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Skin hydration and possible shunt route penetration in controlled estradiol delivery from ultradeformable and standard liposomes

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Abstract

Human skin delivery of estradiol from ultradeformable and traditional liposomes was explored, comparing occlusive and open application, with the aim of examining the role of skin hydration. Partially hydrated epidermis was used for open hydration, but fully hydrated membranes were used for occluded studies. In addition, we developed a novel technique to investigate the role of shunt route penetration in skin delivery of liposomal estradiol. This compared delivery through epidermis with that through a stratum corneum (SC)/epidermis sandwich from the same skin with the additional SC forming the top layer of the sandwich. This design was based on the fact that orifices of shunts only occupy 0.1 % of skin surface area and thus for SC/epidermis sandwiches there will be a negligible chance for shunts to superimpose. The top SC thus blocks most shunts available on the bottom membrane. If shunts play a major role then the delivery through sandwiches should be much reduced compared with that through epidermis, taking into consideration the expected reduction owing to increased membrane thickness. After open application, both ultradeformable and traditional liposomes improved estradiol skin delivery, with the ultradeformable liposomes being superior. Occlusion reduced the delivering efficiency of both vesicle types, supporting the theory that a hydration gradient provides the driving force. Shunt route penetration was found to play only a very minor role in liposomal delivery. In conclusion, full hydration of skin reduces estradiol delivery from liposomes and the shunt route is not the main pathway for this delivery.

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Introduction

Although the transdermal route of drug delivery can offer many advantages over other pathways of administration, the barrier nature of skin makes it difficult for most drugs to penetrate into and permeate through it. The strategy of using liposomes to overcome this problem and control drug delivery has gained attention in the last two decades (Mezei & Gulasekharam 1980, 1982; Mezei 1992; Touitou et al 1994). Recently, a new type of lipid vesicle (Transfersome) was reported to penetrate intact skin carrying therapeutic concentrations of drugs, but only when applied under non-occluded conditions (Cevc & Blume 1992). Transfersomes are described as freely movable carriers having their own non-metabolic means of locomotion through partly hydrated skin. They are reported to penetrate intact skin because of a transdermal hydration force (Cevc & Blume 1992). Phospholipids have a tendency to avoid any dry surroundings (xerophobia). Accordingly, when a

suspension of phospholipid-based liposomes is applied on the skin surface non-occlusively, it will partially dehydrate by evaporation. For vesicles to remain maximally swollen under such conditions, they follow the local transdermal hydration gradient. In doing so they penetrate into the more strongly hydrated and deeper skin strata. Vesicles can only do this if they are sufficiently deformable. Traditional liposomes in this situation will confine themselves to the skin surface, dehydrate completely and fuse, whereas Transfersomes, as deformable vesicles (also referred to as ultradeformable), may distort and enter the tissue (Cevc 1992; Cevc & Blume 1992; Cevc et al 1995). According to such a scheme, occlusion would thus inhibit the action of these deformable vesicles. It was recently reported that sodium cholate-containing deformable phospholipid vesicles when applied to fully hydrated mouse skin only improved the skin deposition of ciclosporin, compared with improved permeation and deposition when freshly excised, non-hydrated skin was used (Guo et al 2000). On the other hand, intact traditional liposomes were reported to penetrate into the skin and deposit in the dermis after occluded application (Foldvari et al 1990).

We have previously compared estradiol skin delivery from deformable vesicles and traditional liposomes after open application in-vitro, using human epidermal membranes hydrated by an open hydration protocol, which could maintain the transepidermal hydration gradient (El Maghraby et al 1999). Both deformable and standard liposomes improved the transepidermal maximum flux (J_{max}) as well as the skin deposition of estradiol compared with saturated aqueous estradiol (maximum thermodynamic activity). Under these conditions, deformable vesicles were superior to standard liposomes with respect to J_{max} only, although the difference was not great.

