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Ethinylestradiol-loaded ultraflexible liposomes: pharmacokinetics and pharmacodynamics

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Abstract

This study aimed to develop ultraflexible liposomes as an alternative to the oral route, which would enhance the bioavailability and reduce the toxicity of ethinylestradiol. Ultraflexible liposomes of ethinylestradiol using an optimized concentration of surfactants were prepared and characterized in vitro. The effect of surfactant type under non-occlusive conditions on transdermal permeability was assessed. A histopathological study was performed to assess the action of ethinylestradiol on the uterus and ovaries. The pharmacokinetics of free ethinylestradiol (following single oral administration and one day of application to the skin), ultraflexible liposomal ethinylestradiol and non-flexible liposomal ethinylestradiol were studied in female Sprague–Dawley rats. Insignificant differences in size between the ultraflexible liposomal formulations containing optimized concentrations of different surfactants were observed. Ultraflexible liposomes can penetrate through pores much smaller than their own diameter. The transdermal permeability of lipophilic surfactant was greater than that of hydrophilic surfactant. The release of ethinylestradiol from the proposed formulation through rat skin was found to be constant. The histopathological study showed that the ultraflexible liposomal transdermal drug delivery system for ethinylestradiol provided effective contraception by follicular cell lysis, depletion of zona granulosa and ova, and by increasing the uterine mucosal and endometrial proliferation. Encapsulation of ethinylestradiol in ultraflexible liposomes modified the pharmacodynamics and pharmacokinetics of the contraceptive agent, resulting in a marked improvement in bioavailability and optimized therapy.

Introduction

Estradiol is a commonly used steroidal contraceptive. Oral administration of estradiol has a serious limitation of extensive first pass hepatic metabolism. Ethinylestradiol is frequently used orally, either alone or with a progestin in oral contraceptives; the ethinyl substitution in the C17 position inhibits first-pass hepatic metabolism. There is some initial conjugation by the gut wall and the systemic bioavailability is about 40% (Williams & Stancel 1990). Transdermal administration of ethinylestradiol was found to be effective in reducing this conjugation. Not only this, a transdermal patch of ethinylestradiol was able to provide sustained release of the hormone and more constant blood levels as compared to oral administration (Henzl & Loomba 2003). Transdermal administration of ethinylestradiol offers a well-tolerated, effective, reversible and easy-to-use method of hormonal contraception with an increased likelihood of compliance relative to oral contraceptives (Goa et al 2003). Thus transdermal administration is one of the ‘sought after’ methods for enhancing ethinylestradiol efficiency.

Many reports indicating the utility of ultraflexible liposomes in transdermal drug delivery are available. Cevc & Blume (1992) proposed the reduced deformability of liposomes in comparison to ultraflexible liposomes. They also reported that liposomes were less capable of passing through barriers with pores smaller than their own diameter. It is a well-established fact that these elastic lipid vesicles permeate the skin more effectively than conventional rigid liposomes. Cevc et al (1995) studied transfersome-based transfer across the skin and proposed the concept of a transdermal osmotic gradient, which creates a substantial transdermal flux for the loaded drug,

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and reported the efficacy of drug transport across the intact skin by properly optimized transfersomes. Essa et al (2002) investigated the impact of the combined use of ultradeformable liposomes and iontophoresis on the penetration of estradiol. They found that the use of ultradeformable liposomes increased passive skin delivery of estradiol over that of saturated aqueous solution under occluded conditions. The effect of propylene glycol and supersaturation on estradiol permeation through human skin and silastic membrane was reported (Megrab et al 1995). IDEA (Munich, Germany), the innovative product and drug delivery company, developed and commercialized non-invasive, targeted therapeutics based on stable, ultra-deformable carriers that are typically applied to the skin (Cevc 2001a, b, c). There are many reports available on in-vitro liposome and transfersome-mediated estradiol delivery (Megrab et al 1995; El Maghraby et al 1998, 1999, 2000; Essa et al 2002), however no studies have been published concerning the pharmacokinetic and pharmacodynamic effects of ethinylestradiol on ultraflexible-liposome-mediated transdermal administration. The present study aimed to investigate the comparative pharmacokinetic and pharmacodynamic effects of ethinylestradiol on oral (plain drug solution), ultraflexible liposome, non-flexible liposome and plain drug solution mediated transdermal administration to female Sprague–Dawley rats.

Materials and Methods

Materials

Ethinylestradiol was received as a gift sample from M/S Wyeth Lederle Ltd, Mumbai, India. Soya-phosphatidylcholine (PC, 99% purity), Sephadex G50, 6-carboxyfluorescein, Triton X-100, phosphotungstic acid, sodium deoxycholate(DC), Tween 60, Span 60 and Span 65 were purchased from Sigma Chemicals, USA. Polycarbonate membrane filters were purchased from Millipore USA. Polyethyleneglycol-200 (PEG-200) (Loba Chemie, India), acetonitrile (HPLC), methanol, chloroform, xylene (E. Merck, India) and other reagents used in the study were of analytical grade. Double-distilled water was used for all experiments.

Preparation of ultraflexible liposomes

Ethinylestradiol-loaded ultraflexible liposomes were prepared by the conventional rotary evaporation sonication method as described by Jain et al (2003). Briefly, soyaphosphatidylcholine, surfactant in different ratios (95–75:5–25% w/w PC:surfactant) and ethinylestradiol were dissolved in a solvent mixture of chloroform:methanol (2:1) in a clean dry round-bottomed flask. The solvent was removed completely by rotary evaporation (Rotary evaporator, York Scientific Ltd, India) under vacuum above the transition temperature of lipid. Final traces of solvents were removed under vacuum at room temperature before hydration. The thin, uniform lipid film thus produced was hydrated with PBS (pH 6.4) for 1 h at room temperature with continuous rotation at 60 rev min^{-1}. The resulting vesicles were allowed to swell for 2 h at room temperature to produce large multilamellar vesicles (LMLVs). The LMLVs were probe sonicated (Titanium probe, Ultrasonicator; Imeco Ultrasonics, India) at 4°C for 20 min at 40% output frequency (40 W) to cause them to form small unilamellar vesicles. The sonicated vesicles were extruded through a stack of polycarbonate membrane filters (200–450 nm) with the finest at the bottom.

