) and other formulations which might be due to an increased vesicle–skin interaction because of the electrostatic attraction between the positive and negative charge of vesicles and skin surface, respectively.[34,41] The higher deposition of exemestane in skin layers with proliposome gels compared with control reveals the potential of these formulations in avoiding the barrier function of the SC and delivering the drug efficiently into the viable regions of the skin for improved bioavailability.

Pharmacokinetic Study

The objective of the formulation of proliposomes for exemestane is to check the feasibility of transdermal delivery so as to improve the bioavailability. As exemestane absorption is highly variable and depends on the nature of food, a transdermal route provides a means for the sustained delivery of exemestane with minimal fluctuations in the systemic circulation. The plasma concentration of exemestane following peroral administration

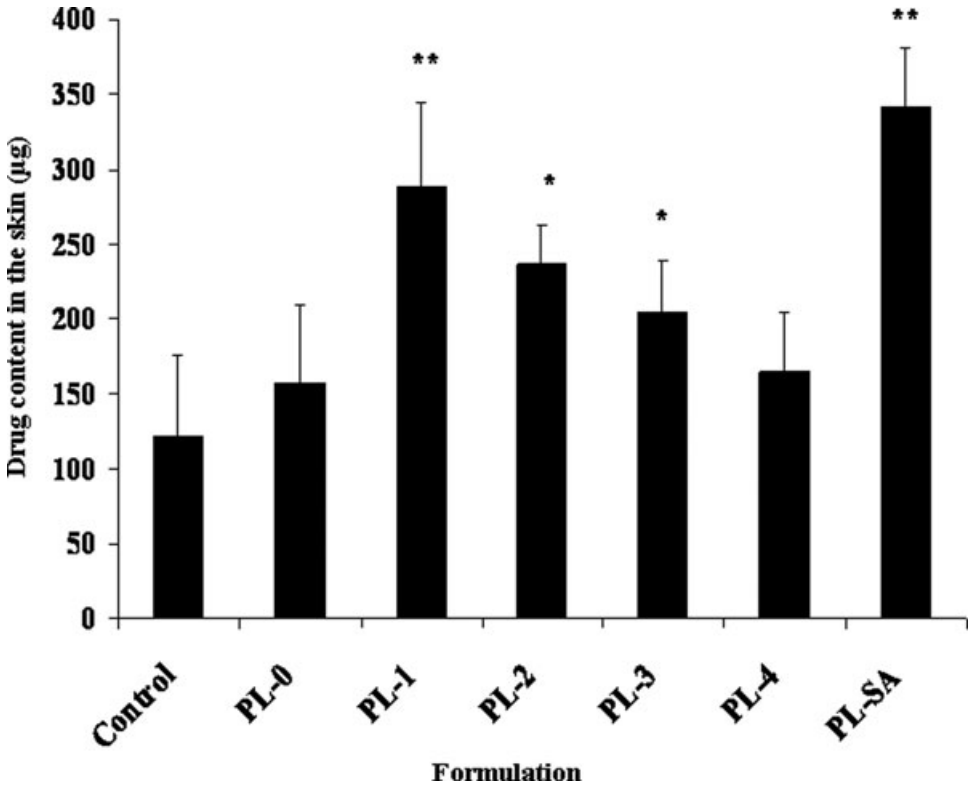

Figure 5. Drug deposited in the skin layers following treatment with different proliposome formulations after 24 h (mean ±SD; $n = 3$); * and ** represent significant difference versus control at $p < 0.05$ and $p < 0.01$, respectively.

