Proultraflexible Lipid Vesicles for Effective Transdermal Delivery of Levonorgestrel: Development, Characterization, and Performance Evaluation

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ABSTRACT

The present investigation aimed at formulation, performance evaluation, and stability studies of new vesicular drug carrier system protransfersomes for transdermal delivery of the contraceptive agent, levonorgestrel. Protransfersome gel (PTG) formulations of levonorgestrel were prepared and characterized for vesicle shape, size, entrapment efficiency, turbidity, and drug permeation across rat skin and were evaluated for their stability. The system was evaluated in vivo for biological assay of progestational activity including endometrial assay, inhibition of the formation of corpora lutea, and weight gain of uterus. The effects of different formulation variables (type of alcohol, type and concentration of surfactant) on transdermal permeability profile were assessed. The optimized PTG formulation showed enhanced in vitro skin permeation flux of 15.82 ± 0.37 μg/cm^2/hr as compared to 0.032 ± 0.01 μg/cm^2/hr for plain drug solution. PTG also showed good stability and after 2 months of storage there was no change in liquid crystalline nature, drug content, and other characteristic parameters. The peak plasma concentration of levonorgestrel (0.139 ± 0.05 μg/mL) was achieved within 4 hours and maintained until 48 hours by a single topical application of optimized PTG formulation. In vivo performance of the PTG formulation showed increase in the therapeutic efficacy of drug. Results indicated that the optimized protransfersomal formulation of levonorgestrel had better skin permeation potential, sustained release characteristic, and better stability than proliposomal formulation.

KEYWORDS: contraception, transdermal delivery, protransfersomes, levonorgestrel, stability.

INTRODUCTION

Levonorgestrel is a potent contraceptive agent given alone or in combination with ethinylestradiol in a daily oral dose of 150 μg. Although the oral route for progestogens is widely accepted, it is associated with contraindicative manifestations such as gastrointestinal (GI) disturbance, weight gain, irregular bleeding, headache, and depression of mood mainly a result of excess drug level in blood.[1] Transdermal delivery of levonorgestrel is a better option to overcome problems associated with its oral delivery. The transdermal route, besides being convenient and safe, offers several advantages over conventional ones, such as avoidance of GI incompatibility, variable GI absorption, avoidance of first pass metabolism, improved bioavailability, reduced frequency of administration, improved patient compliance, and rapid termination of drug input. In addition, transdermal delivery can maintain a suitable plasma concentration through a noninvasive zero-order delivery, which would enhance the efficacy of contraceptive agent with high patient compliance.[2]

Despite decades of research, the barrier function of the stratum corneum still remains a problem, which makes the development of new transdermal drug delivery systems an interesting challenge. Vesicular systems have been widely studied as vehicles for dermal and transdermal drug delivery. Their benefits in enhancing drug permeation have been well established.[3] However, the effectiveness of a vesicular system is strongly dependent on its physicochemical characteristics, in particular its thermodynamic state. Liquid-state vesicles have been found to be more effective in enhancing drug transport as compared to gel-state vesicles.[4,5] In the early 1990s a novel series of liquid-state vesicles with elastic lipid membranes were developed.[6] It was suggested that high elasticity of these vesicles could facilitate drug transport across the skin as compared to vesicles with rigid membranes.[7] Phosphatidyl choline (PC)-Span 80 elastic vesicles were recently reported to be more effective in enhancing the skin permeation of dexamethasone, diclofenac, zidovudine, and norgestrel as compared to PC-cholesterol rigid vesicles.[8-12]

Despite the strong rationale for use of vesicles in transdermal drug delivery, the major problem in the development of vesicular systems at industrial and clinical levels is their poor stability. Different approaches have been proposed to enhance the stability of vesicles. Proliposomes offer a versatile delivery concept for improving the stability

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with potential for use with a wide range of active compounds, eg, amphotericin B, propanolol, 5-fluorouracil, ibuprofen, and indomethacin.[13] Stimulated by these findings it was envisaged to extend the concept of proliposomes to systemic delivery of drugs across the skin.

The enhanced transdermal delivery of levonorgestrel was reported using proniosomal formulation.[14] In the present study the provesicular approach has been extended to the transfersomes, which are reported to have superior skin penetration ability. Liquid crystalline protransfersome gel (PTG) will be converted into the ultraflexible vesicles, transfersomes also known as elastic liposomes, in situ by absorbing water. The proposed PTG is a liquid crystalline gel in which the drug is intercalated within phospholipids. The purpose of this study was to examine the feasibility of PTG formulation as a transdermal drug delivery system.

Materials and Methods

Materials

Soya PC, cholesterol (Ch), sodium cholate, sodium deoxycholate, Sephadex-G-50, 6-carboxyfluorescein, Triton X-100, and phosphotungstic acid were purchased from Sigma Chemicals (St Louis, MO). Polyethylene glycol-200, 400, and 4000; Briz-35; sodium chloride; hard paraffin; wax; wool fat; cetostearyl alcohol; white soft paraffin; and copper acetate were purchased from Loba Chemie (Mumbai, India). Ethanol, isopropyl alcohol, butanol, xylene, chloroform, acetonitrile, glycerol, and hematoxylin and eosin were purchased from E. Merck (Mumbai, India). All other reagents used in the study were of analytical grade. Double distilled water was used for all experiments. Sprague-Dawley rats were used for all the animal experiments.