Non-flexible liposomes were prepared using film hydration method (New 1990). The lipids (PC and cholesterol 7:3) were dissolved in organic solvent (chloroform: methanol 2:1) and the method was the same as given for ultraflexible liposomes.

Characterization

Vesicle size and shape

The particle size of the vesicles was determined by dynamic light scattering using a computerized inspection system (Malvern Zetamaster, ZEM 5002, Malvern, UK). Ultraflexible liposomes were visualized using a transmission electron microscope (Philips, Japan), with an accelerating voltage of 100 kV. A drop of the sample was placed on a carbon-coated copper grid to leave a thin film on the grid. Before the film dried, it was negatively stained with 1% phosphotungstic acid. A drop of the staining solution was added to the film and the excess solution was drained off with a filter paper. The grid was allowed to air-dry thoroughly and viewed on a transmission electron microscope. Ultraflexible liposomes vesicles (without sonication) were visualized using an optical microscope (Leica DMLB, Germany). A thin film of ultraflexible liposomes was spread on a slide and after placing the coverslip was observed under the optical microscope.

Entrapment efficiency

Entrapment efficiency was determined after separation of unentrapped drug by the mini-column centrifugation method (New 1990; Fry et al 1978). Sephadex G-50 was allowed to swell in 0.9% NaCl at room temperature with occasional shaking for at least 5 h, after which the gel was formed and stored at 4°C. To prepare the mini-column, Whatman paper pads were placed at the bottom of the barrels of a 1.0 mL syringe, which were filled with the gel. Excess water was removed by centrifugation (Remi R8C Laboratory Centrifuge, Mumbai, India) at 3000 rev min^{-1} for 3 min. A 200-μL ultraflexible liposome suspension was applied dropwise to the centre of the column, followed by centrifugation and collection of vesicles. Distilled water was added to the mini-column and centrifugation was repeated. Ultraflexible liposomes (depending on their type and size) can be recovered from the first or the second stage of centrifugation. Here two stages were necessary to recover the vesicles. When a saturated drug solution was used instead of the ultraflexible liposomes, the entire drug remained bound to the gel. This confirmed that there would be no free drug present after recovering the vesicles. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% v/v

Triton X-100 and filtering it, and the drug amount was determined in a UV spectrophotometer (Shimadzu 1601) at 283.8 nm. Ultraflexible liposomes disrupted with 0.1% v/v Triton X-100 were used as a blank. The entrapment efficiency of the non-flexible liposomal formulation was determined in a similar way.

Permeation capability

The permeation capability was determined using a home-built device as described by Jain et al (2003) and Cevc et al (1998). In this study, the flux of vesicles through a large number of pores of known size (through a stack of different polycarbonate filters with pore diameters between 200 and 50 nm) driven by an external pressure of 2.5 bar was determined. The vesicle size was monitored by dynamic light scattering measurement pre- and post-filtration. The experiment was performed in triplicate and each sample was analysed twice.

Skin permeation study

In-vitro permeation of ethinylestradiol from the ultraflexible liposomal system was studied using a locally made Keshary–Chien-type diffusion cell. This study was performed to evaluate the permeation influx and percentage cumulative drug permeation. The effective permeation area of the diffusion cell was 1 cm^2 and the receptor cell volume was 10 mL. The receptor medium was 50 mL of 30% v/v PEG-200. The receptor compartment was surrounded by a water jacket to maintain the temperature at $37 \pm 1°C$, using a thermostatic hot temperature control on a magnetic stirrer. The receptor fluid was stirred by a magnetic bead on a magnetic stirrer (Expo India Ltd, Mumbai, India). The top of the donor compartment was left open for air circulation.

Female albino rat skin with an area of 1 cm^2 (4 to 5 weeks old, Sprague–Dawley strain) was mounted between the donor and receptor compartments. The optimized ultraflexible liposomal formulation with 500 μg drug (1 mL), non-flexible liposomal formulation and plain drug solution in 30% v/v PEG-200 with an equivalent amount of drug was applied to the epidermal surface of the rat skin. At each sampling interval of 1 h over 48 h, 0.1 mL samples were withdrawn from the sampling port then replaced with the same volume of fresh receptor fluid every time, maintaining the same temperature. The samples were analysed by HPLC assay. Every experiment was performed six times.

In-vivo characterization

Pharmacodynamic study

Female albino rats (Sprague–Dawley strain) weighing between 100 and 130 g were used for the in-vivo studies. The animals were fed on a commercial pellet diet (Hindustan Lever, Bangalore, India) and water ad libitum. The animals were acclimatized to laboratory hygienic conditions for 10 days prior to commencement of the experiment. Permission from the institutional Animal Ethics Committee was obtained for all animal experimentation (Registration Number 379/01/ab/CPCSEA, India). Forty-five rats were divided into five groups of nine each.

The first group was kept as control. The hair on the dorsal side of 36 rats (i.e. groups II, III, IV and V) was cut using scissors. Depilatory (Anne French, India) was applied to this area for 10 min and wiped with wet cotton to clean the skin (Vora et al 1998). All 45 rats were synchronized by injecting 1 mL of 0.01% w/v copper acetate, intraperitonially.

After 24 h, 100 μL of sonicated ultraflexible liposomal formulation (1 mg drug/5 mL of UL-DC), non flexible liposomal formulation and free drug solution in 30% v/v PEG-200 with an equivalent amount of drug were applied topically to the rats of groups II, III and IV, respectively, with a micropipette and left to dry. After the drug administration, the animals were kept individually in separate cages to prevent them from licking the skin or ingesting a part of the topical dose. Group V rats received a plain solution of 30% v/v PEG-200 orally containing the same amount of drug.

Three rats from each group were sacrificed by excessive chloroform inhalation after the first, fourth and ninth days of application.

