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Iontophoretic estradiol skin delivery and tritium exchange in ultradeformable liposomes

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Abstract

This work evaluated the in vitro transdermal iontophoretic delivery of tritiated estradiol from ultradeformable liposomes compared with saturated aqueous solution (control). Effects of current density and application time on tritium exchange with water were also determined. Penetration studies used three Protocols. Protocol I involved occluded passive steady state estradiol penetration from ultradeformable liposomes and control. The effect of current densities on drug penetration rates was also assessed (Protocol II). In Protocol III, three consecutive stages of drug penetration (first passive, iontophoresis and second passive) through the same human epidermal membranes were monitored. Such an experimental design investigated the possible effect of high current density (0.8 mA/cm^2) on skin integrity. The tritium exchange study showed that extent of exchange correlated well with current density and time of application, with some shielding of estradiol by the liposomal structure. Liposomes enhanced estradiol passive penetration after occlusion. Protocol II showed that estradiol flux increased linearly with current density, although being delivered against electroosmotic flow. In Protocol III, reduction in flux of the second passive stage to near that of the first reflected a reversibility of the structural changes induced in skin by current.

Keywords: Skin delivery; Estradiol; Liposomes-ultradeformable; Transfersomes; Iontophoresis; Tritium exchange

1. Introduction

The distinctive architecture of the stratum corneum with its unique nature of an interstitial lipoidal environment plays the major role in regulating the barrier function of the skin. Several strategies have been developed to overcome such skin resistance, because of the benefits of using transdermal drug delivery over other routes of administration e.g. avoidance of hepatic first pass metabolism, fewer side effects and improved patient compliance (Barry, 1983). One such approach, iontophoretic transdermal delivery, uses electric current mainly to enhance the movement of ionized drugs that are driven into intact skin by electrostatic repulsion (Banga, 1998). The enhancement results from mechanisms such as: the ion-electric current interaction (electrorepulsion); electroosmotic flow (Pikal, 1990; Pikal and Shah,

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1990) and current-induced increase in skin permeability (Jadoul et al., 1999).

Many reports on enhancing transdermal drug delivery focus on the use of liposomes as they can aid the transport of hydrophilic and lipophilic compounds, proteins and other macromolecules through skin (Mezei and Gulasekharam, 1982; Berrnard et al., 1995; Codrech et al., 1996; Fresta and Puglisi, 1996; El-Maghraby et al., 2000). Ultradeformable (ultraflexible) vesicles, also known as Transfersomes™, are a modified type of liposome with special tailored properties, due to the incorporation of so-called edge activators into the vesicular membrane. Surfactants such as sodium cholate, sodium deoxycholate, Span 80 and Tween 80 have been used as edge activators (Cevc and Blume, 1992; Cevc et al., 1995, 1996, 1997, 1998; El-Maghraby et al., 1999, 2000). It was suggested that Transfersomes™ could respond to external stresses by rapid shape transformations requiring low energy. This high deformability allows them to deliver drug across barriers, including skin, when applied non-occlusively, as it is proposed that the main driving force for Transfersome™ penetration is the transdermal hydration and osmotic gradients (Cevc and Blume, 1992).

There are many reports on the separate use of iontophoresis and liposomes, however, few studies have been published concerning the iontophoretic delivery of drugs formulated into liposomes (Kulkarni et al., 1996; Vutla et al., 1996; Badkar et al., 1999; Fang et al., 1999). The combined use of liposomes and iontophoresis reduced degradation and enhanced transdermal delivery of enkephalin (Vutla et al., 1996), and encapsulation of neutral colchicine into positively charged liposomes increased the iontophoretic flux of the drug by two to three-fold (Kulkarni et al., 1996).

In the present work, we investigated the impact of the combined use of ultradeformable liposomes and iontophoresis on the penetration of a model lipophilic drug (estradiol). As radioactive material was used, the relationship between current densities and time of application on the degree of tritium exchange of estradiol in ultradeformable liposomes and saturated aqueous solution was evaluated. The correlation between current density and drug penetration rates was also assessed. Additionally, sequential passive and iontophoretic delivery of estradiol through the same pieces of skin was monitored to evaluate the possible effect of the maximum current used (0.8 mA/cm^2) on skin integrity.

2. Materials and methods

2.1. Materials

Estradiol [2,4,6,7,^{3}H(N)] was obtained from DuPont Chemical Company as an ethanolic solution of 1.0 mCi/ml, phosphatidylcholine (PC) from soybeans (purity 99%), 17β-estradiol (98%) and sodium cholate were purchased from Sigma Chemical Company, St. Louis, MO, USA. Silver wire (99.99%) and sodium azide were obtained from Aldrich Chemical Company. Scintillation cocktail, OptiPhase Hisafe 3 was obtained from Fisher Chemical, UK. Chemicals were used without further purification. Water was deionised, double distilled.