As occlusion is believed to abolish the natural hydration gradient in the skin and thus should inhibit the action of deformable vesicles, we have investigated skin delivery of estradiol from deformable and traditional liposomes after occluded application. The delivery obtained under occlusive conditions was compared with that obtained after open application. Thus the effect of skin hydration and the role of a hydration gradient in vesicular skin delivery of estradiol was investigated. Additionally, the possible role of shunt route penetration in the skin delivery of estradiol from selected liposome formulations was studied using a novel technique. To date, all literature reports investigating the role of the shunt route in liposomal skin delivery have been concerned with localization of drug into these shunts, especially to the pilosebaceous glands (Lieb et al 1992, 1994; Lauer et al 1996; Bernard et al 1997). The preparations used included deformable vesicles as well as traditional liposomes. Deformable vesicles included three optimized formulations (El Maghraby et al 2000), which contained soybean phosphatidylcholine (PC) as the main component together with edge activators, which provide the deformability of these vesicles (Cevc et al 1995). Edge activators included the reported sodium cholate (Cevc et al 1995) and two other surfactants (Span 80 and Tween 80) used at optimum concentrations (El Maghraby et al 2000). Accordingly, deformable formulations were D1, which contained soybean PC and sodium cholate (86:14, w/w), D2, comprising soybean PC and Span (86.7:13.3, w/w) and D3, comprising soybean PC and Tween (84.5:15.5, w/w). Traditional liposome formulations included T1, prototype pure soybean PC liposomes (non-rigid, i.e. with low membrane transition temperature; T_m) and T2, soybean PC mixed with cholesterol, a membrane stabilizer (1:1 molar ratio). In addition, two rigid formulations were used: T3 (pure dipalmitoylphosphatidylcholine; DPPC) and T4 (DPPC and cholesterol, 2:1 molar ratio). Thus, the selected systems provided a range of vesicles comprising non-rigid, deformable, membrane-stabilized and rigid liposomes. Saturated radiolabelled aqueous estradiol solution was used as the control.

Materials and Methods

Materials

Estradiol (2,4,6,7-^{3}H(N)) was obtained from NEN Life Science Products. Soybean PC (purity 99%), 17β-estradiol (purity 98%), DPPC, sodium cholate, sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80) and cholesterol were purchased from Sigma Chemical Company, St Louis, MO. Scintillation fluid (OptiPhase HiSafe 3) was obtained from LKB Scintillation Products Ltd (UK). All chemicals were used without further purification. Water was double-distilled.

Preparation of lipid vesicles

Vesicles were prepared by bath sonication (New 1990) and homogenized by manual extrusion. Briefly, the lipid mixtures were dissolved in ethanol, except for cholesterol- or DPPC-containing formulations, which were

dissolved in chloroform–ethanol (2:1, v/v). Radiolabelled estradiol, sufficient to produce 1 mg mL^{-1} (25 μCi mL^{-1}) in the final preparations, was added. Organic solvent was removed by rotary evaporation above transition temperatures (ambient for deformable and pure soybean PC vesicles or 50°C for cholesterol- or DPPC-containing formulations) and traces were vacuumed overnight. Deposited films were hydrated with either 7% v/v ethanol in water (deformable vesicles) or water (traditional formulations) by rotation at 60 rpm for 1 h at the corresponding temperatures. Resulting vesicles were swollen for 2 h at room temperature. These liposomes were sonicated in a bath at ambient temperature or 50°C for 30 min (New 1990), before homogenization by extrusion 10 times through a sandwich of 200 and 100 nm polycarbonate membranes. The final lipid concentration in suspensions was 5%, w/v. These procedures produced vesicles with sizes ranging from 127 to 146 nm with no significant difference between formulations ($P > 0.05$) (El Maghraby et al 1999). Estradiol saturated all formulations and thus ensured equal maximum thermodynamic activity (El Maghraby et al 1999).

Preparation of skin samples

Midline abdominal post-mortem skin samples were obtained from 24 Caucasian donors (9 male) with an average age of 73.1 ± 10.3 years. Samples were flattened and stored in vacuum-sealed double polythene bags at −20°C (Harrison et al 1984). Epidermal membranes were prepared by a heat separation technique (Kligman & Christophers 1963); fat and connective tissue were removed, the skin was heated for 45 s in a water bath at 60°C after which the epidermis was gently teased off the underlying dermis.

To prepare stratum corneum (SC) samples, epidermal membranes were floated overnight on an aqueous solution of trypsin (0.0001%, w/v) and sodium bicarbonate (0.5%, w/v) at 37°C. Membranes were squeezed between two filter papers and any remaining digested material was removed by washing with water and gentle swabbing. The SC was then floated on aqueous sodium azide (0.002%, w/v) for 1 h to remove any remaining digested matter. Membranes were dried on a mesh screen for 24 h, rinsed with cold acetone for 10 s and stored in a desiccator.