Biological assay for progestational activity

The sacrificed rats were dissected. The ovaries and uterine horns were harvested and assayed for progestational activity. The organs were immediately fixed into fixative solution (3:1, absolute alcohol:chloroform) for 3 h and then transferred to pure absolute alcohol for ½ h and further treated with absolute alcohol and xylene for 1 h. After this, wax scrapings were added to this solution till saturation was reached and kept for 24 h for histopathological studies. After 24 h, paraffin blocks were made by embedding the tissue in hard paraffin, matured at 62°C. After attaching these blocks to carriers (wood cubes) the sections were cut using the microtome (Model 3880/A88, Beck, London) at 5 μm, and then mounted on slides and stained with Ehrlich's haematoxylin and eosin for nucleus and cytoplasm, respectively. Histological changes in the uterus and ovaries were examined (Galiher & Kozioff 1971).

Endometrial assay method

The morphological changes produced by the direct action on the uterine endometrium of the rats were recorded (Bhowmik & Mukherjea 1988; Thompson 1990). The uterine thickness in each group of rats was determined by calibrated stage and ocular micrometer (Elico Instruments, Hyderabad). Photomicrographs of the selected sections were taken using a microscope (Leica DMLB, Germany).

Assay involving interference with the formation of corpora lutea

The ovaries were examined microscopically for the presence of the ovulation point. The anti-ovulatory effect of ethinylestradiol-loaded ultraflexible liposomes, non-flexible liposomal formulation, free drug solution in 30% v/v PEG-200 with an equivalent amount of drug applied topically and plain drug solution given orally on the ovary and uterus was compared with the ovary and uterus of control rats.

Fluorescence microscopy

Fluorescence microscopy was performed to confirm the deposition of the selected vesicular drug carrier system into the dermis region. 6-carboxyflourescein (0.5 $\mu g\,mL^{-1}$) was used as a fluorescent marker and loaded into ultraflexible liposomes. Florescent-marker-loaded ultraflexible liposomes were applied topically to six rats. After 2 h the rats were sacrificed and their skin was removed, cut into pieces and washed with Ringer's solution then blotted between filter papers and wiped with tissue paper. The wiped tissue was fixed in the same manner as given above for ovaries and uterus. The sections were viewed under a fluorescence microscope (Leica DMLB, Germany).

Pharmacokinetic study

Forty rats were divided into four groups of 10 each. The hair on the dorsal side of animals in both groups was cut using scissors. Depilatory (Anne French, India) was applied in this area for 10 min and wiped with wet cotton to clean the skin. A 100-μL portion of sonicated ultraflexible liposomal formulation UL-DC (1 mg 5 mL^{-1}) was applied topically to the rats of group I whereas rats of group II received a plain solution of 30% v/v PEG-200 orally containing same amount of drug. To the skin of rats of groups III and IV were applied non-flexible liposomal formulation and plain drug solution in 30% v/v PEG-200 with an equivalent amount of drug, respectively. Blood samples withdrawn at different time intervals from the retro-orbital plexus (2, 4, 6, 8, 10, 12, 24 and up to 72 h) were mixed with heparin sodium (anticoagulant) as 100 IU mL^{-1} in saline (0.9% NaCl) to achieve a minimum workable volume of 1 mL and centrifuged at 2000 rpm for 10 min to separate serum and cell debris. The supernatant was collected and 1 mL of acetonitrile was added to precipitate the proteins that were settled by centrifugation at 2000 rpm for 15 min. The collected supernatant was diluted with acetonitrile and water such that the final ratio of acetonitrile:water was 50:50, similar to the ratio of mobile phase. The drug concentration in samples was estimated by HPLC (column ZORBAX SB-C18; mobile phase acetonitrile:water (50:50); flow rate 1 mL min^{-1}; room temperature; detector Jasco UV and Lamp D2; detection at 280 nm) (Sandor 2004). The area under the plasma concentration–time curve (AUC) and the area under the first moment of the concentration–time curve (AUMC) were calculated using the log trapezoidal rule. C_{max} (maximum drug concentration in serum) and T_{max} (maximum time corresponding to maximum serum drug concentration) were determined. The mean residence time (MRT) was calculated as AUMC/AUC.

Statistical analysis

Statistical analysis was performed with GraphPad InStat software (version 3.00, GraphPad Software, San Diego, CA) using one-way ANOVA followed by the Tukey–Kramer multiple comparison test. Differences with $P < 0.05$ were considered statistically significant.

Results and Discussion

From the 20 formulations prepared using different concentrations of PC and surfactants, the optimized formulation obtained was that prepared using PC:surfactant ratio of 85:15% w/w. This is in accord with the results already published from our laboratory (Jain et al 2003). As we increase the surfactant concentration from 5% w/w (surfactant:PC) to 15% w/w, the formulation becomes dense and colloidal. On increasing the surfactant concentration above 15% w/w, however, the formulation starts to become clear as 25% w/w of surfactant is reached. This may be due to the formation of micelles. At the optimized ratio the entrapment efficiency of the formulation was greatest. The reduced encapsulation efficiency of formulations with lower surfactant concentration (5–14% w/w) may be due to the formation of vesicles which are less flexible, leading to the fusion of vesicles due to high PC content. High surfactant concentrations (up to 25% w/w), i.e. beyond the optimum concentration ratio, result in the formation of micelles, which entrap a somewhat lower amount of drug. Only the results for the optimized formulation are given here.

Vesicle size and shape

The size of ultraflexible liposomes was expressed as Z average (Table 1). There were insignificant ($P = 0.0971$) differences in size among the ultraflexible liposomal formulations containing optimized concentrations of different surfactants. These results correlate well because a similar method of preparation was involved where the vesicles were homogenized by extrusion through a stack of 200- and 450-nm polycarbonate membranes. Ultraflexible liposomes appeared as multilamellar vesicles, with the lamellae of vesicles evenly spaced to the core as visualized by transmission electron microscope. The phase-contrast microscope revealed the vesicular structure of ultraflexible liposomes (Figure 1).