2.2. Preparation of liposomes

Ultradeformable liposomes as previously optimised by El-Maghraby et al. (2000) were used. The vesicles consisted of PC mixed with sodium cholate as an edge activator. To form a homogenous mixture, liposome components (PC-sodium cholate; 86:14(w/w)) were dissolved in ethanol. Ethanolic radiolabelled estradiol solution sufficient to produce 1 mg/ml (25 μCi/ml) in the final preparation was added. The organic solvent was removed under a stream of nitrogen. Final traces were evaporated under vacuum overnight at room temperature. The deposited lipid film was subsequently disrupted by hydration with 7% (v/v) ethanol in deionised water by vortexing for 15 min. This led to the spontaneous formation of 50 mg/ml multilamellar liposomes of heterogeneous size. The hydrated vesicles were swollen for 2 h at room temperature and then bath sonicated for 30 min using a B12 FTZ bath sonicator (New, 1990). The vesicles were then homogenised by ten times manual extrusion through a sandwich of 200 and

100 nm polycarbonate membranes to produce unilamellar vesicles. Final lipid concentration was 5% w/v.

2.3. Entrapment efficiency

This was determined using a mini-column centrifugation method (New, 1990). To prepare the mini-column, Whatman GF/B filter pads were inserted in the bottom of the barrel of a 2.5 cm^3 syringe, which was then filled with Sephadex G50 gel. Excess water was removed from the gel by centrifugation at 3000 rpm for 3 min using a WIFUG Lab centrifuge (WIFUG, Bradford, UK). Liposomal suspension (200 μl) was added dropwise to the centre of the column, followed by centrifugation as before. Then 250 μl of water was added and centrifugation was repeated. The non-entrapped drug remained bound to the gel, while vesicles traversed the gel and were collected from the first and second stage of centrifugation (New, 1990). When saturated solution was used instead of liposomes, all the drug remained bound to the gel confirming that there will be no free drug present after recovery of the vesicles (El-Maghraby et al., 2000). The amount of drug entrapped in the vesicles was determined by liquid scintillation counting after correction for dilution.

2.4. Determination of vesicle size

Vesicle diameters were determined using photon correlation spectroscopy (PCS) employing a Zetasizer (Malvern Instruments Ltd., Malvern, UK). To avoid interference from particulate matter, samples were diluted with deionised double distilled water passed through a 200 nm filter.

2.5. Determination of zeta potential

The charges on the vesicles' surfaces were determined using a Zetamaster S particle electrophoresis and particle size analyser (Malvern Instrument Ltd.). To avoid interference, excess drug and surfactant were removed by gel filtration before measurement (New, 1990).

2.6. Preparation of epidermal membrane

Human epidermal cadaver membranes were used. Mid-line abdominal samples obtained post-mortem were flattened and stored in vacuum-sealed polythene bags at -20 °C (Harrison et al., 1984). Membranes from seven donors (five female) aged 73 ± 9.0 (S.D.) years were prepared by a heat separation technique (Kligman and Christophers, 1963). Fat and connective tissues were removed, the skin was soaked in a water bath at 60 °C for 45 s after which the epidermis was removed by gentle teasing off the underlying dermis. To hydrate the membrane, the epidermis was floated with the stratum corneum side uppermost on a solution of 0.002% w/v sodium azide in distilled water and left for 24 h.

2.7. Set up of iontophoretic cells

In vitro permeation studies employed glass diffusion cells specially adapted for iontophoretic experiments. Hydrated epidermal membranes cut into discs of 0.8 cm diameter were sandwiched between the donor and receptor chambers of each cell with stratum corneum side up, leaving a 0.126 cm^2 diffusion area of membrane exposed. Receptor chambers were filled with deionised water and were kept at 32 ± 1.0 °C, while the donor chambers were filled with deionised water at room temperature. After 24 h, donor compartments were dried, then 150 μl of donor (5% liposomal suspension or saturated aqueous solution) was added to the donor chambers which were occluded. The receptors were stirred with Teflon coated magnetic bars to minimise diffusion boundary layers at the skin surface. Silver/silver chloride reversible electrodes (to prevent pH shifts) were used. As ultradeformable liposomes are negatively charged due to the cholate anions (as shown by zeta potential results), the cathode was mounted in the donor compartment while the return electrode (anode) was in the receptor. A constant current was supplied from a power source. At pre-set intervals, 1 ml receptor samples were withdrawn and replaced by fresh deionised double distilled water.