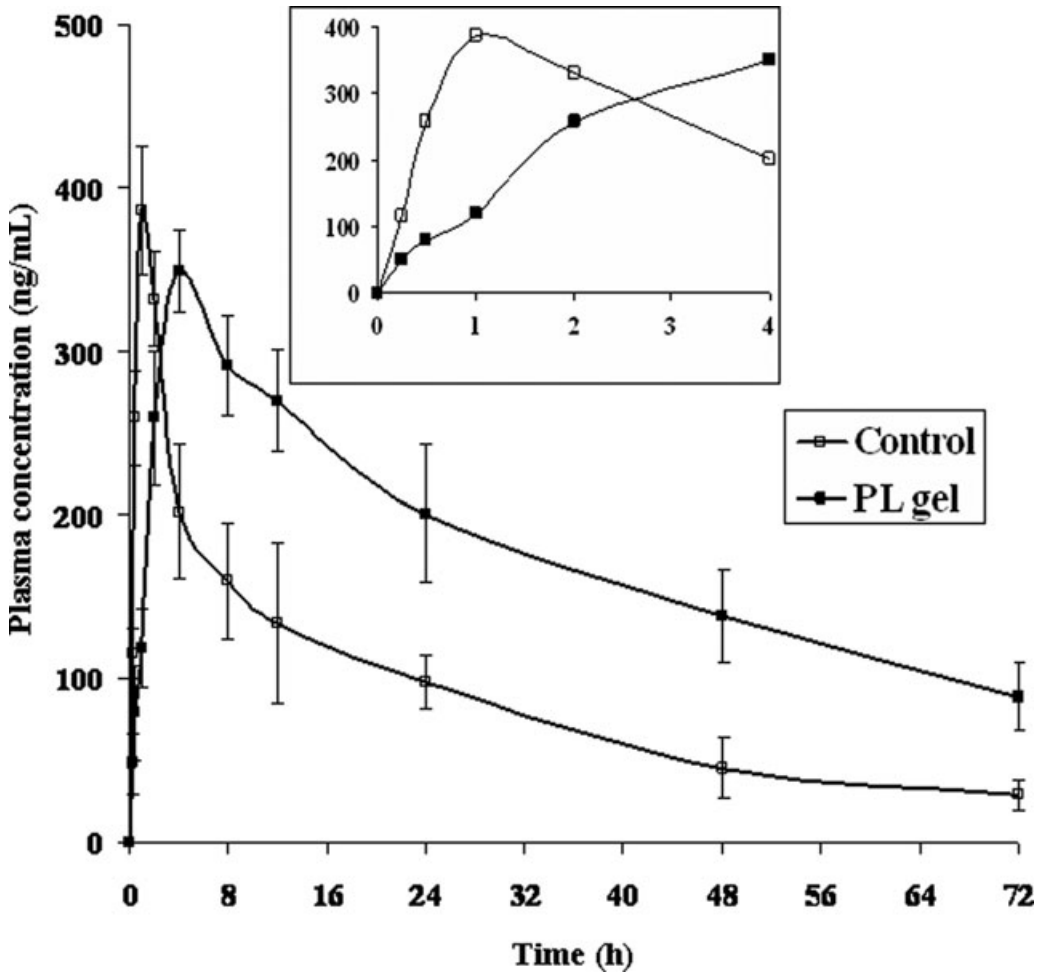

Figure 6. Mean plasma concentration versus time profiles of exemestane following treatment with proliposomal gel (PL-1) and control (mean ±SD; $n = 6$).

and transdermal application of proliposomes is shown in Figure 6. It is evident from the figure that the time to reach maximum concentration (T_{max}) and the C_{max} were higher for the control compared with proliposome. This is obviously due to the ready availability of the drug from suspension with an enhanced effective surface area and good membrane contact for absorption across gastrointestinal (GI) membrane. The slow elimination of exemestane from systemic circulation indicates the sustained delivery of exemestane from proliposomes. The extent of absorption described by AUC was significantly higher for PL gel compared with control ($p < 0.001$) (Table 5). The enhancement in the bioavailability assessed in terms of AUC and F was increased by 2.4-fold with proliposome formulation ($p < 0.001$) (Table 5). The significant enhancement in bioavailability with proliposome compared with control could be due to the avoidance of presystemic metabolism, which is responsible for poor bioavailability of the drug (42%).

The *ex vivo* and *in vivo* correlation was performed between the cumulative amount permeated (μg) and AUC (μg/h/mL^{-1}). The higher regression coefficient with an R^2 value of 0.9926 indicates point–to-point correlation following level A correlation.

Table 5. Pharmacokinetic Parameters of Exemestane in Rats Following Administration of Proliposome Gel (PL gel) and Control (Oral Suspension)

Formulation	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (μg h mL^{-1})	$AUC_{0-\alpha}$ (μg h mL^{-1})	K (h^{-1})	F
Control	349 ± 25	1.33 ± 0.57	6423 ± 1594	7450 ± 1975	0.0287 ± 0.002	2.38 ± 0.117*
PL gel	386 ± 39	4.00 ± 0.00	12888 ± 2246*	17599 ± 3876*	0.0194 ± 0.002*	–

Each value represents mean ±SD, $n = 6$
K, elimination rate constant; F, relative bioavailability.
* indicates significant difference at $p < 0.001$ versus control.

Skin Irritation Studies

The skin irritation studies could not find any marked erythema during 7 days, indicating that the exemestane proliposomal gel is nonirritant.