To obtain partially hydrated membranes, an open hydration protocol was used (El Maghraby et al 1999, 2000). This procedure can maintain a transepidermal hydration gradient, which has been proposed to generate the driving force for Transfersome skin penetration (Cevc & Blume 1992). The membranes were floated, SC side uppermost, on 0.002% aqueous sodium azide and the upper surface was left open to the atmosphere for 24 h. They were equilibrated for a further 12 h on diffusion cells with receptor fluid flowing beneath the membrane and the skin surface exposed. Membranes hydrated in this way were used in all studies except those involving occlusive application.

To study the effect of skin hydration, fully hydrated skin was obtained using an occluded hydration protocol. Membranes were floated, SC side uppermost, on aqueous sodium azide for 48 h in closed Petri dishes. Hydration continued for a further 24 h on the diffusion cells again with the receptor flowing, but the skin surface was covered with aqueous sodium azide solution under occlusion. This solution was removed before applying the donor preparation.

Permeation studies

An automated diffusion apparatus was used (Akhter et al 1984). The validated diffusion cells with a diffusional area of 0.126 cm^2 were used to investigate the effect of skin hydration on liposomal delivery of estradiol (by comparing occluded vs open application). However, large diffusion cells (diffusional area of 0.95 cm^2) were validated and used to monitor shunt route penetration in liposomal skin delivery of estradiol (this was to increase the number of shunts because of the larger surface area). The membranes were mounted, SC side uppermost, with the skin surface at 32°C. Sink conditions were maintained by pumping heated degassed receptor solution through the receptor compartments at 2 mL h^{-1}.

Experiments involving occluded application used 150-μL doses with aqueous sodium azide as receptor fluid. Receptor samples were collected periodically and assayed for drug permeated by liquid scintillation counting. The cumulative amount of drug permeated with time produced the permeation profile. Occluded application yielded typical steady-state profiles (examples are shown in Figure 1). These profiles were used to calculate permeation parameters. The flux (J) was obtained from the slope of the regression line, fitted to the linear portion of the permeability profile.

Experiments involving open application used the low-dose design (20 μL open application) and used aqueous sodium azide as the receptor medium. The cumulative amount vs time plots were used to calculate the rate of drug permeation by taking the slope of the line between