Preparation of Formulations

PTG was prepared by the method reported by Perrett et al[15] for the preparation of proliposomes. Phospholipid, surfactant, alcohol, and drug were taken in a small, clean, dry, wide-mouth glass tube. After mixing all the ingredients, the open end of the glass tube was covered to prevent the loss of solvent. The tube was warmed in a water bath at 60 to 70°C until the ingredients were dissolved. Phosphate buffer (pH 7.4) was added and warmed on a water bath until clear solution was formed. This mixture was converted into protransfersomal gel on cooling. The final composition contained the ratio 5:4:5 wt/wt of lipid + surfactant: alcohol:aqueous phase and the drug concentration in all formulations was 1% wt/wt. Formulations containing different type and concentration of surfactant and alcohol were prepared.

The proliposomal gel (PC + Ch:alcohol:aqueous phase, 5:4:5 wt/wt and drug concentration 1% wt/wt) that was used for comparison was also prepared by using the above method.

The mixed micelle (PC:surfactant; 6:4 wt/wt and drug concentration 1% wt/wt) used for comparison was prepared by the method reported by Dangi et al[16] by dissolving the sodium deoxycholate in phosphate buffer (pH 7.4) with soya PC and then sonicating the system for 3 minutes at 37°C. The solution was then filtered through a 0.45-μm polycarbonate membrane filter (Millipore, Billerica, MA).

In Vitro Characterizations

Visualization of Vesicles

Reconstitution of transfersomes from PTG formulation after hydration was confirmed by transmission electron microscopy (TEM). Samples were prepared by adding phosphate buffer (pH 7.4) to PTG and shaking the mixture manually for 1 minute. A drop of the sample was placed on a carbon-coated copper grid after 15 minutes and negatively stained with 1% aqueous solution of phosphotungstic acid. The grid was allowed to air dry thoroughly and samples were viewed on a TEM (Philips, TEM, New Brunswick, Canada).[17]

A thin layer of PTG was spread on a slide and a drop of phosphate buffer was added through the side of the cover slip into the cavity slide and again observed. Photomicrographs were taken at 400× and 1000×, respectively, before and after addition of water for both plain and polarized light (Leica, DMLB, Bensheim, Germany).

Vesicle Size, Turbidity, and Entrapment Efficiency Measurement

PTG (100 mg) was hydrated with 10 mL of saline solution (0.9% NaCl) using manual shaking for 5 minutes. The vesicle size and turbidity after hydration were determined by dynamic light scattering (DLS) method (Malvern Zetamaster, ZEM 5002, Malvern, UK) and Nephalometer (Superfit, Mumbai, India), respectively. The entrapment efficiency was determined after separating the unentrapped drug through Sephadex G-50 column. The eluted vesicles were lysed using Triton-X 100 (0.1% vol/vol) and analyzed for drug content.[18] Entrapment efficiency was expressed as percentage of total drug entrapped.

In Vitro Skin Permeation Study

Skin permeation of levonorgestrel from different PTG formulations was studied using a Franz glass diffusion cell.

The effective permeation area of the diffusion cell and receptor cell volume was 1 cm^2 and 10 mL, respectively. The temperature of receptor fluid was maintained at 32 ± 1°C. The receptor compartment contained 30% PEG-200 in phosphate-buffered saline (PBS, pH 6.5) (based on the solubility characteristic of the drug). Abdominal skin of female Sprague-Dawley rats (5 to 6 weeks old) was mounted between the donor and receptor compartment. PTG formulation containing 1 mg of drug was applied to the epidermal surface of the rat skin. Samples were withdrawn through the sampling port of the diffusion cell at predetermined intervals over 24 hours and analyzed by high-performance liquid chromatography (HPLC) assay.[2] An equal volume of fresh PBS containing 30% PEG-200 maintained at 32 ± 1°C was replaced into the receptor compartment after each sampling.[19] Cartesian plots of cumulative amount of drug present in receptor compartment versus time were plotted. Flux (Jss, μg/cm^2/hr) was calculated from the slope of the steady state portion of these graphs.

Stability Studies

The formulation PTDF-I_3 was selected for stability studies based on its in vitro characterization. The formulations were stored in glass tubes covered with aluminum foil at 30 ± 2°C and 4 ± 2°C for 2 months and observed visually under microscope for change in consistency, liquid crystalline structure, and appearance of drug crystals. Transfersomes formed from PTG formulation were also characterized for vesicle size and drug content.[20]

In Vivo Evaluation

In vivo studies were performed on female Sprague-Dawley rats weighing 100 to 150 g. Four groups each comprising 9 animals were employed. The first group served as a control while the second, third and fourth groups received plain drug, optimized protransfersomal gel (PTDF-I_3), and proliposomal formulation, respectively. The quantity of levonorgestrel in all the formulations was 500 μg. Rats were synchronized by injecting 1 mL of 0.1% copper acetate intraperitoneally. After 24 hours the formulation was applied to the dorsal surface of rats (1.0 cm^2). The treated areas of animals were protected by using nylon mesh, which was supported by plastic squares having small pores. Treated animals were kept in separate cages and housed in standard laboratory conditions. Food and water were provided ad libitum. Three rats from each group were killed by excessive chloroform inhalation after the first, fourth, and ninth day of application. All investigations were performed after approval by the animals ethical committee of Dr H.S. Gour University (formerly University of Sagar, Madhya Pradesh, India), and in accordance with the disciplinary principles and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Biological Assay for Progestational Activity

The rats were dissected and ovaries and uterine horns were harvested and assayed for progestational activity. Uterine horns were weighed immediately in a single pan balance (Sisco Popular, Varanasi, India) and ovaries were examined visually for the presence of ovulation point and bleeding points. Organs were incised into smaller pieces and fixed into fixative solution (3:1, absolute alcohol: chloroform) for 3 hours. The organs were first transferred to absolute alcohol for 0.5 hour and then in absolute alcohol and xylene for 1 hour. The wax scrapings were added in this solution until saturation and were kept for 24 hours. After 24 hours, the paraffin blocks were made by embedding the tissue in hard paraffin, matured at 62°C. The sections of 5 μm were cut using microtome (Erma Optical Works, Tokyo, Japan) and stained with Ehrlich's hematoxylin and eosin for nucleus and cytoplasm, respectively. Histological changes in the uterus and ovaries were examined under optical microscope (Leica, DMLB).