Stability Studies

The physical appearance, vesicle size, and leakage of drug were monitored for the proliposome formulations for a period of 30 days. The microscopical studies indicate that the vesicles formed from proliposomes were multilamellar and we could not observe any appreciable change in the morphological behavior. The liposomes formed after hydration was evaluated for size and percent retention of exemestane in the vesicles. We could not notice any appreciable change in size and percent retention of the drug for PL-1 formulation when stored at refrigerated conditions (Figs. 7a and 8a). However, a significant increase in size and drug leakage has been observed after a period of 30 days ($p < 0.05$). In contrast, the size was increased and the percent retention of drug was reduced upon storing the preparations at room temperature (Figs. 7b and 8b). The data clearly indicate the influence

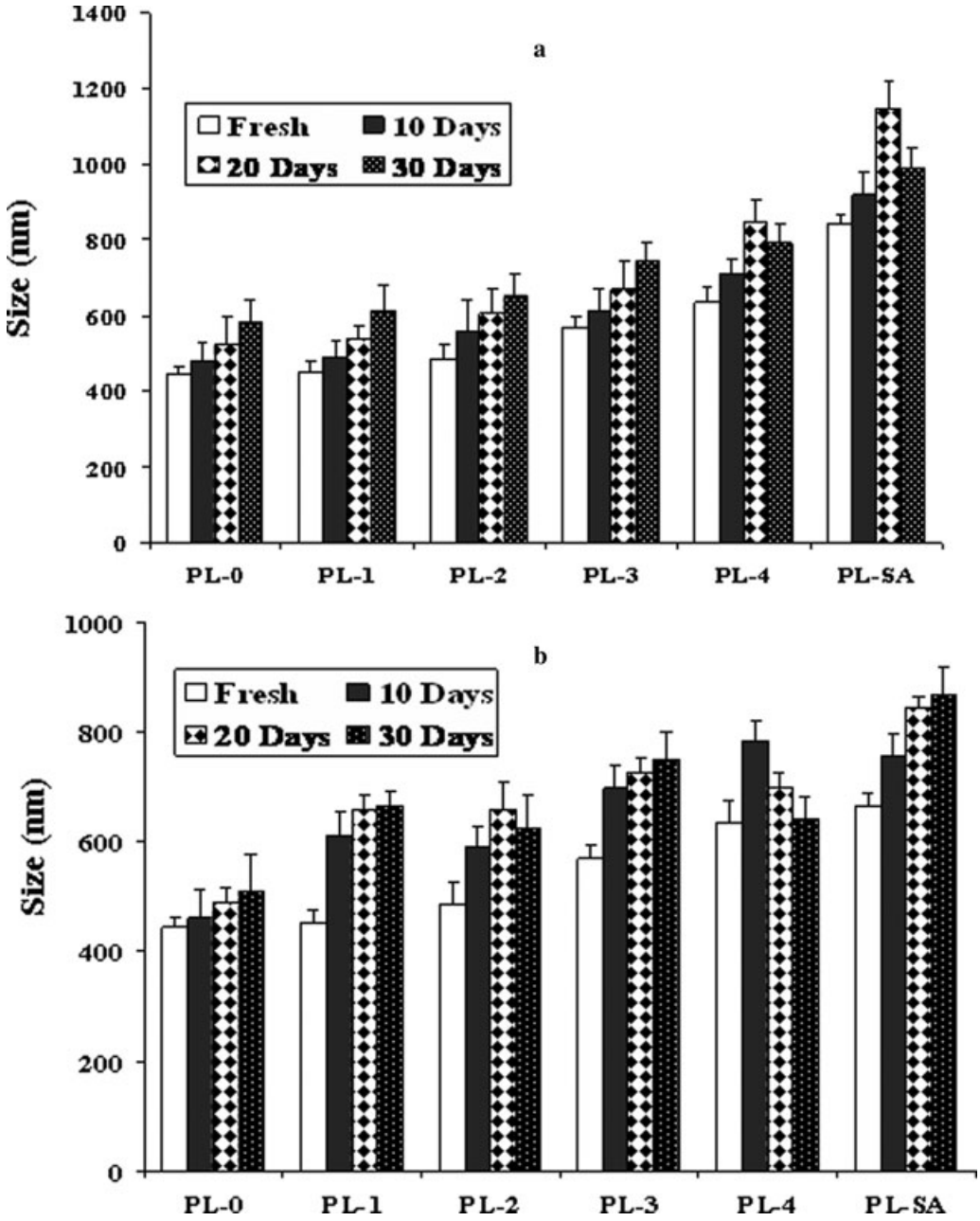

Figure 7. Change in size of proliposome formulations upon storage in (a) refrigerator and (b) room temperature.