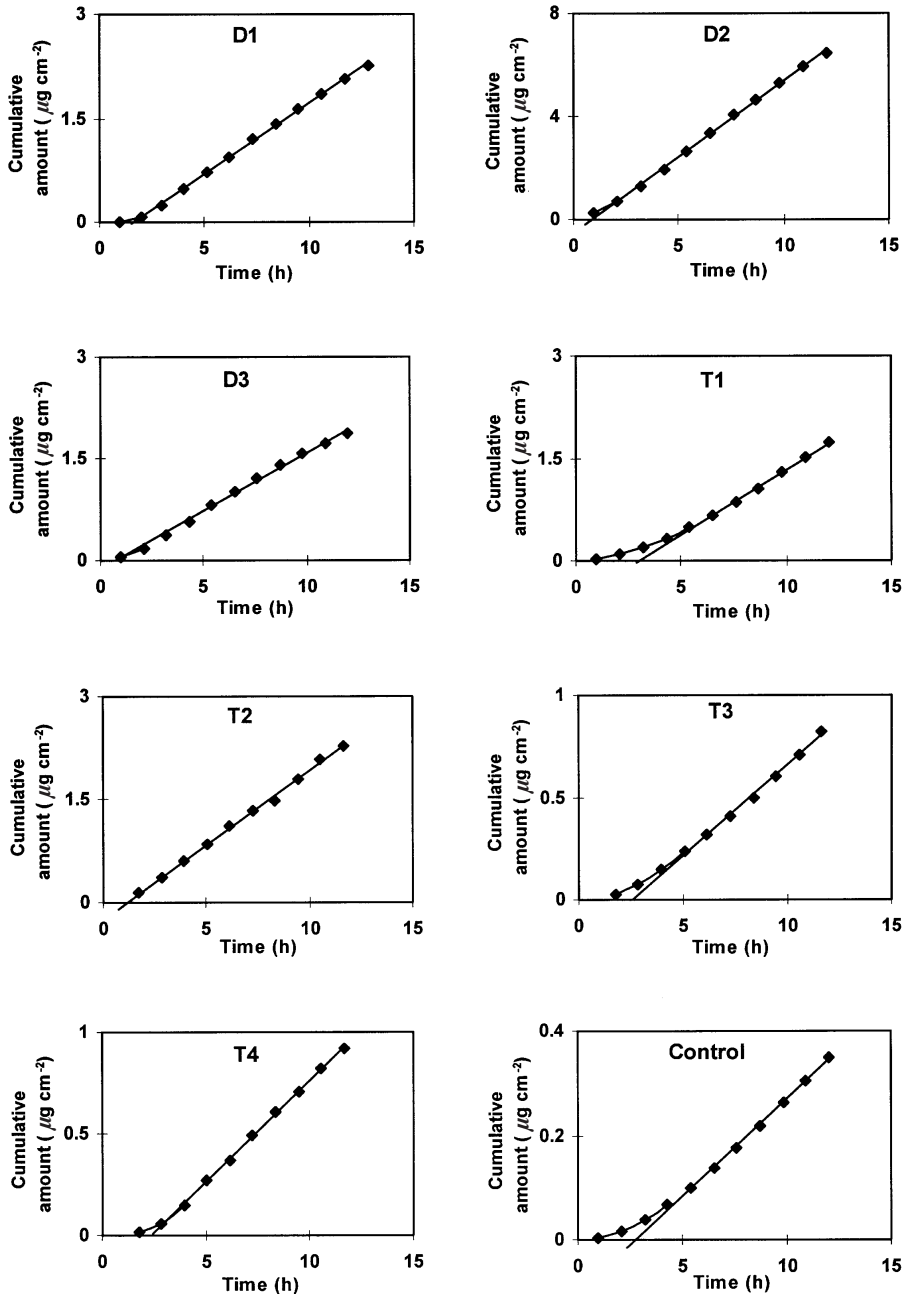

Figure 1 Examples of the transepidermal permeation profiles obtained after occlusive application of 150 μL of estradiol liposomes or saturated aqueous solution (control). D1, D2 and D3 are sodium cholate-, Span- and Tween-containing deformable formulations, respectively. T1, T2, T3 and T4 are soybean PC, soybean PC and cholesterol, DPPC, and DPPC and cholesterol traditional formulations, respectively.

adjacent points. Rates were plotted at midtime points to produce the flux profile which was a typical finite dose profile with the flux increasing with time to a maximum, before decreasing again. These profiles were used to calculate the maximum fluxes, J_{max} (El Maghraby et al 1999, 2000).

Investigation of the role of shunt route penetration in vesicular skin delivery of estradiol

A novel in-vitro technique using human abdominal skin was used to explore the role of the appendageal route on liposomal skin delivery of estradiol. The study involved

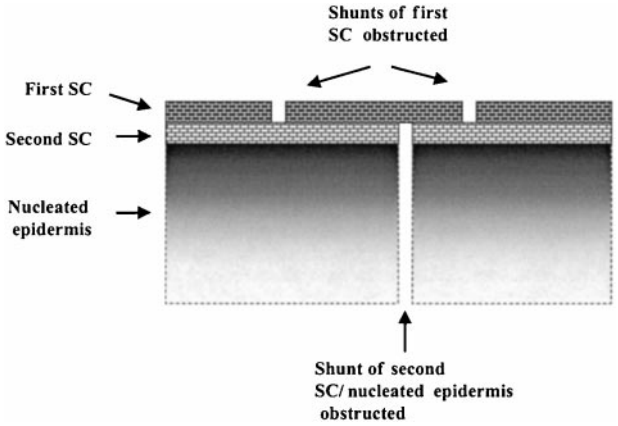

Figure 2 Illustration of a sandwich of stratum corneum (SC) and epidermis, showing how the additional SC layer should block nearly all shunts in the epidermis (SC plus nucleated epidermis). Not to scale.

monitoring the liposomal delivery of estradiol through epidermal membranes and comparing this with penetration through a sandwich of SC and epidermis, where the additional SC formed a top layer. As the orifices of the shunts occupy only about 0.1% of the total skin surface area (Scheuplein 1967; Illel & Schaefer 1991), there is a negligible chance that shunts in the two membranes will superimpose. It was therefore assumed that the top layer of SC would block most of the shunts available in the bottom membrane. This is illustrated in Figure 2.