Endometrial Assay

The uterine thickness in each group of rats was determined by calibrated stage and ocular micrometer (Elico Ltd, Hyderabad, India). The morphological changes produced by the direct action of levonorgesteral on uterine endometrium of rats were recorded using phase contrast microscope, and photomicrographs of the selected sections were taken.

Assay for Interference with the Formation of Corpora Lutea

The mean number of corpora lutea present in the ovarian section of each group of rats were counted and percent inhibition was calculated with respect to control. Photomicrographs of the selected sections were taken.

Plasma Concentration Determination

The blood samples were collected from retro orbital plexus of the eye at defined time intervals. Each blood sample was centrifuged at 2000 rpm for 10 minutes and drug concentration after deproteinization with acetonitrile was determined by HPLC.

Fluorescence Microscopy

Fluorescence microscopy was performed to confirm the skin penetration ability of flexible PTG formulation. The fluorescent labeling was carried out by preparing the optimized PTG formulation (PTDF-I_3) and proliposomal formulation as described earlier with fluorescence marker 6-carboxyfluorescein. Fluorescence marker loaded formulation was applied topically to rats. After 6 hours of application the rats were humanely killed and skin was removed, cut into small pieces, fixed by the conventional procedure as described earlier for fixation of the tissue for progestational activity, and examined under a fluorescence microscope (Leica, DMRBE, Bensheim, Germany).[9]

HPLC Assay

The quantitative determination of drug was performed by HPLC using acetonitrile/water (50/50 vol/vol) as mobile phase delivered at 2.0 mL/min by LC 10-AT vp pump (Shimadzu, Tokyo, Japan). An injection volume of 20 μL was eluted in LUNA 54, C18, 4.6 × 150 mm column (Phenomenex, Tokyo, Japan) at room temperature. The column eluant was monitored at 243 nm using SPD-M10A vp diode array UV detector (Shimadzu).[2]

Statistical Analysis

Statistical significance of all the data generated was tested by analysis of variance (ANOVA) followed by studentized range test. A confidence limit of $P < .05$ was fixed for interpretation of the results using the software PRISM (Graphpad, San Diego, CA).

Results and Discussion

Preparation and In Vitro Characterization of Formulations

The PTG when observed under cross polarizer showed birefringent streaks lamellar structures in liquid crystalline form (Figure 1a). Hydration of this gel formed spherical vesicular structure (Figure 1b). The transformation of lamellar liquid crystalline PTG to transfersomes can be ascribed to different degree of hydration of surfactant and phospholipid molecules. Initially, due to the presence of limited solvent, the PTG formed was a mixture of lamellar liquid crystals resembling palisades and vesiculating lamellas linked together. Further addition of water resulted in swelling of the lipid bilayer due to interaction of water with polar groups of surfactants and above a limiting concentration of solvent, the bilayers formed spherical structures randomly giving rise to vesicular structures.[14,21] Morphological characterization of the protransfersomes as well as existence of their vesicular structure after hydration

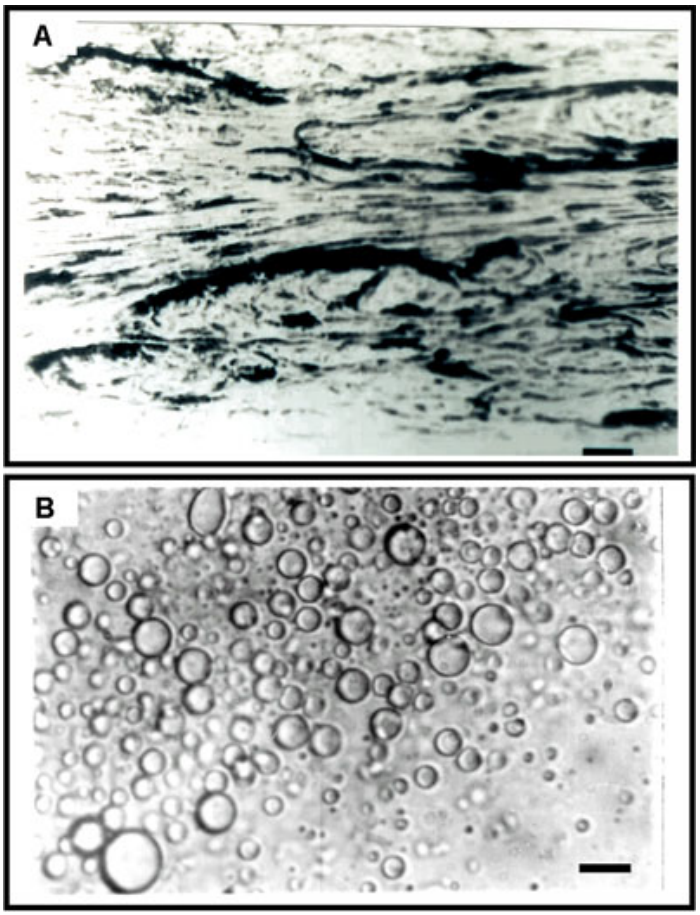

Figure 1. Lamellar liquid crystalline structure of protransfersomal gel (Photomicrograph A, X 400), Scale bar = 500 μm and vesicular structure of transfersomes formed upon hydration of protransfersomal gel (photomicrograph ×1000). Scale bar = 1 μm.