2.8. Penetration studies

As tritium-labelled estradiol was used in penetration studies, it was necessary to ensure that any results obtained were true and not falsely elevated due to tritium exchange with water. Such a phenomenon becomes more important when using iontophoresis (Prausnitz et al., 1995). Thus, we started our work by investigating the possibility of tritium exchange, while the in vitro penetration studies employed three Protocols (I, II and III).

2.8.1. Investigation of tritium exchange

This experiment investigated the effect of current density and time of application on the extent of tritium exchange of ^{3}H-labelled estradiol prepared in saturated aqueous solution and ultradeformable liposomes. Current densities of 0.0, 0.2, 0.5, 0.8 mA/cm^2 were applied separately for 6 h. Receptor samples from the iontophoretic cells were withdrawn at time intervals and divided into halves. One half was dried under vacuum (so that any tritium exchanged with water would evaporate) and the residue reconstituted with fresh receptor solution (deionised water). Both halves were mixed with 5 ml of scintillation cocktail, counted and penetration data compared (El-Maghraby et al., 2000). Concomitantly, the effect of current densities on drug penetration rates was also assessed (see Protocol II).

2.8.2. Protocol I

Passive steady state penetrations of estradiol from ultradeformable liposomes and control (saturated aqueous solution) were studied under occlusion. Samples were collected at time intervals for 12 h.

2.8.3. Protocol II

The effect of current densities (0.0, 0.2, 0.5 and 0.8 mA/cm^2) on iontophoretic drug penetration was evaluated. Current was separately applied for 6 h during which drug penetration was monitored.

2.8.4. Protocol III

This protocol involved three, 4-h stages; an initial period of passive penetration (first passive), then cathodic iontophoresis using 0.8 mA/cm^2, and finally another period of passive penetration (second passive). This design investigated not only passive and iontophoretic drug penetration but also assessed the possible effect of the current density on skin barrier properties (second passive stage compared with first passive).

Drug concentration was determined by radioactive counting using a Tri-Crab® Packard counter after mixing with 5 ml of scintillation cocktail, Optiphase 'Hisafe' 3. Cumulative amounts versus time plots were used to calculate the rate at the mid-time points to produce flux plots. Each experiment was conducted at least in triplicate, and all data plotted were mean $\pm$ standard error of the mean, allowing for tritium exchange. Data were compared for significance by Student's t-test.

3. Results and discussion

3.1. Entrapment efficiency

Entrapment efficiency is the percentage of the initial drug incorporated into liposomes. As estradiol is a lipophilic drug it was expected to have a high percentage of entrapment. The entrapment efficiency of estradiol in the ultradeformable vesicles was found to be $94 \pm 1.2\%$ (about 99.6% calculated with respect to dissolved drug), in agreement with El-Maghraby et al., 1999. This indicates that the amount of drug added was sufficient to saturate the lipid, and the remaining non-entrapped drug was enough to maintain equal maximum thermodynamic activity for both liposomes and saturated drug solution.

3.2. Particle size analysis

The unilamellar vesicles had a Z Average mean diameter of 126 ± 15 nm ($n = 3$). The particle size distribution was monomodal with a polydispersity index (which is the width of the particle size distribution curve) of less than 0.1, indicating a narrow size distribution of the liposomal suspension and consequently a homogenous distribution.

3.3. Zeta potential

The ultradeformable liposomes were negatively charged due to the cholate anions. Zeta potential was determined to be -29 ± 1.4 mV ($n = 3$).

3.4. Skin delivery of estradiol from ultradeformable liposomes versus saturated aqueous solution

3.4.1. Results of tritium exchange

As proposed earlier, it is necessary to test for tritium exchange whenever dealing with ^{3}H-labelled drugs (especially when using iontophoresis), to ensure that the results obtained are true and not falsely elevated. The phenomenon is simply the movement of the tritium atom from the ^{3}H-labelled drug and its replacement with a hydrogen atom from the bulk solution, leaving behind a tritium labelled water molecule. As a result, some of the bulk water now becomes tritiated. Thus, when using radioactivity counting to determine drug concentration in the receptor, the counts may be falsely elevated due to the tritiated water molecules, not the drug. As such errors may be facilitated by external stresses such as electrical current, it was important to investigate this during iontophoresis. Accordingly, we studied the possible effect of iontophoretic parameters (current density and application time) on the degree of tritium exchange of estradiol prepared in saturated solution and also encapsulated in ultradeformable vesicles. Current of 0.0, 0.2, 0.5 and 0.8 mA/cm^2 densities were applied for 6 h. Plots of cumulative amount of the analyte versus time were used to calculate the percentage loss (error), due to tritium exchange, in the cumulative amount of drug penetrated, as shown in Fig. 1A and B. Fig. 1C represents the protective effect of liposomes to the drug relative to control.