To increase the number of shunts available in the bottom membrane, flow-through diffusion cells with a surface area of 0.95 cm^2 were used instead of the usual surface area of 0.126 cm^2. However, the diffusion cells with 0.126 cm^2 surface area were also used and compared with the large cells to further validate the latter.

The studies involved a low-dose design (20 μL open application for the small cells and 150 μL open application for the large cells). The doses were calculated to maintain equal dose per unit area.

The study tested complex liposome formulations and used a low-dose design in which the donor evaporated within about 45 min, but the mathematical model of a finite dose was applied. Thus, it was necessary to test the validity of the SC/epidermis sandwiches using a simple design. Accordingly, a steady-state experiment was used to study the effects of shunt routes and membrane thickness on the delivery of estradiol from a simple saturated aqueous solution of estradiol (i.e. in the absence of liposomes). In addition, a saturated solution was also used in a low-dose design.

Mathematical treatments of skin permeation data have been reviewed for steady-state (infinite dose) and finite dose experimental design (Barry 1983). For a simple membrane without shunts, the flux (J) in a steady-state experiment is governed by the following equation:

$$J = (DPC)/h \quad (1)$$

where D is the diffusion coefficient, P is the partition coefficient of the diffusant between the membrane and the bathing solution or the vehicle, C is the donor concentration, and h is the thickness of the membrane.

In traditional finite dose experiments, a small amount of the drug dissolved in a volatile solvent is applied to the skin. The solvent readily evaporates leaving a solid thin film (finite thickness, δ) of the penetrant on the skin surface. The maximum flux (J_{max}) of the penetrant is given by the following equation:

$$J_{max} = (1.85DC_o\delta)/h^2 \quad (2)$$

where C_o is the concentration of the diffusant in the first layer of the membrane.

This equation was applied here in a simple comparative way for the low-dose design, assuming that δ is relatively small and constant.

In the present study we used SC/epidermis sandwiches and therefore the thickness of the membrane was increased. This design did not simply double the actual membrane thickness. This is because the sandwiches contain SC and epidermis (SC plus nucleated epidermis), which was compared with epidermal membrane alone (SC plus nucleated epidermis). The resistance of the SC to estradiol permeation was found to be 224 h cm^{-1}, and for the epidermis (SC plus nucleated epidermis) the resistance was 272 h cm^{-1} (no significance difference) (Williams & Barry 1991). Accordingly, it was concluded that the SC is the main barrier to estradiol permeation. Based on this report, the presence of nucleated epidermis was neglected and considered simply as a mechanical support, which did not add significantly to the resistance of a membrane. Thus for SC/epidermis sandwiches, the membrane thickness (resistance) was considered to be doubled compared with the single epidermal membrane.

For steady-state experiments, based on equation 1, doubling the membrane thickness was expected to reduce the steady-state flux by 50%, compared with the flux through a single epidermal membrane. For the low-dose design, based on equation 2, doubling the membrane thickness for SC/epidermis sandwiches was expected to reduce the maximum flux by approximately 75% compared with that through a single epidermal membrane.

Any significant deviation (much greater reduction in flux) from these ratios may be taken to imply that shunt route permeation is important and should be taken into

account with respect to liposomal delivery across skin. The Student's *t*-test was used as the test for significance.

Results and Discussion

Occluded vs non-occluded application in liposomal skin delivery of estradiol

Table 1 presents the in-vitro transepidermal permeation parameters of estradiol obtained after occlusive and open application of deformable and traditional small unilamellar liposomes. When comparing the application methods, values relative to the saturated aqueous control were used to overcome the differences in the permeation profiles obtained with occlusive application (steady state) and open application (typical finite dose profile). The relative flux values obtained after open or occlusive application of estradiol liposomes are also presented in Table 1.

The skin delivery of estradiol from deformable and traditional liposomes has been previously described in detail (El Maghraby et al 1999). After open application, both types of lipid vesicles improved skin delivery of estradiol compared with saturated aqueous control, deformable vesicles being superior to traditional liposomes with respect to the transepidermal maximum flux of estradiol.