was confirmed by TEM (Figure 2). Entrapment efficiency of the PTG formulation was high (**maximum 96.9 ± 0.4 for PTDF-I_3**) because of the lipophilic nature of the drug. Hence, drug molecules seem to be intercalated almost completely within the lipid bilayer forming a part of the bilayer (Table 1). This result was consistent with the entrapment efficiency of levonorgestrel in proniosome formulation as reported in previous publication.[14]

In Vitro Skin Permeation Study

The flux value obtained from PTDF-I_3 (15.82 ± 0.37 μg/cm^2/hr) is 3-fold higher than that of the proliposomal formulation (5.90 ± 0.20 μg/cm^2/hr), nearly 8-fold higher than the mixed micelle formulation (1.82 ± 0.12 μg/cm^2/hr), and 494.4-fold higher than that attained by the saturated levonorgestrel solution in water (0.032 ± 0.01 μg/cm^2/hr). The transdermal flux of saturated plain drug solution is consistent with that reported by Chen et al[2] (0.03 ± 0.01 μg/cm^2/hr) (Table 2). The very low skin permeability of plain drug solution is due to extreme hydrophobicity and low solubility of levonorgestrel in water (1.42 ± 0.2 μg/mL,

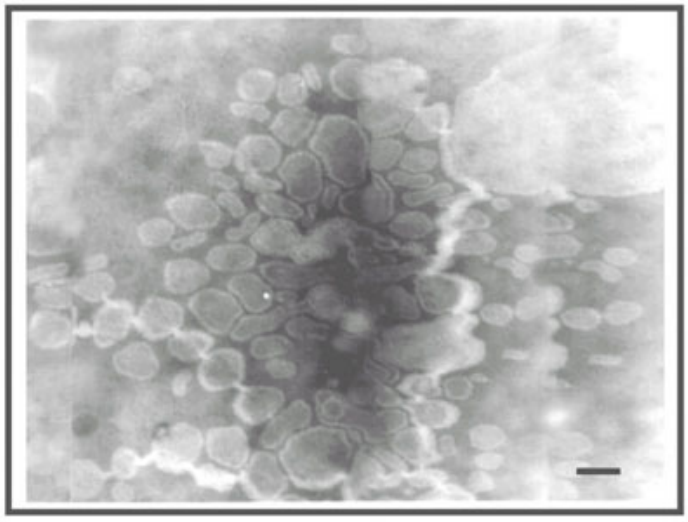

Figure 2. TEM photomicrograph of PTG formulation after hydration (×1,20,000). Scale bar = 500 nm.

Table 2. Steady State Flux (Jss) of Levonorgestrel Across Rat Skin*

Formulation Code	Jss (μg/cm^2/hr)	ER[a]
PTF-I_3	11.32 ± 0.32	353.7
PTDF-I_3	15.82 ± 0.37	494.4
PTBF-I_3	8.88 ± 0.36	277.6
PTDF-I_1	6.28 ± 0.28	196.2
PTDF-I_2	9.28 ± 0.22	290.0
PTDF-I_3	15.82 ± 0.37	494.4
PTDF-I_4	5.74 ± 0.23	180.6
PTDF-I_5	2.12 ± 0.12	66.25
PTDF-E_3	11.50 ± 0.25	359.0
PTDF-B_3	13.63 ± 0.30	425.9
PTDF-I_3	15.82 ± 0.37	494.4
Pro-liposome	5.90 ± 0.20	184.3
Mixed micelles	1.82 ± 0.12	56.87
PD	0.032 ± 0.01	

*Values represented as mean ± SD (n = 3). ER[a] indicates enhancement ratio (ratio of transdermal flux from protransfersomal gel to plain drug).

at 32°C). Better transdermal flux and no lag phase with PTG gel was perhaps a result of the combination of one or more of following mechanisms: (1) increased solubility of levonorgestrel, (2) high association of drugs with vesicle bilayers, (3) increased partitioning of vesicles into the stratum corneum, (4) penetration enhancement effect of the short chain alkanols, and (5) elasticity of vesicle membrane.

Levonorgestrel is a hydrophobic drug and its aqueous solubility (1.42 ± 0.2 μg/mL) is very poor, which leads to a very low concentration gradient across the skin resulting in very poor skin permeation (0.032 ± 0.01 μg/cm^2/hr). Levonorgestrel solubility in PTG was 715-fold (1000 ± 15.2 μg/mL) higher than in PBS leading to a greater concentration gradient across the skin and this subsequently improved permeation. The probable reason for increased solubility of drug in PTG is the presence of short chain alkanols (ethanol, isopropanol, and butanol) together with PC-surfactant mixture.

For optimum drug delivery, a high degree of drug-vesicle association is essential so that appreciable quantity of drug could be carried by elastic vesicles across the stratum corneum. Subsequently, larger quantities of drugs will be released from the vesicles, thereby increasing the amount of free drug available for diffusion into the deeper skin

Table 1. Composition and Characterization of Different Protransfersomal Gel Formulations*