The amount penetrated for both the evaporated and non-evaporated halves at zero current was essentially identical for both saturated solution and ultradeformable liposomes for all time intervals, thus excluding significant tritium exchange during passive drug penetration (data

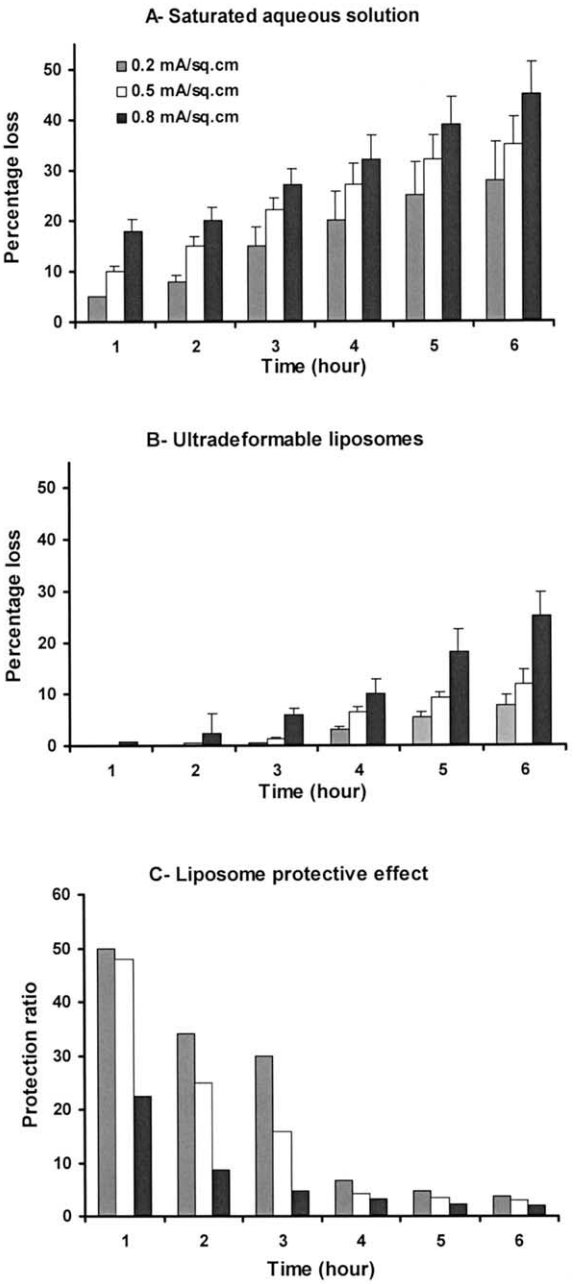

Fig. 1. Percentage loss (or error) in the calculated cumulative amount of estradiol penetrated from saturated aqueous solution (A) and ultradeformable liposomes (B) through human epidermal membrane as a result of tritium exchange with water. (C) Shows the protective effect of liposomes compared with saturated solution as illustrated by the ratios of B to A (the protection ratio).

not shown) in agreement with El-Maghraby et al., 2000. Upon applying iontophoresis, tritium exchanged with water, the degree of which correlated well with the current density and time of application. The behaviour of estradiol to the effect varied with the formulation.

For saturated aqueous solution, the exchange could be detected after 1 h of current application (Fig. 1A). The percentage loss was up to 17% for 0.8 mA/cm^2. At the end of the experiment (6 h) the loss increased to 45%.

With ultradeformable liposomes, there was a trend of reduced drug concentration for the evaporated halves relative to the non-evaporated samples in the first 2 h for the three current densities. However, such reductions were statistically non-significant ($P > 0.05$). Upon further application of current (3–5 h), tritium exchanged with water, the extent being lowest with 0.2 mA/cm^2 and highest with 0.8 mA/cm^2. After 6 h of current application, percentage losses of approximately 8, 15 and 24 were obtained for 0.2, 0.5 and 0.8 mA/cm^2, respectively (Fig. 1B).