As expected, the transepidermal steady-state flux of estradiol from the saturated aqueous control (occluded) was higher than the maximum flux obtained after the low-dose open application (Table 1). This may be explained on the basis that after low-dose application the donor dries before achieving the steady-state flux values. The values obtained for the steady-state flux agree with previously reported values (Megrab et al 1995a, b). Thus, if the hydration gradient mechanism for liposome delivery is invalid, it would be expected that, when liposomes are used under occlusion, the relative estradiol flux will be higher than that obtained after open application of the same liposomal formulation.

Occluded application of deformable vesicles (D1, D2 and D3) increased the transepidermal flux of estradiol compared with control, as has been found in general with liposomes in our studies (Table 1). Comparing the delivery after open application with that obtained after occlusive application, the delivering efficiency decreased after occlusive application compared with open application (taking the relative transepidermal flux as a measure for this efficiency). The delivering efficiency was decreased by 62, 47 and 50% after occluded ap-

Table 1 In-vitro transepidermal permeation parameters and their values relative to the control (ratio) obtained for estradiol after occlusive and open application of deformable and traditional small unilamellar liposomes.

Formulation	Occluded application J (ng cm^{-2} h^{-1})	Ratio	Open application J_{max} (ng cm^{-2} h^{-1})	Ratio
D1	220 (37, 5)**	6.6	171 (17, 17)***	17.3
Control	33.2 (9.6, 6)		9.88 (1.1, 17)	
D2	251 (70, 5)*	9.3	161 (33, 10)**	17.4
Control	27.0 (5.8, 6)		9.26 (1.6, 10)	
D3	187 (52, 3)*	6.9	128 (12, 9)***	13.8
Control	27.0 (5.8, 6)		9.26 (1.6, 10)	
T1	176 (34, 5)*	6.5	98.8 (18, 9)**	8.6
Control	27.0 (5.8, 6)		11.5 (1.3, 12)	
T2	160 (27, 4)*	5.9	74.1 (12, 12)**	8.2
Control	27.0 (5.8, 6)		9.04 (1.5, 10)	
T3	117 (42, 4)*	4.3	109 (21, 10)**	9.8
Control	27.0 (5.8, 6)		11.1 (1.6, 11)	
T4	127 (16, 4)**	4.7	83.8 (15, 9)**	8.2
Control	27.0 (5.8, 6)		10.2 (1.7, 9)	

Data of the open application protocol are taken from El Maghraby et al (1999). D1, D2 and D3 are sodium cholate-, Span- and Tween-containing deformable liposome formulations, respectively. T1, T2, T3 and T4 are soybean PC, soybean PC and cholesterol, DPPC, and DPPC and cholesterol traditional liposome formulations, respectively. Values in parentheses are the s.e.m. and number of replicates, respectively. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, significantly different compared with the corresponding saturated aqueous controls.

plication compared with open application of sodium cholate-containing (D1), Span-containing (D2) and Tween-containing deformable vesicles, respectively. These results provide support for the hydration gradient theory.

Application of traditional liposomes (T1, T2, T3 and T4) under occlusion also increased the transepidermal flux of estradiol compared with the saturated aqueous control (Table 1). Comparing open application procedures with the occluded application, the relative flux value was considered as a measure for the delivering efficiency. This efficiency was decreased by 24, 28, 66 and 43% after occluded application compared with open application of traditional liposomes T1–T4, respectively.

Non-occluded application has been recommended for efficient skin delivery of drugs using deformable vesicles (Transfersomes). Occlusion always inhibited the highly effective delivery obtained from deformable vesicles, producing an efficiency similar to that of traditional liposomes. Thus, instead of most of the applied dose permeating through the SC and reaching deeper skin strata and blood as after open application, most of the applied Transfersome dose was recovered from the skin surface and surface layers after occluded application (Cevc 1992; Cevc & Blume 1992; Cevc et al 1995). In our study, occlusion decreased the efficiency of deformable vesicles, supporting the reports of their proponents, but the reduction was not as marked as in their in-vivo studies. However, it should be noted that researchers from the same group reported successful anaesthetic effects after occluded application of local anaesthetic Transfersomes (Planas et al 1992).