Code	Composition PC:S wt/wt	Alcohol Used	Vesicle Size, nm	Turbidity, NTU	% Entrapment
1.With Sodium cholate					
a. PTF[a]-E_3	85:15	Ethanol	1192 ± 45	315 ± 12	89.4 ± 0.1
b. PTF-B_3	85:15	Butanol	1151 ± 42	329 ± 15	92.6 ± 0.26
c. PTF-I_3	85:15	Isopropanol	1073 ± 38	345 ± 15	93.3 ± 0.26
2.With Brij 35					
a. PTBF[b]-E_3	85:15	Ethanol	1201 ± 49	287 ± 11	87.8 ± 0.34
b. PTBF-B_3	85:15	Butanol	1165 ± 36	296 ± 12	91.5 ± 0.50
PTBF-I_3	85:15	Isopropanol	1083 ± 35	310 ± 14	92.6 ± 0.22
3.With Sodium Deoxycholate					
a. PTDF[c]-E_3	85:15	Ethanol	1175 ± 37	316 ± 15	90.2 ± 0.45
b. PTDF-B_3	85:15	Butanol	1146 ± 41	327 ± 18	94.8 ± 0.40
c. PTDF-I_1	95:05	Isopropanol	1041 ± 35	305 ± 13	94.4 ± 0.34
d. PTDF-I_2	90:10	Isopropanol	1073 ± 36	324 ± 10	95.9 ± 0.17
e. PTDF-I_3	85:15	Isopropanol	1120 ± 40	362 ± 12	96.9 ± 0.40
f. PTDF-I_4	80:20	Isopropanol	935 ± 30	196 ± 9	80.6 ± 0.3
g. PTDF-I_5	75:25	Isopropanol	862 ± 30	105 ± 10	68.4 ± 0.45

*Values represented as mean ± SD (n = 3). PC indicates phosphatidyl choline (Soya); S, surfactant; PTF[a], protransfersomal gel formulation containing sodium cholate; PTBF[b], protransfersomal gel formulation containing Briz 35; PTDF[c], protransfersomal gel formulation containing sodium deoxycholate.

layers. High entrapment efficiency of protransfersome gel (96.9% ± 0.4%) is probably the reason for its better skin permeation.

Furthermore, higher skin permeation of PTG could be a result of better partitioning across the stratum corneum and to deeper layers of skin under the influence of transepidermal osmotic gradients.[22] The osmotic gradient is developed because skin penetration barrier prevents water loss through the skin and maintains a water activity difference in viable parts of the epidermis (75% water content) and nearly completely dry stratum corneum near the surface (15% water content).[4] PTG consists of polar lipids (PC) that have a tendency to attract water because of the energetically favorable interaction between the hydrophilic lipid residues and proximal water molecules. Hence, when PTG is applied on skin surface that is partly dehydrated by water loss due to evaporation, the lipid vesicles feel this osmotic gradient and try to escape complete drying by moving along this gradient resulting in faster partitioning of vesicles into the stratum corneum and other deeper layers of the skin. The above hypothesis is well supported by the report of Kirjavainen et al,[23] which states that phospholipids influence the stratum corneum lipid bilayer fluidity, thus improving drug partitioning into the bilayers.

PTG formulations contain short-chain alkanols (ethanol, isoproproyl alcohol, and butanol) in their composition and these alkanols are known to enhance penetration by increasing the fluidity of stratum corneum, thereby reducing the resistance of the intracellular lipoidal barrier.[24] Hence, the role of short-chain alkanols in enhancing the permeation of levonorgestrel, a hydrophobic drug, cannot be ruled out.

Mixed micelle formulation contains a higher amount of detergent than optimized PTG (PTDF-I_3) formulation (PC/Surfactant; 60/40 and 85/15 wt/wt % for micelles and optimized PTG formulation, respectively). Thus, if penetration enhancement due to solubilization of the skin lipids by the surfactant had been the reason for the superior penetration capability of PTG, one would expect an even better penetration performance of the micelles. In contrast to this postulate, the higher detergent concentration in mixed micelles did not improve the efficiency of material transport into the skin, whereas optimized PTG formulation (PTDF-I_3) showed 8-fold higher transdermal flux (15.82 ± 0.37 μg/cm^2/hr) as compared to mixed micelle formulation (1.82 ± 0.12 μg/cm^2/hr). These results correlated well with the report of Cevc et al[25] and van-Kuijk Meuwissen et al[26] who compared the penetration ability of transfersomes, liposomes, and mixed micelles by confocal laser scanning microscopy (CLSM) and suggested that mixed micelles were restricted to the top-most part of stratum corneum and transfersomes penetrated to deeper layers of skin.[27]

It is evident from Figure 3 that the effect of different alcohols on skin permeation rate followed the following order: isopropanol > butanol > ethanol. These observations do not correlate well with the previous report of Friend et al[28] who found permeation to increase with increase in the chain length of alcohols from C_2 to C_4 (ethanol to butanol). The results of this study showed maximum permeation of levonorgestrel for isopropanol formulation (358.53 ± 9.8 μg after 24 hours). This was possibly owing to the branched chain structure of isopropanol that acted as a cosurfactant and might have loosened the bilayer packing resulting in better skin penetration.

Figure 4 shows the effect of different surfactants on release rate and it was found to follow the following order: sodium deoxycholate > sodium cholate > Briz 35. Sodium deoxycholate showed better skin permeation (highest transdermal flux 15.82 ± 0.37 μg/cm^2/hr for formulation PTDF-I_3) as compared to other surfactants possibly due to better interaction with phospholipid bilayers, which attributes better deformability to the vesicles resulting in better skin permeation.[9,18]

In Vivo Study

The in vivo performance evaluation of the optimized PTG formulation (PTDF-I_3) containing levonorgestrel for progestational activity was carried out by biological assay. The method reported by Hebborn[29] was slightly modified. Mature rats were taken and were synchronized by injecting copper acetate intraperitoneally.