Comparing ultradeformable liposomes with control, it is clear that at every time point and current density, tritium exchange was significantly lower in liposomes than in aqueous solution. This may be due to the fact that in simple aqueous solution, the drug molecules were more susceptible to the current and consequently more ready to exchange their tritium atoms with water. To illustrate further the protective effect of liposome structure, the percentage losses in cumulative amount of estradiol from saturated solution (Fig. 1A) were divided by those from ultradeformable liposomes (Fig. 1B), to produce the liposome protective effect histogram (Fig. 1C). The overall results reflect the possible shielding and protective effect of the liposomal structure for the drug, especially within the first few hours. Continuing current application reduces such protection. Based on these results, all iontophoretic fluxes for Protocols II and III were calculated by taking into consideration the effect of tritium exchange.

3.4.2. Penetration study using Protocol I

Cevc et al., (1993) recommended an open application protocol to obtain the maximum effect from Transfersomes™, stating that the main driving force for such vesicle penetration is the transdermal hydration gradient that is usually eliminated by occlusion. However, iontophoresis needs a sufficient volume of liposome suspension in which the electrode can be inserted. Consequently, to evaluate the impact of iontophoresis in enhancing drug penetration, the first passive estradiol steady state penetration from ultradeformable liposomes and control was studied under occlusion (hydration) for 12 h, to give enough time for the drug to achieve steady state penetration. The penetration profiles (cumulative amount versus time plots) produced typical steady state penetration profiles as shown in Fig. 2.

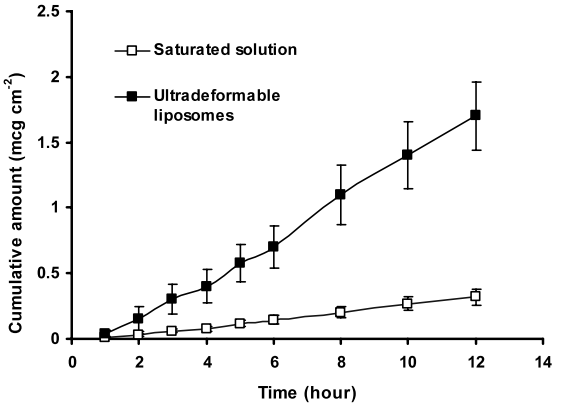

Fig. 2. Steady state passive penetration of estradiol from saturated solution and ultradeformable liposomes, through human epidermal membrane.

Penetration rates (fluxes) were calculated from the slope of the regression lines, fitted to the linear portion of the permeability profiles. The enhancement ratio of estradiol penetration from ultradeformable liposomes relative to control was calculated by dividing flux of the former by that of the latter. Occluded application of ultradeformable liposomes increased estradiol flux in comparision to control by about seven-fold (transepidermal fluxes were 181 ± 34.0 and 24 ± 8.0 ng/cm^2 per h for ultradeformable liposomes and control, respectively). Although the enhance-

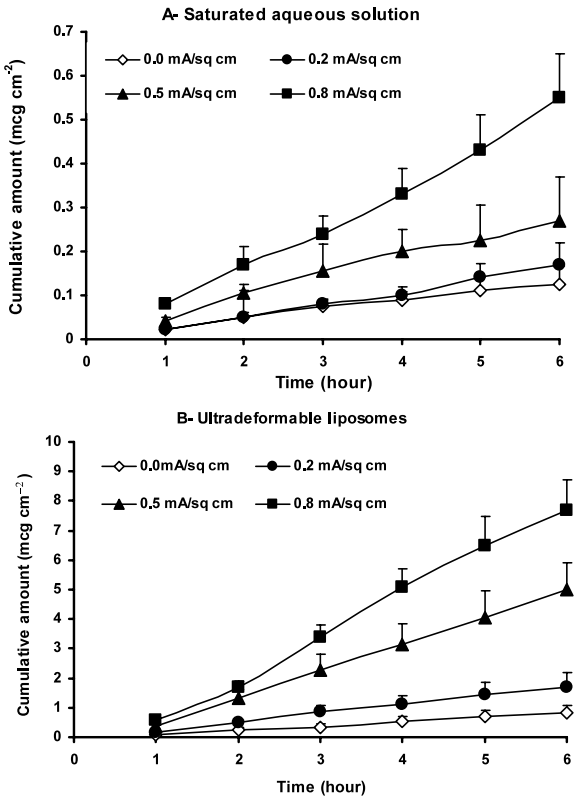

Fig. 3. Iontophoretic delivery of estradiol through human epidermal membrane from saturated aqueous solution and ultradeformable liposomes using different current densities.

ment in flux is not as high as that after open application of a finite dose (17-fold increase in flux- El-Maghraby et al., 1999), ultradeformable liposomes improved drug penetration compared with saturated solution, indicating that occlusion did not completely abolish the vesicular effect.