Table 1 Composition and characterization of different ultraflexible liposomal formulations

Formulation code	Particle size (nm)	Entrapment efficiency
UL-DC	165 ± 10.00	84.3 ± 1.64
UL-TW	160 ± 8.00	80.1 ± 1.01
UL-S60	174 ± 9.65	90.3 ± 1.20
UL-S65	180 ± 8.20	92.1 ± 1.32
LF	184 ± 11.6	72.1 ± 1.42

UL-DC, ultraflexible liposomes with sodium deoxycholate; UL-TW, ultraflexible liposomes with Tween-60; UL-S60, ultraflexible liposomes with Span 60; UL-S65, ultraflexible liposomes with Span 65; LF, non-flexible liposomes. All values are expressed as mean ± s.d. (n = 3).

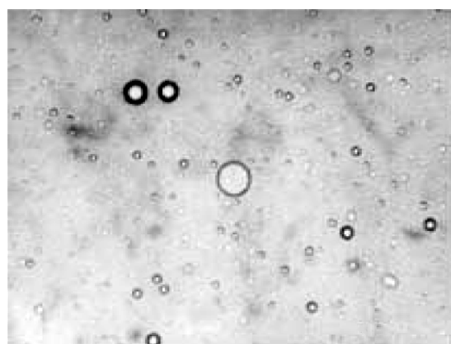

Figure 1 Phase contrast microscopy of ultraflexible liposomes (× 450).

Entrapment efficiency

Entrapment efficiency is the percentage fraction of total drug incorporated into the ultraflexible liposomes. The entrapment efficiency depends on the surfactant type. An insignificant difference ($P > 0.05$) between the entrapment efficiencies of Span 60 and Span 65 was observed (Table 1). This may be due to the lipophillic nature of these surfactants. Span 60 and Span 65 had significant differences ($P < 0.01$) in their entrapment efficiencies as compared to those of Tween 60 and sodium deoxycholate. Vesicles prepared with sodium deoxycholate entrapped a significant ($P < 0.05$) amount of drug as compared to Tween 60. This could be due to the steroidal structure of sodium deoxycholate, which is similar to ethinylestradiol, which displaces a part of the latter from the bilayered vesicles. This displacement involves competition between the drug and surfactant. These results clearly indicate that the amount of drug ($0.5\,mg\,mL^{-1}$ of ultraflexible liposomal formulation; 85 mg soyaphosphatidyl choline and 15 mg Span 65) added is sufficient to saturate the lipids. Only 3.9% of the initial drug added remains free ($19.5\,\mu g\,mL^{-1}$), which is above the saturation solubility of ethinylestradiol. When $1\,mg$ drug mL^{-1} of ultraflexible liposomal formulation was used, 52% of drug added initially remained free. Non-flexible liposomes entrapped a significantly ($P < 0.001$) lower amount of drug. This may be due to the addition of fluidity buffer (cholesterol), which resulted in closed packing with decreased fluidity and entrapment efficiency.

Permeation capability

The permeation capability is the most important parameter of ultraflexible liposomal formulation because it differentiates ultraflexible liposomes from other vesicular carrier systems, such as liposomes, which are not able to cross the intact stratum corneum (Gomper & Kroll 1995; Berge Van den et al 2001).

Ultraflexible liposomes with an average diameter of 200 nm can be transported through pores four times smaller. Such a high penetration capability is only seen, however, when the stress suffered by ultraflexible liposomes (flow driving pressure) is sufficiently high. An insignificant difference ($P > 0.05$) between the permeation capabilities of Span 60 and Span 65 was observed. This may be due to the lipophillic nature of these surfactants. Span 60 and Span 65 had significant differences ($P < 0.01$) in their permeation capabilities compared to Tween 60 and sodium deoxycholate for all sizes examined (Figure 2). Sodium deoxycholate and Tween 60 also showed insignificant ($P > 0.05$) differences in permeation capability. This property of the ultraflexible liposomal membrane is due to the presence of surface-active agent in proper ratios. The resulting permeability of the ultraflexible liposome membrane minimizes the risk of complete vesicle rupture in the skin. In comparison to ultraflexible liposomes, non-flexible liposomes are less deformable. They are less capable of passing through barriers with pores smaller than their own diameter (Cevc et al 1995). When liposomes are larger than the pore diameter, sieving sets in. Exclusion of non-flexible liposomes occurs when this mismatch exceeds a factor of approximately 1.5 (Figure 2).

The extremely high flexibility of ultraflexible liposomal membranes permits them to squeeze themselves even through pores much smaller than their own diameter. This is because of the high flexibility of the ultraflexible liposomes membrane and is achieved by judiciously combining at least two lipophilic/amphiphilic components (phospholipid plus surfactant) with sufficiently different packing characteristics into a single bilayer. The resulting high aggregate deformability permits ultraflexible liposomes to penetrate the skin spontaneously. This tendency is supported by the high ultraflexible liposomes surface hydrophilicity that enforces the search for surrounding high water activity (Cevc et al 1993). The resulting deformability of ultraflexible liposomes membrane minimizes the risk of complete vesicle rupture in the skin.

Skin permeation study

The in-vitro skin permeation study provides valuable information about the product behaviour in vivo. The drug

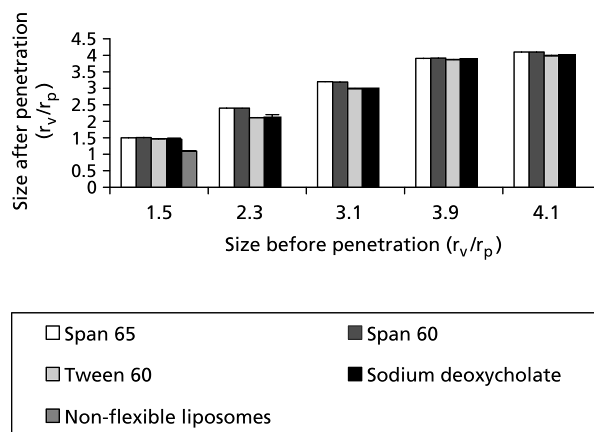

Figure 2 Penetration-induced changes in vesicle sizes. Ultraflexible liposomes composed of different surfactants with radius r_v penetrating through pores of diameter r_p.