It is obvious that levonorgestrel provided contraception by producing morphological changes in uterine mucosa,

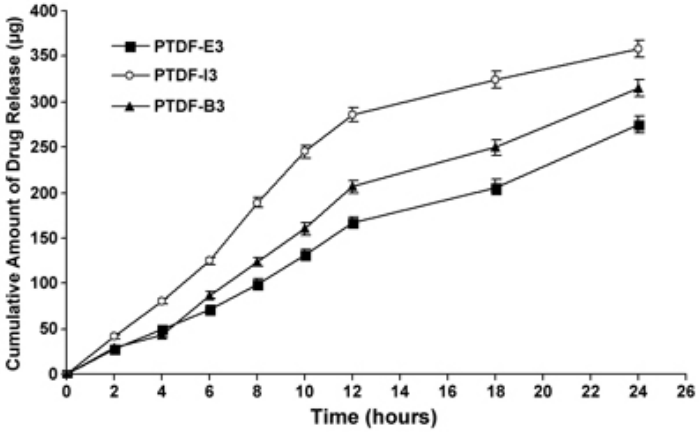

Figure 3. Effect of different alcohols on drug permeation across rat skin from different protransfersomal gel formulations. Mean ± SD (n = 3).

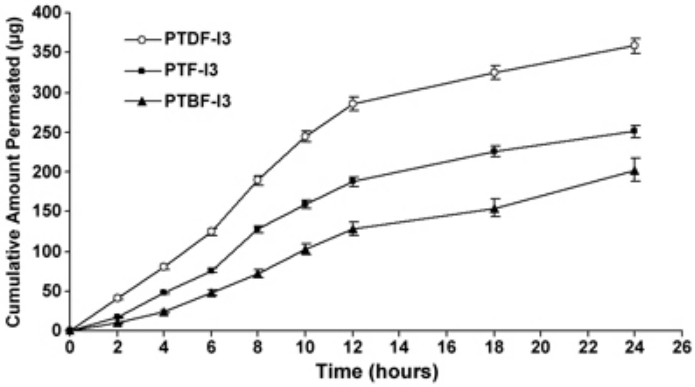

Figure 4. Effect of different surfactants on drug permeation across rat skin from different protransfersomal formulations. Mean ± SD (n = 3).

endometrium, and ovary as well as by inhibiting the secretion of luteinizing hormone that is required for ovulation and for transformation of the follicle in a functioning corpus luteum. The histological studies give a better prediction of the in vivo performance of the developed PTG formulation. The PTG formulation PTDF-I_3 was selected for in vivo studies based on its in vitro performance.

Table 3 shows an increase in uterine mucosal thickness and endometrial weight. The mucosal thickness in the case of plain drug, proliposomal, and PTDF-I_3 formulation–treated rats were 15 ± 0.50 μm, 26.3 ± 1.4 μm, and 64.8 ± 3.5 μm, respectively. Uterine mucosal thickness in rats treated with PTG formulation was 4.3-fold higher as compared to that after treatment with plain drug.

It is clear from Figure 5a-c that more villi are present in uterine mucosa and uterine glands in rats of the control group as well as the proliposomal formulation–treated group as compared to protransfersomal gel–treated group. These results clearly indicated that application of the PTG formulation efficiently inhibited the formation of corpora lutea as compared to treatment with plain drug. The ovulation points were not observed in PTG formulation–treated female rats indicating better therapeutic performance of the proposed system.

Table 4 shows the inhibition of luteinization in rat ovary for parameters like number of ovulation points and number of