3.4.3. Penetration study using Protocol II

Iontophoretic studies involved the application of constant current densities of 0.0, 0.2, 0.5 and 0.8 mA/cm^2 for ultradeformable liposomes and saturated aqueous solution for 6 h. Plots of cumulative amount versus time for estradiol penetrated are in Fig. 3. Fluxes were calculated from the slopes of the regression lines fitted to the linear parts of the plots and are presented in Table 1, together with the enhancement ratios of iontophoretic estradiol delivery from liposomes relative to the control.

Neutral compounds are usually iontophoresed under the anode to obtain the benefit of electroosmotic flow, that is the bulk fluid flow in the direction of counter ion movement carries drug molecules with it. This mechanism was suggested to be the main driving force for uncharged species during iontophoresis (Pikal, 1990; Pikal and Shah, 1990). However, as liposomes were negatively charged, it was necessary to keep the same experimental conditions and estradiol in saturated aqueous solution was therefore, driven under the cathode. Surprisingly, although estradiol was driven against electroosmotic flow, that was calculated to be 21.6 ng/cm^2 per h (as determined from Essa et al., 2001), fluxes actually increased during cathodic iontophoresis. This increase was not significantly different from passive (0.0 mA/cm^2) flux when 0.2 mA/cm^2 was used ($P > 0.05$); however, there were about two and four-fold increases in flux following application of 0.5 and 0.8 mA/cm^2 current densities, respectively (Table 1). Such increases in neutral drug penetration under the cathode may be due to a reduction in the skin

Table 1
Passive and iontophoretic delivery of estradiol through human epidermal membrane from saturated solution and ultradeformable liposomes at increasing current densities, together with the enhancement ratios

Current density (mA/cm^2)	Flux (ng/cm^2 per h)		Enhancement ratio
	Saturated solution	Ultradeformable liposomes	
0.0	23.4 ($\pm$3.00, 5)	173.0 ($\pm$22.3, 5)	7.4
0.2	24.8 ($\pm$4.70, 5)	306.0 ($\pm$53.0, 5)	12
0.5	53.0 ($\pm$7.81, 5)	811.0 ($\pm$104, 5)	15
0.8	91.0 ($\pm$11.6, 5)	1417 ($\pm$170, 5)	15

Values between brackets are the SEM and number of replicates, respectively.

barrier properties during iontophoresis. This supports the suggestion that an electric field can perturb the intercellular lamellar ordering in stratum corneum, giving rise to a more permeable membrane (Jadoul et al., 1999).

For ultradeformable liposomes, there were about two, five and nine-fold increases in fluxes relative to passive steady state flux after using 0.2, 0.5 and 0.8 mA/cm^2, respectively. Such enhancement in flux indicates that the electrostatic repulsion force between the negatively charged liposome vesicles and the negative cathode overcomes the skin cation permselectivity and also the reverse electro-osmotic flow. The following inferences and findings can explain Table 1 results. The repulsion force between the applied current and the negatively charged liposome moved liposomes towards and into the skin surface, aided by any enhancement effect of free phospholipids. As phospholipids can adhere to and fuse with the stratum corneum, altering the lipid barrier properties and producing a more permeable structure (Blume et al., 1993; Hofland et al., 1995), the presence of liposomes in high concentration due to iontophoresis may increase this effect. At the same time, current disorganises the lipid layer stacking (Jadoul et al., 1996). Consequently, phospholipids and electric current synergistically enhance the transdermal drug flux, agreeing with Kirijavainen et al., 2000, whether or not some of the ultradeformable liposomes move through the skin (see later).

The increase in drug penetration rates linearly correlated with the applied current densities. This characteristic gives iontophoresis its unique property of providing a programmable delivery system (Banga, 1998).

To clarify the role of ultradeformable liposomes in improving transdermal iontophoretic estradiol penetration through the skin, enhancement ratios were calculated by dividing fluxes of ultradeformable liposomes by those of control under the same experimental conditions (see Table 1). It is clear that liposomes enhance drug penetration many fold over that of the control following passive occluded application, and that iontophoresis produces about a further two-fold effect compared with zero current. Such results show the benefits gained from the combined use of iontophoresis and ultradeformable liposomes in transdermal drug delivery.

3.4.4. Penetration studies using Protocol III

This Protocol involved three consecutive 4-h stages; first passive, iontophoresis and second passive. This sequence of drug delivery was developed to assess the effect of current on the skin barrier properties. Theoretically, if the applied electric current did not dramatically affect skin integrity, then the flux of the second passive stage, following iontophoresis, should eventually fall to the same as that of the first passive stage. During the iontophoresis stage, a current of 0.8 mA/cm^2 was used for both ultradeformable liposomes and control. Such a relatively high current density was selected to provide the high repulsion force required for additional significant movement of the multilamellar vesicles.