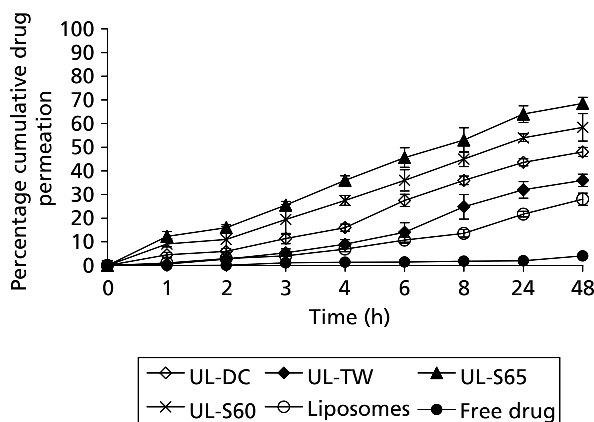

Figure 3 Percentage cumulative drug permeation of different optimized ultraflexible liposomal formulations through albino rat skin.

permeated dictates the amount of drug available for absorption. Figure 3 shows the effect of different optimized ultraflexible liposomal formulations on the percentage of cumulative drug permeation. The permeation was found to have the following order: Span 65 > Span 60 > sodium deoxycholate > Tween 60. The data for each permeation profile of ethinylestradiol through excised rat skin were linearly regressed and fitted into straight-line equations. The regression coefficient values for the formulations were very close to 1, showing a zero-order release profile. The percentage cumulative drug permeation in 48 h was significantly ($P < 0.001$) high ($68.5 \pm 4.1\%$) compared to non-flexible liposomes and the plain drug solution, indicating better skin permeation of the ultraflexible liposomal vesicular system. The transdermal flux from control (i.e. the solution of ethinylestradiol in 30% v/v PEG-200) was less ($0.42 \pm 0.02\,\mu g\,h^{-1}\,cm^{-2}$). Ultraflexible liposomes, when applied under suitable conditions, can transfer 6 to 7.5 μg of drug per hour, per cm^2 area across intact skin (Table 2). This value is substantially higher than that of the control and other previous liposomal approaches for transdermal delivery of other drugs (Mezei & Gulasekharam 1982; Kim et al 1997). Comparison of the individual ultraflexible liposomal formulations (Table 2) revealed no significant ($P > 0.05$) differences between them.

The high flux rate has been attributed to naturally occurring transdermal osmotic gradients (Bhowmik & Mukherjea 1988). This osmotic gradient developed because the skin penetration barrier controls water loss through the skin and maintains a water activity difference in the viable part of the epidermis (75% water content) and nearly completely dry stratum corneum, near to the skin surface (15% water content) (Sparr & Wennerstrom 2001; Cevc 2003). This gradient is very stable because ambient air is a perfect sink for the water molecule even when the transdermal water loss is unphysiologically high. All polar lipids attract some water due to the energetically favourable interaction between the hydrophilic lipid residues and their proximal water. Most lipid bilayers thus spontaneously resist an induced dehydration (Warner et al 1988; Cevc et al 1995). Most lipids have an excess higher free energy when packed within a bilayer membrane since in isolation they pack into high curvature assemblies with polar groups on the concave side (Rand 2002). Consequently, all lipid vesicles made from polar lipid vesicles move from a rather dry location to sites with a sufficiently high water concentration. When a lipid suspension (transfersomes) is placed on a skin surface that is partly dehydrated by water evaporation loss, the lipid vesicles feel this 'osmotic gradient' and try to escape complete drying by moving along it (Schalzlein & Cevc 1995). They can only achieve this if they are sufficiently deformable to pass through the narrow pores in the skin because ultraflexible liposomes composed of surfactant have more suitable rheologic and hydration properties, which are responsible for their greater deformability (Cevc & Blume 1992; Cevc et al 1995). However, further evidence is necessary to conclusively prove the mechanism and efficiency of skin penetration by ultraflexible liposomes. PC has permeation enhancer activity. Cholesterol added to the non-flexible liposomal composition tends to stabilize the structure thereby generating more rigid liposomes. Their delivery mechanism is reported to be associated with accumulation of the liposomes and associated drug in the

Table 2 Steady-state transdermal flux for the transport of ethinylestradiol across albino rat skin

Formulation code	Transdermal ($\mu g\,cm^{-2}\,h^{-1}$)	Relative maximum flux	Slope	Regression coefficient	Correlation coefficient
UL-DC	6.38 ± 1.23***	15.15 ± 2.01	0.9363 ± 0.09	0.9668 ± 0.11	0.8245 ± 0.51
UL-TW	6.28 ± 0.05***	14.91 ± 0.16	0.7596 ± 0.12	0.9245 ± 0.21	0.8664 ± 0.31
UL-S65	7.52 ± 0.21 ***	17.85 ± 0.62	1.1807 ± 0.06	0.9938 ± 0.25	0.7795 ± 0.16
UL-S60	7.24 ± 0.54***	17.20 ± 0.68	1.0554 ± 0.17	0.9911 ± 0.09	0.8010 ± 0.09
LF	3.57 ± 0.21***	8.50 ± 0.31	0.5721 ± 0.16	0.9076 ± 0.21	0.9310 ± 0.01
Control	0.42 ± 0.02	–	0.0752 ± 0.01	0.8487 ± 0.06	0.9128 ± 0.02

All values are expressed as means ± s.d. (n = 14). ***$P < 0.001$ compared with control (solution of ethinylestradiol in 30% v/v PEG-200). Relative maximum flux means transdermal flux of the formulations with respect to control. Slope can be used to determine the rate of drug permeation. Permeation profile or the nature of relationship between two variables can be determined from regression coefficient. Correlation coefficient is a measure of the quantitative linear relationship between two variables (percentage cumulative drug release and time).