For traditional liposomes, an occluded application protocol has been used by many researchers (e.g. Gesztes & Mezei 1988; Foldvari et al 1990; Foldvari 1994; Hung et al 1997). Non-occluded application has also been used (e.g. Egbaria et al 1990; Egbaria & Weiner 1992; Fresta & Puglisi 1997). However, occlusion vs non-occlusion comparisons for traditional liposomes have not been reported. Our results showed a trend of decreasing the delivery efficiency of estradiol from traditional liposomes after occluded application compared with the non-occluded process (taking the relative flux values as a measure for the delivering efficiency), although absolute amounts delivered apparently increased with occlusion.

The trend of decreasing skin delivering efficiency of liposomes after occlusion compared with open application may support the hypothesis that the transdermal hydration gradient can provide the driving force for transdermal vesicular permeation (Cevc & Blume 1992). An alternative explanation for the observed trends could be that the shunt route can have a significant influence on the liposomal delivery of drugs. As occlusion leads to full hydration and swelling of the cells with subsequent partial closure of shunts, occlusion might decrease liposomal delivery of drugs if shunt route penetration was significant in the non-occluded state. Accordingly, we decided to investigate the influence of shunt route on the skin delivery of estradiol from liposomes.

Validation of the large diffusion cells

To validate the larger diffusion cells (diffusional area of 0.95 cm^2), their performance was compared with the well-validated small cells (diffusional area of 0.126 cm^2). The skin delivery of estradiol from sodium cholate-containing deformable vesicles (D1) was studied using the small and large diffusion cells and skin samples from the same donor. The study used a low-dose design (open application) with the applied doses maintaining equal dose per unit area. The J_{max} values were 156 ± 36 and 134 ± 6.1 ng cm^{-2} h^{-1} (mean $\pm$ s.e.m., $n = 6$) for small and large cells, respectively. No significant differences were found ($P > 0.05$) when the J_{max} values obtained from small diffusion cells were compared with values obtained from the large cells. In addition, the transepidermal permeation profiles of estradiol obtained using both cells were similar. This indicates the validity of the large diffusion cells. It should be noted that the large diffusion cells produced less variable results as indicated by the low s.e.m. values compared with small cells. This lower variability was expected given the larger surface area available.

Validation of the SC/epidermis sandwiches

The specific role of hair follicles in percutaneous transport remains difficult to elucidate owing partly to the lack of an adequate animal model to distinguish follicular from non-follicular transport (Lauer et al 1996). This route and the animal models used have recently been reviewed by Lauer (1999). Some authors used hairless rodents in comparison with hairy skin to probe follicular delivery (Du Plessis 1992). However, the skin of hairless rodents cannot exclusively represent the transepidermal pathway as it is not follicle-free, but contains underdeveloped follicles (Lauer et al 1996). Follicle-free skin was created in hairless rats under anaesthesia by immersing an area of the dorsal skin in water at 60°C for 1 min. The epidermis was removed and allowed to redevelop into follicle-free skin (scar formation after burning) (Illel & Schaefer 1988). This