To measure the actual enhancement of iontophoresis over passive drug penetration, it is simpler to compare fluxes under approximately steady state conditions. However, to gather data in a reasonable time, the duration of the first passive stage needed to be shorter than that required for the drug to reach its steady state penetration. Therefore, the passive steady state estradiol penetration profiles from ultradeformable liposomes and control in Protocol I were used to correct the data in the first passive stage so as to produce approximate pseudo-steady state values in Protocol III. Corrections were made by measuring the approximate flux during the first 4 h (pseudo-steady state flux) and after reaching steady state penetration (steady state flux) from penetration profiles in Fig. 2. The ratios of steady state to pseudo-steady state fluxes were calculated to be 1.2 and 1.4 for liposomes and control, respectively. These ratios were then used to correct the first passive flux values of Fig. 4A, so producing the theoretical first passive fluxes in Table 2.

To delineate better the effect of iontophoresis on skin integrity, the instantaneous fluxes (those at the mid-time points obtained from the slopes of the lines between each two successive values in the cumulative amount plots) were calculated from

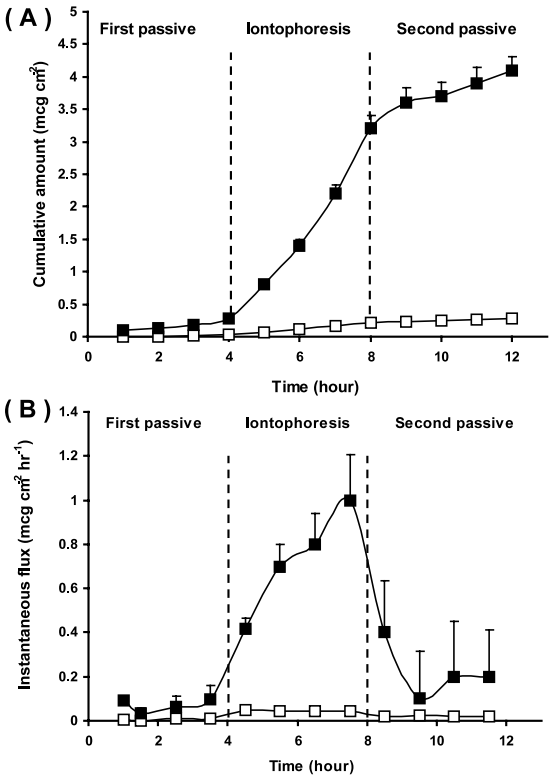

Fig. 4. Penetration profiles through human epidermal membrane of estradiol from saturated aqueous solution (□) and ultradeformable liposomes (■) using the three stage Protocol III, represented as cumulative amount plots (A) and instantaneous flux plots; (B). Error bars, if not shown, are within the symbols.

the cumulative amount plots in Fig. 4A to produce the instantaneous flux plots in Fig. 4B.

Flux values represented in Table 2 are the theoretical steady state (first passive) fluxes and the maximum fluxes obtained for the iontophoretic stage, as this provides the best estimate of the effect. For the second passive stage, the final datum point was used, to measure possible membrane recovery. In addition, the increase in drug penetration from liposomes relative to control in every stage was calculated and presented as enhancement ratios.

For saturated aqueous estradiol solution, there was a slow increase in drug penetration in the first passive stage (Fig. 4A). Upon application of iontophoresis, the penetration rate increased to a steady value with time of current application. Switching off the current reduced the delivery. The instantaneous fluxes plot (Fig. 4B) shows that during iontophoresis, estradiol penetration reached a maximum flux of 45.5 ± 16.5 ng/cm^2 per h, about two-fold higher than the theoretical steady state flux (see Table 2). As current disorganises the intercellular lipid lamella, the main pathway for a lipophilic molecule such as estradiol, so drug penetration increased within the first hour of current application, overcoming the reverse electro-osmotic flow. The constancy of fluxes throughout the iontophoretic stage, as shown in Fig. 4B, indicates that the skin barrier resistance did not progressively reduce with further current application. Immediately after switching off the current, fluxes fell to a minimum value of 23.8 ± 7.10 ng/cm^2 per h, which was similar to the theoretical passive flux ($P > 0.05$) and published data (Megrab et al., 1995; El-Maghraby, 2000). Such a reduction in flux reflects the fact that the skin may restore its in vitro barrier function shortly after current termination, and not be permanently damaged.