stratum corneum and upper skin layers, with minimal drug penetrating to the deeper tissues and systemic circulation (Mezei & Gulasekharam 1982). However, in our study the non-flexible liposomes contain PC in a proportion almost similar to that of ultraflexible liposomes. Despite this, the permeation enhancement was less in the former than in the latter. This means that the surfactants present in ultraflexible liposomes rather than PC are responsible for permeation enhancement. The hydration gradient could be the main driving force for the transport of highly deformable liposomes as the 17-fold increase in estradiol flux reduced to six- to nine-fold increase under occlusion (El Maghraby et al 2001). All aggregates capable of crossing semipermeable barriers with normally confining pores have a highly flexible membrane. This also holds true for skin crossing by ultraflexible liposomes. This could be due to the capability of such aggregates to deform and fit into pores in the skin, widened by penetrants into sufficiently broad hydrophilic channels (Cevc & Gebauer 2003).

In-vivo study

Pharmacodynamic study

Biological assay for progestational activity. Ultraflexible liposomes prepared with sodium deoxycholate were used for the in-vivo study because of their biocompatibility (Patel & Chen 2001).

In the section of the ovary all conditions of the developing ovum were seen in the slide of the control animals (Figure 4A, B). The stroma, developing oocytes, larger blood vessels and compressed cells of the stroma were seen, in both stages graffian follicles were present and the ovum was surrounded by granular cells. On application of ethinylestradiol-loaded ultraflexible liposomes there was no change after the first day except that the theca externa was not uniform and was wavy in some places. Secondly, there was no new formation of ova and all these layers were also not seen adequately. Thirdly, the germinal epithelium was not active, and a reduced number of graffian follicles and space were formed around the developing oocytes, which may be due to the lysis of the cells. All these effects on the ovary may be due to the effect of ethinylestradiol on follicle-stimulating hormone (FSH), which changes ovulation. The axis of ethinylestradiol through ultraflexible liposomes to the organs studied seemed to be very quick. The disintegration of the graffian follicle starts right from the first day. Follicular cell lysis and depletion of the zona granulosa and ova are evident, as shown in the photomicrographs (Figure 4C). After the ninth day no new follicles could be found developing and the ovary was filled with remnants of theca externa, theca interna and stromal tissue (Figure 4D). This is because of the inhibition of luteinizing hormone (Hebborn 1971). In rats treated with the non-flexible liposomal formulation, although the depletion of follicular cells and ova is visible it seems that the lower amount of drug approaches the ovary and uterus, and hence a delayed process of disintegration of the ova and

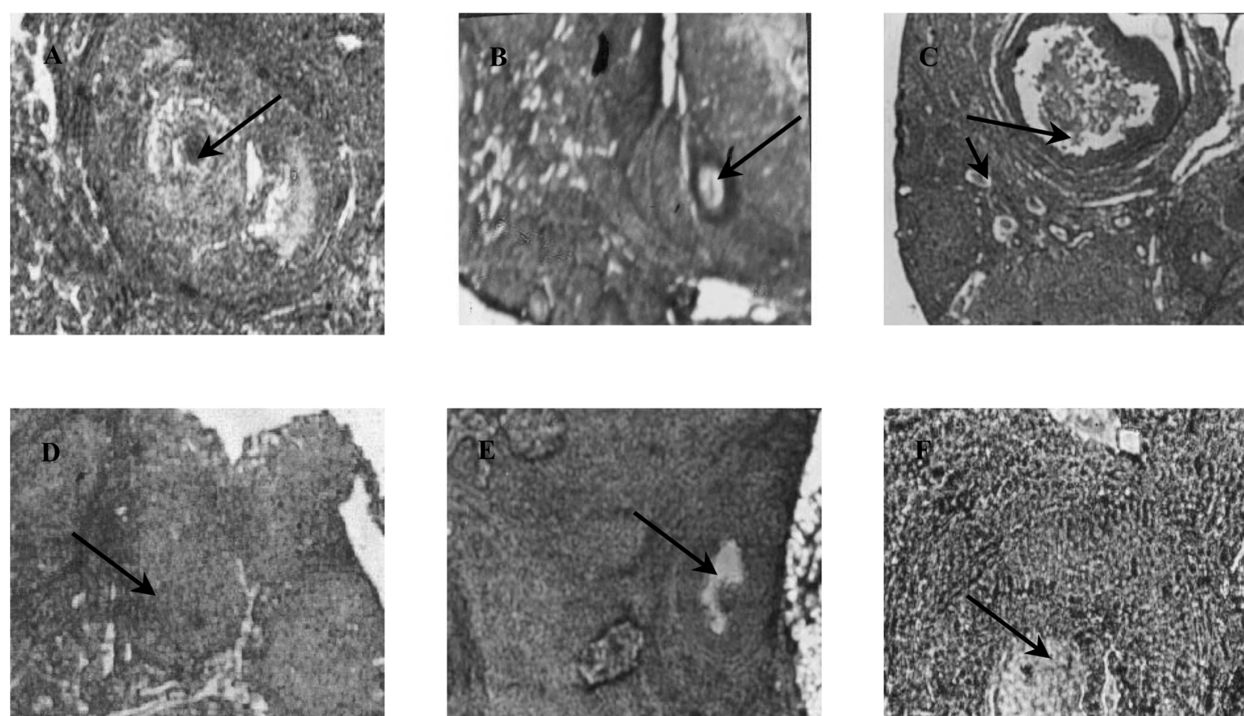

Figure 4 Photomicrographs showing the ovaries of rats after different treatments and different days (×450). (A) Control rats after one day, (B) control rats after nine days, (C) ultraflexible liposomal formulation treated rats after one day, (D) ultraflexible liposomal formulation treated rats after nine days, (E) plain drug solution administered orally to rats after one day, and (F) plain drug solution administered orally to rats after nine days. Arrow in the figures indicate ovary and stage of graffian follicle.

inhibition of the development of follicles is observed in comparison to ultraflexible liposomes. Depletion of ova is yet to complete after the ninth day with liposomal delivery of drug (photomicrograph not shown). The ovary of the rats with plain drug solution applied to the skin did not show any prominent effect of steroid. Significant ($P < 0.001$) anti-ovulatory activity of ultraflexible liposomes was observed as compared to non-flexible liposomes and plain drug solution given orally and topically (Table 3). Plain drug solution applied on the skin exhibited significantly low ($P < 0.05$) anti-ovulatory activity as compared to non-flexible liposomes. Non-flexible liposomes showed significantly ($P < 0.05$) low anti-ovulatory activity as compared to plain drug solution given orally. From these results it may be concluded that the liposomal formulation may have poor penetration potential through the skin compared to ultraflexible liposomes.