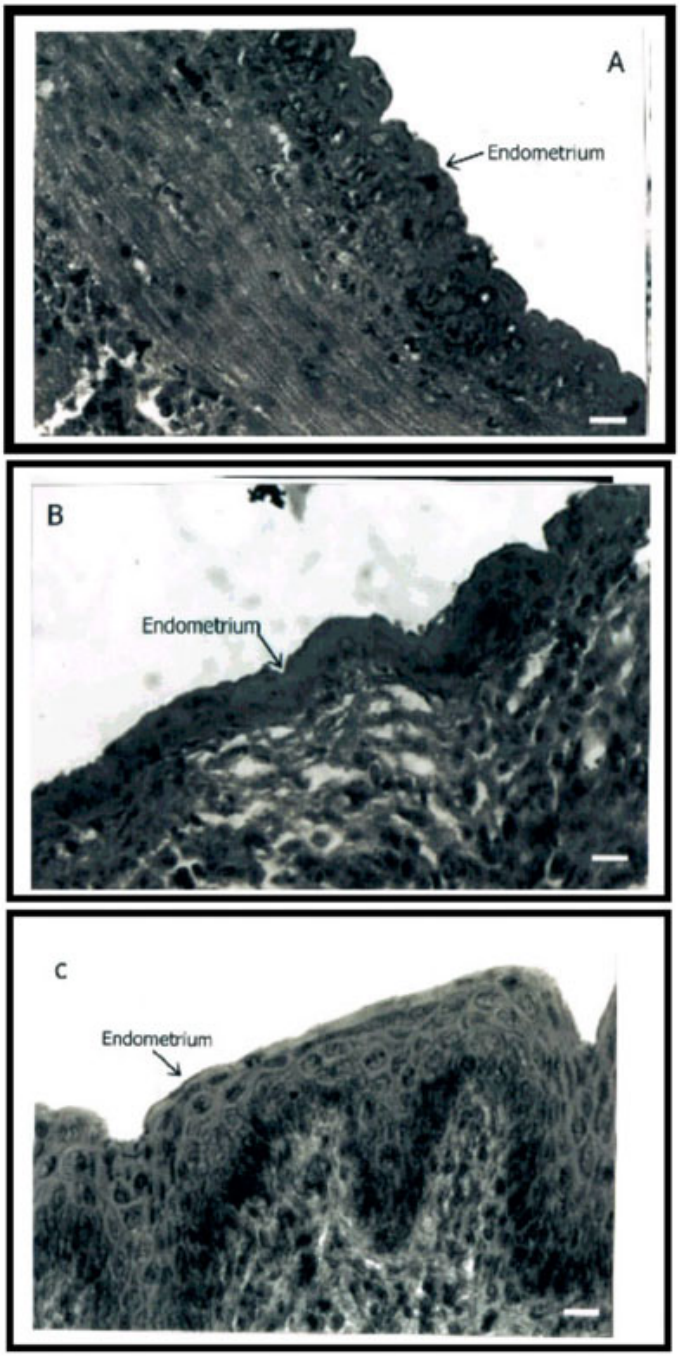

Figure 5. Photomicrograph showing histology of uterine mucosa and endometrium of a. Control rat (×450). b. Proliposomal gel formulation–treated rat (×450). c. Protransfersomal gel formulation–treated rat (×450). (Scale bar = 500 μm.)

Table 3. Endometrial Assay of Levonorgestrel in Rats*

Group	Formulation	Endometrial Weight, mg			Mucosal Thickness, μm
		First Day	Fourth Day	Ninth Day	
I	Control	2.45 ± 0.14	5.05 ± 0.35	6.14 ± 0.50	3.2 ± 0.26
II	Plain drug	15.6 ± 0.2	21.2 ± 0.11	33.2 ± 0.34	15.0 ± 0.50
III	Protransfersomes	23.7 ± 1.8	51.5 ± 2.7	94.2 ± 5.4	64.8 ± 3.5
IV	Proliposomes	17.2 ± 1.3	29.3 ± 1.8	42.3 ± 2.0	26.3 ± 1.4

*Values represented as mean ± SD (n = 3).

Table 4. Inhibition of Luteinization in Rat Ovary by Levonorgestrel*

Formulation	Interference in Ovulation			Mean No. of OP[a]	Mean No. of CL[b]	Inhibition, %
	First Day	Fourth Day	Ninth Day			
Control				10.5 ± 1.2	21.0 ± 2.0	
Plain drug		+	+	7.5 ± 1.1	18.0 ± 1.5	14.28 ± 1.60
Protransfersomes	+	+++	++	1.0 ± 0.3	3.0 ± 0.5	85.71 ± 7.20
Proliposomes		++	+	4.0 ± 1.0	11.5 ± 1.0	45.23 ± 2.80

*Values represented as mean ± SD (n = 3). OP[a] indicates ovulation point; CL[b], corpora lutea; −, no interference (OP >10); +, slight interference (OP, 7-10); ++, moderate interference (OP, 4-7); +++, strong interference (OP, 0-4).

points related to formation of corpora lutea. The interference with formation of corpora lutea was greater with PTG formulation as compared to proliposomal formulation containing levonorgesteral. The percent inhibition compared to control with PTG-treated rats after 9 days was 85.7% ± 5.2%, while for proliposomal formulation treated rats it was 45.2% ± 2.8%.

Pharmacokinetic studies in rats revealed very low concentration (0.015 ± 0.005 μg/mL) of levonorgestrel in plasma following topical application of plain drug. However, application of optimized PTG formulation produced 8-fold higher plasma concentration (0.139 ± 0.05 μg/mL) within 4 hours that was maintained over 48 hours. These results established the sustained and prolonged delivery of levonorgestrel from PTG formulation and ability to maintain the constant drug concentration over 48 hours (Figure 6). The area under the curve $AUC_{0\text{-}24\ h}$ after application of PTG formulation was nearly 4 times higher (8.384 μg h/mL) than that after application of proliposomal gel formulation (2.146 μg h/mL) and nearly 18 times higher than after plain drug application (0.451 μg h/mL). Based on in vivo results, it can be concluded that the investigated vesicular carrier system provided a controlled and prolonged delivery of levonorgestrel.

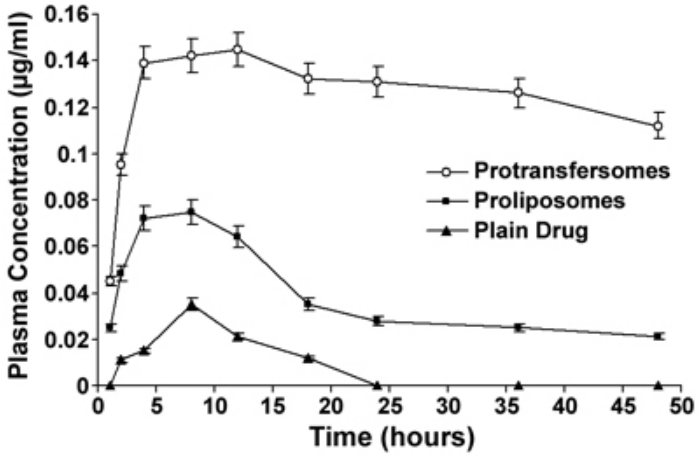

Figure 6. Comparison in plasma concentration of drug after administered in the form of control and protransfersomal gel and proliposomal gel formulations. Mean ± SD (n = 3).