Table 2
Transdermal estradiol fluxes from saturated aqueous solution and ultradeformable liposomes through human epidermal membrane using the three-stage Protocol III

Stage of penetration	Flux (ng/cm^2 per h)		Enhancement ratio
	Saturated solution	Ultradeformable liposomes	
Theoretical first passive	20.3 ($\pm$3.81, 6)	158.0 ($\pm$27.5, 5)	7.7
Iontophoretic	45.5 ($\pm$16.5, 6)	1014 ($\pm$131, 5)	22
Second passive	23.8 ($\pm$7.10, 6)	177.3 ($\pm$49.0, 5)	7.3

ER is enhancement ratio of liposomes relative to control. Current density was 0.8 mA/cm^2. Values between brackets are the SEM and number of replicates, respectively.

With ultradeformable liposomes, the cumulative amount plot shows an increase during the first passive stage, which was markedly raised during the iontophoretic stage. However, the increase in drug penetration was not linear with time (as evidenced by the curved line during the iontophoresis period in Fig. 4A), indicating a possible continuous change in the membrane barrier as the time of current supply increased. Termination of current reduced the rate of increase of cumulative amounts. The instantaneous fluxes plot (Fig. 4B) shows that during the iontophoretic period the flux continuously increases to a maximum of 1014 ng/cm^2 per h following 4 h of iontophoretic delivery. This was about six-fold higher than the theoretical passive flux. This progressive increase in flux may be explained by the penetration enhancing effect of phospholipids released from liposomes, which fluidise intercellular lipid lamella. This disruption was now aided by the disorganising effect of current. These processes led to progressively higher estradiol fluxes delivered through liposomes. The longer the time of current application, the more vesicles move towards and into the skin and the greater the enhancing effect of the phospholipid molecules fusing with the intercellular lipids. This may explain the continuous increase in estradiol flux from liposomes as the time of current application increased. After termination of current, some hours were required for the membrane to release excess drug and for the flux to fall to its final value (177 ng/cm^2 per h), which was similar to the theoretical passive flux ($P > 0.05$).

Comparing ultradeformable liposomes with saturated estradiol solution, liposomes always showed improved drug penetration by many fold relative to control (Table 2). For the first occluded passive penetration stage, there was a 7.7-fold higher estradiol flux from liposomes relative to control, agreeing with the results of Protocol II. With iontophoresis, liposomes were superior to saturated aqueous solution, with a maximum of about 22-fold higher flux at the end of the iontophoretic stage. During the second passive phase, after current termination, the flux from liposomes did not rapidly reduce as for saturated solution (Fig. 4B). The membrane was loaded with phospholipid and excess drug molecules that needed time to be released into the receptor before normal passive penetration kinetics could operate.

The overall results suggest two important conclusions. Firstly, iontophoresis using 0.8 mA/cm^2 did not permanently affect the in vitro skin barrier properties. Secondary, ultradeformable liposomes may penetrate intact into and at least partially through the skin during iontophoresis (with some vesicles possibly disrupting), as evidenced by high fluxes during the iontophoretic period, followed by reduction at the end of the second passive stage to near that of the theoretical steady state flux. Such vesicular penetration may be aided by many factors. Firstly, the synergistic effect of current and phospholipids on the intercellular lipid lamella, that were suggested to be the main pathway for liposomal drug penetration (El-Maghraby, 2000). Secondary, the external force imposed by the current on the liposomal vesicles may aid the deformability of the vesicular membrane and enable liposomes to squeeze through the already temporarily impaired skin.

3.5. General conclusion

Tritium exchange with water should be taken into consideration when dealing with ^{3}H-labelled compounds, especially during iontophoresis. To counteract the problem, low current densities or minimum times of current application should be used, but allowances should also be made for the effect. It was also found that liposomes partially protected estradiol from tritium exchange. The use of ultradeformable liposomes increased passive skin delivery of estradiol over that of saturated aqueous solution under occluded (hydrated) conditions. Iontophoresis (up to 0.8 mA/cm^2) further promoted liposomal delivery in a proportional fashion i.e. increased current densities raised fluxes. Such improvements may be due to high deformability of the vesicular membrane that enables the liposomes to penetrate intact through the skin, and/or the negative charge on the vesicular surface. The disorganising effect of current on the intercellular lipid lamella overcomes the reverse electroosmotic flow as evidenced by increasing iontophoretic fluxes of estradiol from

saturated aqueous solution. Additionally, the greatest applied current density used (0.8mA/cm^2), considered relatively high for in vivo use, decreased in vitro skin barrier properties, but in a reversible fashion.

4. Uncited references

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