The ovaries of rats treated with plain drug solution given orally did not show much effect of steroid on their tissues. Development of follicles in the ovary continued after the ninth day. Whatever amount of drug reaches the ovary exhibits its effect on the vacuolization and regression of follicular cells at a low degree (Figure 4E, F).

The control rat uterus is shown in Figure 5A. Ultraflexible liposomes significantly ($P < 0.001$) increase the endometrial weight and mucosal thickness (compared with plain drug solution applied to the skin), as shown in Table 3. In the ultraflexible liposomal formulation treated uterus, the endometrium seems to be slightly thickened and the cells started sclerosing. The sclerosis of the endometrium gradually increases after the ninth day. Uterine foldings gradually subside. The connective tissue, i.e. the mesometrium, showed a reduced supply of blood and a gradual regression from the first day to the ninth day in connective tissue (Figure 5B, C). In the case of non-flexible liposomes, the thickness of the endometrium from the first day to the ninth day gradually increases but in comparison to ultraflexible liposomes, the sclerosis of the endometrium is very low. In the uterus of rats treated with plain drug solution applied to the skin negligible sclerosis was observed (photomicrograph not shown).

The endometrium and mesometrium were almost normal in plain drug solution given orally. A little sclerosis was observed with this delivery system after the ninth day (Figure 5D, E).

Photomicrographs showing the pharmacodynamic effect after the fourth day of ultraflexible liposomal formulation and plain drug solution given orally are not shown but the anti-ovulatory activity and endometrial weight are recorded in Table 3.

Fluorescence microscopy

The better penetration potential of the developed ultraflexible vesicular carrier than that of non-flexible liposomes was evaluated by fluorescence microscopy. The object of the study was to confirm the deposition of 6-carboxyfluorescein-loaded ultraflexible liposomal formulation in the dermal layer. This is due to the fact that ultraflexible liposomes face the nearly infinite sink below the stratum corneum, which results in extensive dye dilution and elimination through the lymphatic drainage system. From the results of this study it may be concluded that ultraflexible liposomal formulation penetrates the stratum corneum and reaches the subcutaneous tissue, from where the drug is released slowly in a sustained manner (photomicrograph not shown).

Pharmacokinetic study

The incorporation of ethinylestradiol in ultraflexible liposomal vesicles strongly altered its distribution. Serum ethinylestradiol concentrations following a single application of the ultraflexible liposomal formulation, non-flexible liposomes, plain drug solution applied topically and after administration of oral formulation are shown in Figure 6. To compare the data presented in Figure 6 repeated measures of ANOVA have been used. The individual effects of time and the formulations were determined using the Newman–Keuls multiple comparison test (GraphPad Prism version 4.03, USA). A significant ($P < 0.05$) difference in the serum drug concentration obtained from ultraflexible liposomes as compared to non-flexible liposomes

Table 3 Anti-ovulatory activity, endometrial assay and inhibition of luteinization by ethinylestradiol

Formulation	Number of rats with ovulation points (after days)			Mean no. of corpora lutea	Percentage inhibition	Endometrial weight (mg) after days			Mucosal thickness (μm) after nine days
	1	4	9			1	4	9	
Control	3	3	3	24.5 ± 2.0	–	2.52 ± 0.2	5.68 ± 0.4	6.38 ± 0.9	2.8 ± 0.2
Ultraflexible liposome	0	0	0	2.5 ± 0.5***	89.79 ± 6.8	22.5 ± 1.2***	47.8 ± 1.9***	57.4 ± 2.9***	49.2 ± 2.1***
Non-flexible liposome	3	2	2	20.2 ± 0.2***	17.55 ± 1.5	10.1 ± 0.4***	16.2 ± 1.3***	19.6 ± 0.4***	8.9 ± 0.2***
Plain drug solution (topical)	3	3	3	23.1 ± 2.3	5.71 ± 1.1	3.1 ± 1.3	6.0 ± 0.3	7.11 ± 0.3	2.9 ± 0.1
Plain drug solution (oral)	2	3	3	17.0 ± 1.0**	30.61 ± 3.1	14.1 ± 0.8***	20.7 ± 1.6***	24.5 ± 1.4***	11.8 ± 0.9***

All values are expressed as ± s.d. ***$P < 0.001$ (significant) compared with plain drug solution applied to the skin.