The better skin penetration potential of developed vesicular carrier was further confirmed by the fluorescence microscopy study. Six-carboxyflourescein is a hydrophilic fluorescence marker and does not normally get into the deeper layer of the skin when applied in the form of proliposomal formulation (Figure 7a). However, this dye was transported extensively and reached the dermal layer

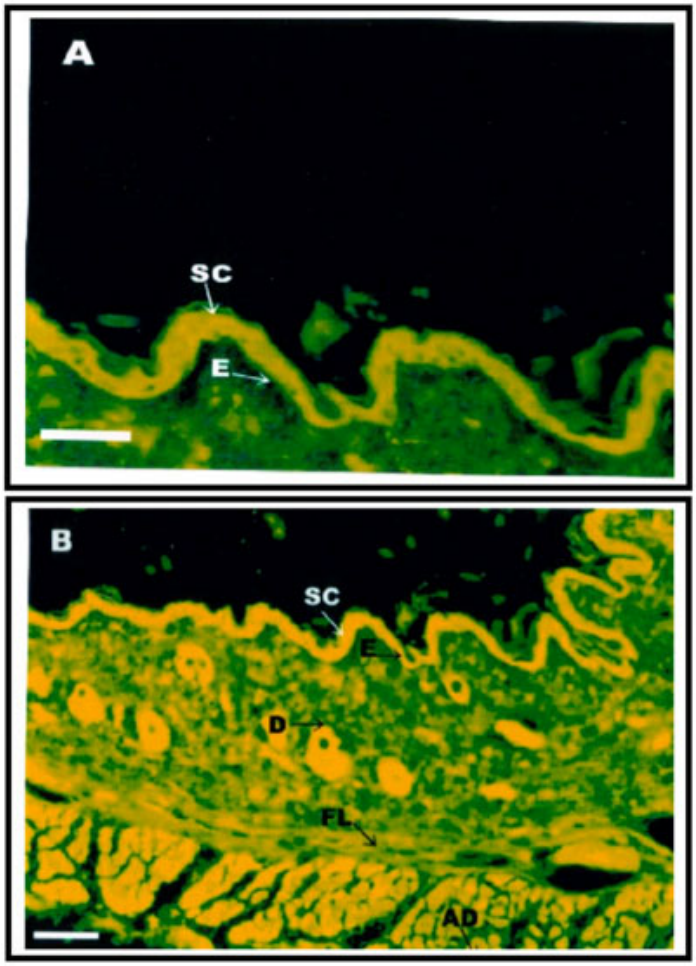

Figure 7. Penetration of 6-carboxyfluorescein as fluorescence probe after 6-hour application of proliposomal formulation (photomicrograph A ×450), scale bar = 250 μm, and protransfersomal gel formulation (photomicrograph B ×450), scale bar = 500 μm; SC indicates stratum corneum; E, epidermis; D, dermis; FL, fibrous layer; AD, adipose tissue.

Table 5. Stability Study of Optimized PTG Formulation (PTDF-I_3) and Proliposomal Formulations*

StorageTime (Days)/ Temp, °C	Vesicle Size, nm	Percentage Entrapment	Consistency	Liquid Crystalline Structure	Appearance of Drug Crystal
Initial	[a]1120 ± 40	[a]96.9 ± 0.4			
	[b]1250 ± 45	[b]85.2 ± 1.2			
20/4°C	[a]1136 ± 44	[a]96.0 ± 0.5	*	NC	NA
	[b]1279 ± 48	[b]83.7 ± 1.0	*	NC	NA
20/30°C	[a]1168 ± 49	[a]95.2 ± 0.4	*	NC	NA
	[b]1308 ± 55	[b]82.4 ± 0.9	*	NC	NA
40/4°C	[a]1150 ± 46	[a]95.4 ± 0.3	*	NC	NA
	[b]1305 ± 57	[b]81.5 ± 0.7	*	NC	NA
40/30°C	[a]1206 ± 52	[a]94.0 ± 0.5	*	NC	NA
	[b]1368 ± 60	[b]79.6 ± 0.7	*	NC	A
60/4°C	[a]1162 ± 46	[a]94.5 ± 0.3	**	NC	NA
	[b]1332 ± 62	[b]79.7 ± 0.6	*	C	A
60/30°C	[a]1241 ± 55	[a]92.5 ± 0.1	**	NC	NA
	[b]1405 ± 65	[b]74.2 ± 0.4	*	C	A

*Values represented as mean ± SD (n = 3). [a] indicates PTG formulation PTDF-I_3; [b], proliposomal formulation; *, unaltered; **, increase; NC, no change; C, change; NA, not appeared; A, appeared.

when applied in the form of PTG formulation (Figure 7b). The presence of fluorescence marker in the deeper layer of skin shows better skin penetration ability of the PTG formulation.

Stability Studies

The liquid crystalline nature of PTG was not affected after 2 months of storage. Drug crystals were not observed after 2 months of storage both at room temperature and cold temperature (4°C ± 2). The increase in consistency of PTG was probably a result of the molecular interaction of polar head groups of surfactants with the solvent and permeation of solvent into bilayer. However, the solvent diffused into the bilayers did not appear to disturb the liquid crystalline structure. It, rather, seems to have resulted in complete bilayer formation due to saturation of lipid polar head groups. This is expected to increase the bilayer distance eventually resulting in overall increase in consistency. Size and size distribution studies showed that vesicle size increased indicating complete swelling of bilayer and hence formation of more uniform vesicles upon storage. The effect of aging on entrapment efficiency was not too much. However, the observed slight reduction in entrapment efficiency after storage may be a result of leaching of drug from the preparation (Table 5).

CONCLUSION

PTG formulation developed for transdermal delivery of levonorgestrel possessed better skin permeation potential, better stability, and higher entrapment efficiency than proliposomal formulation. An 8-fold increase in peak plasma concentration of levonorgestrel and its maintenance over 48 hours after topical application of optimized PTG formulation as compared to plain drug solution suggested that PTG formulation provided a better mode of systemic delivery of levonorgestrel. However, detailed in vivo studies in human volunteers for contraceptive use are suggested.

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