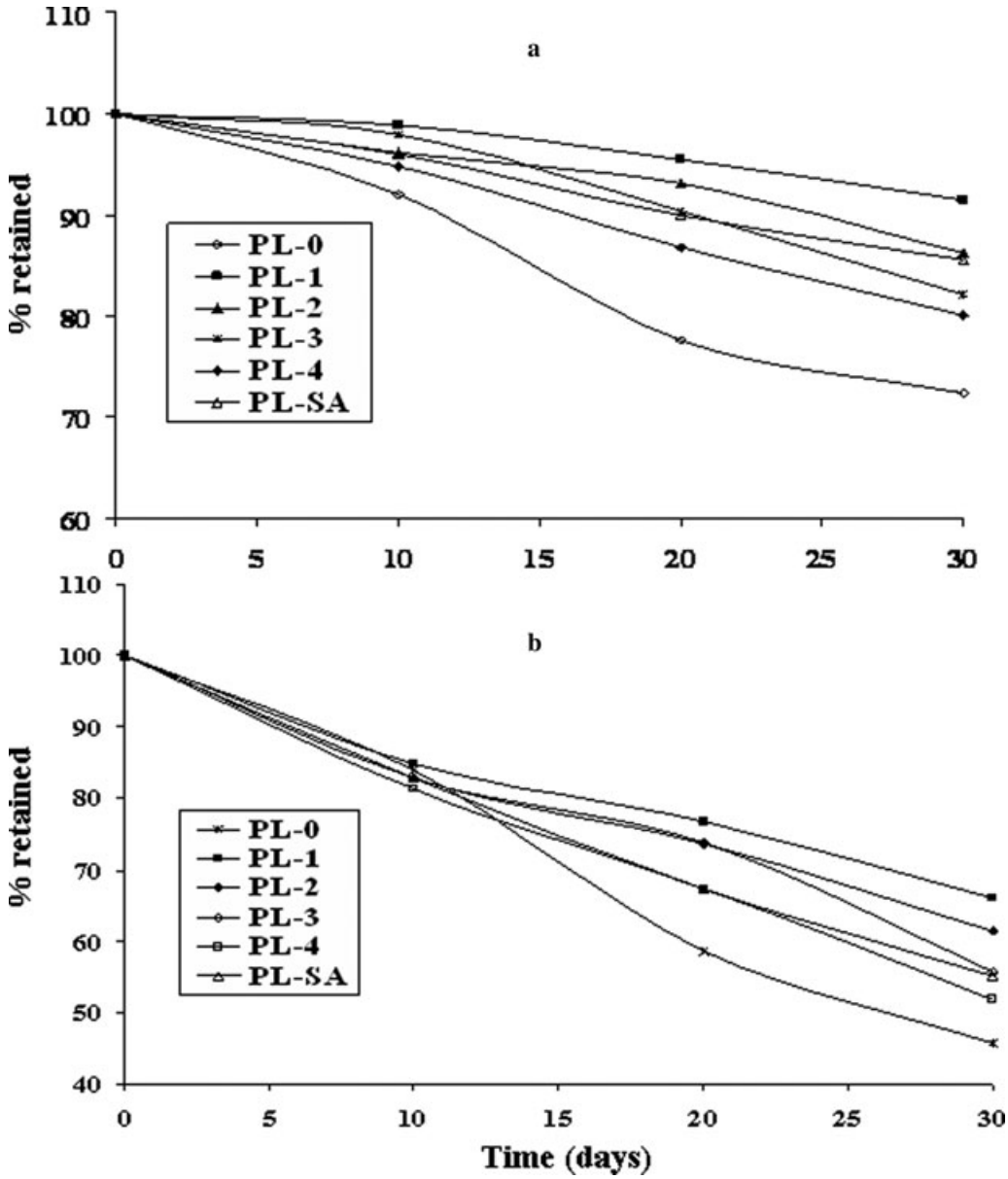

Figure 8. Percentage retention of exemestane in proliposome formulations upon storage in (a) refrigerator and (b) room temperature.

of temperature on the stability of proliposomes. In comparison, the formulation devoid of cholesterol (PL-0) was more stable with respect to vesicle size when stored in refrigerator and room temperature ($p > 0.05$). On the contrary, the percent retention was lower, which suggest the importance of the composition of vesicles. Overall, the formulations stored in refrigerator at 4°C were comparatively more stable than the formulations stored at room temperature.

CONCLUSION

The proliposome drug carriers could be successfully developed for the transdermal delivery of exemestane. The physicochemical evaluation of proliposomes clearly reveal the importance of vesicle composition and the formulation containing PC and cholesterol at a ratio of 2:1 seems to be more stable with desired physicochemical and permeability characteristics. The SA containing vesicles (PL–SA) did not show a significant improvement in the permeation compared with optimized formulation (PL-1), but the drug deposited in the epidermal layers was conspicuous. The pharmacokinetic study revealed the potential of proliposome gel with 2.4-fold improvement in bioavailability and sustained delivery of exemestane. In conclusion, the proliposome gel proves to be an efficient carrier and further studies has to be conducted in order to optimize the stability for the efficient transdermal delivery of exemestane.

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