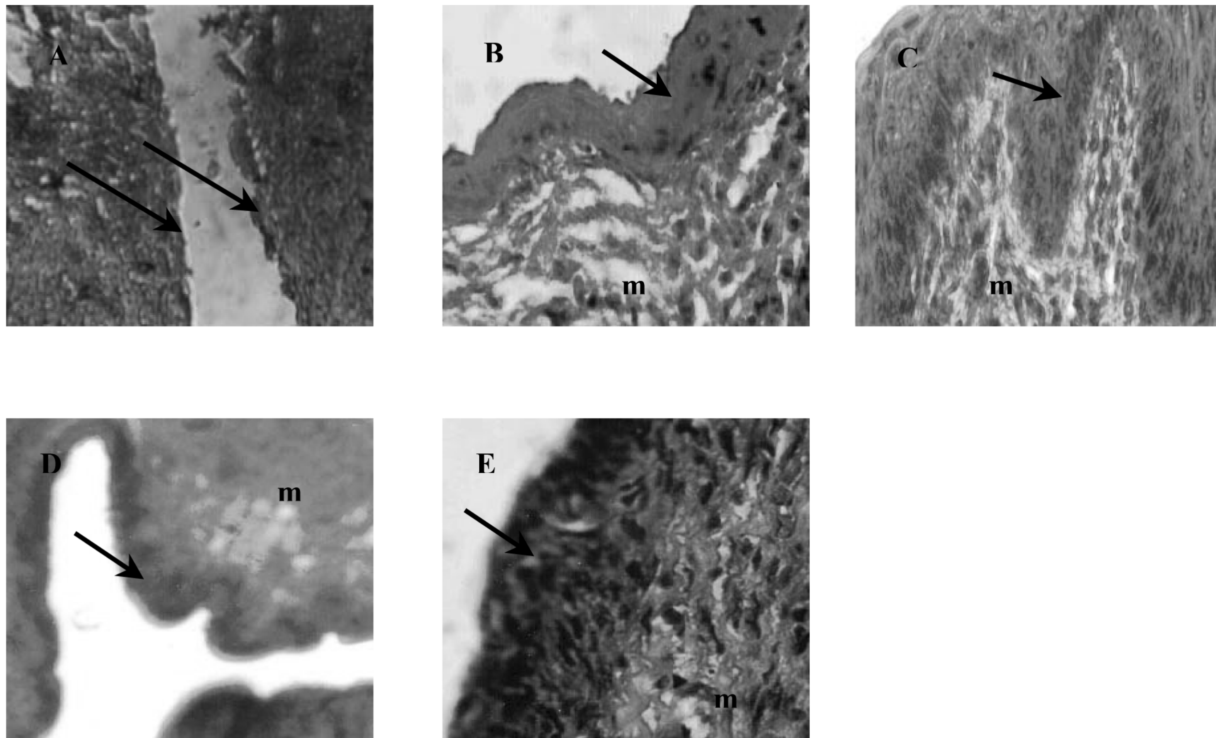

Figure 5 Photomicrographs showing histology of uterine mucosa and endometrium after different treatments and different days (×450). (A) Control rats, (B) ultraflexible liposomal formulation treated rats after one day, (C) ultraflexible liposomal formulation treated rats after nine days, (D) plain drug solution administered orally to rats after one day and (E) plain drug solution administered orally to rats after nine days. Arrow in figures shows endometrium; m, mesometrium (inner to endometrium).

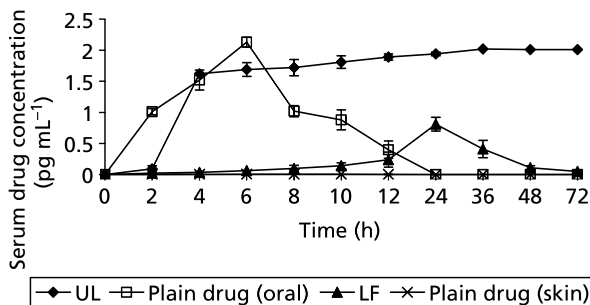

Figure 6 Serum ethinylestradiol concentrations following application of the ultraflexible liposomal formulation, non-flexible liposomal formulation, plain drug solution and administration of oral formulation (n = 10).

and plain drug solution given orally and applied topically was observed whereas non-flexible liposomes showed an insignificant ($P > 0.05$) difference compared to plain drug solution given orally and applied to the skin. An insignificant ($P > 0.05$) difference between the serum drug concentrations obtained from different formulations at different times was observed. The maximum concentrations achieved with ultraflexible liposomes applied topically, non-flexible liposomes applied to the skin, and plain drug solution given orally and after application to the skin were 2.02 ± 0.36, 0.81 ± 0.01, 2.13 ± 0.23 and $0.007 \pm 0.002\,pg\,mL^{-1}$ at times 36 ± 0.20, 24 ± 0.25, 10 ± 2.11 and $8.02 \pm 0.05\,h$, respectively. C_{max} obtained with ultraflexible liposomes was less while T_{max} was more compared to plain drug solution given orally. In the case of non-flexible liposomes the C_{max} obtained was 2.5 and 2.6 times less than that of ultraflexible liposomes and plain drug solution given orally, respectively. In the case of plain drug solution applied to the skin, C_{max} was less than that obtained from other formulations. The poor permeation capability of plain drug solution may be the reason for this. AUC (0–72 h) obtained after transdermal application of ultraflexible liposome ($134.91 \pm 2.11\,pg\,h\,mL^{-1}$) was significantly high ($P < 0.001$) when compared with plain drug solution given orally ($15.94 \pm 0.32\,pg\,h\,mL^{-1}$), non-flexible liposomal formulation ($19.61 \pm 0.86\,pg\,h\,mL^{-1}$) and plain drug solution applied to the skin ($0.16 \pm 0.02\,pg\,h\,mL^{-1}$). The MRT of the drug in serum determined after transdermal application of ultraflexible liposomes ($38.25 \pm 1.21\,h$) was significantly high ($P < 0.001$) compared to that of plain drug solution given orally ($6.89 \pm 0.12\,h$), non-flexible liposomal formulation ($29.07 \pm 1.38\,h$) and plain drug applied to the skin ($3.01 \pm 0.06\,h$). This suggests that drug levels were maintained in blood for much longer time after transdermal application, indicating a longer plasma half-life for the ultraflexible liposomal formulation.

Conclusion

Ultraflexible liposomes can be used to bring drugs across biological permeability barriers, such as skin. It can be concluded from the results obtained that the ultraflexible liposomal transdermal drug delivery system for ethinylestradiol has greater bioavailability compared to plain drug solution given orally. In addition to this, it provides effective contraception by inhibiting ovulation (reduction in the mean number of corpora lutea), by inhibiting the release of luteinizing hormone and by increasing the uterine mucosal layer and endometrial proliferation.

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