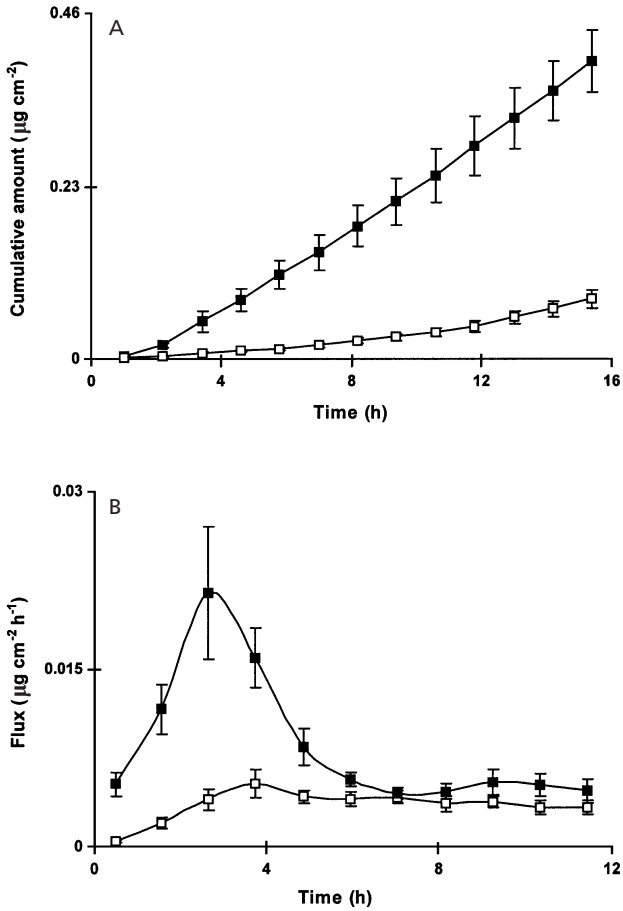

Figure 3 Steady-state permeation profiles (A) of estradiol obtained after occluded application and the flux profiles (B) of estradiol obtained after low-dose open application of saturated aqueous solution to SC/epidermis sandwiches (□) or epidermis (■).

Table 2 Steady-state flux (J; occluded application) and maximum flux (J_{max}; low-dose open application) of estradiol through SC/epidermis sandwiches and epidermis, obtained after application of saturated aqueous solution.

Parameter	Sandwich	Epidermis	Ratio (sandwich/epidermis)
Steady-state studies			
J (ng cm^{-2} h^{-1})	12.6 (2.3, 6)	29.6 (3.0, 7)	0.428
Low-dose studies			
J_{max} (ng cm^{-2} h^{-1})	5.15 (0.95, 4)	19.3 (3.4, 4)	0.267

Values in parentheses are the s.e.m. and number of replicates, respectively.

model was used to probe the follicular pathway and the importance of sebaceous glands in skin delivery (Bernard et al 1997). However, it is recommended that the scar tissue should be further checked to ensure that it is not different from normal skin with hair follicles with respect to permeation (Lauer et al 1996). The Syrian hamster ear has been reported to be an excellent model for human sebaceous glands (Plewig & Luderschmidt 1977). This was used to research liposomal delivery to the pilosebaceous unit by Lieb et al (1992) and Niemiec et al (1995).

In this study, we used a novel technique using human skin, whereby delivery through SC/epidermis sandwiches was compared with that through epidermis. To validate the technique of using a double membrane, it was necessary to use a simple solution and a simple experimental design. Accordingly, we investigated the delivery of estradiol from a saturated aqueous solution through SC/epidermis sandwiches in comparison with the delivery though epidermal membrane alone. The simple steady-state experimental design (150 μL occluded application, small cells) was used first and then the low-dose design was investigated. Figure 3A shows the permeation profiles obtained after steady-state experiments and Figure 3B shows the flux profiles obtained after the low-dose experimental design. The results are summarized in Table 2.

The data of the simple steady-state design (Table 2) revealed a reduction in flux of approximately 57% after using the SC/epidermis sandwiches compared with epidermis alone. These results are acceptable when compared with the expected 50% reduction in flux. The sandwiches can thus be considered valid with respect to the concept of doubling the thickness of the membrane (h) in equation 1. Taking into consideration that we neglected the presence of nucleated epidermis and assumed doubling of the thickness, the results indicated a negligible effect for the shunt route on the steady-state permeation of estradiol. This was expected as occlusion can maintain full hydration of the membrane, swelling of the cells, and, thus, possible blocking of the available shunts. Additionally, estradiol was expected to penetrate skin mainly via the intact SC.

A low-dose study was also performed to further validate the use of SC/epidermis sandwiches to investigate the shunt route. Estradiol permeation through SC/epidermis sandwiches and epidermis was monitored after open application of a low dose, using the large flow-through diffusion cells (diffusional area of 0.95 cm^2). The flux profile of estradiol through epidermis (Figure 3B) showed a typical low-dose profile where the transepidermal drug flux increased to a maximum after

which the flux decreased, but remained above background levels. At the late stages of the flux profile, the flux values tend towards a steady value with time. This presumably indicates that flux at this stage may depend on drug dissolution from deposited crystals. When using the SC/epidermis sandwich, a similar profile was obtained, but the value corresponding to the maximum flux decreased. Both profiles showed similar flux values at the late stages of the profile (after the maxima), again indicating that dissolution limited delivery of estradiol.

The data in Table 2 show that using the SC/epidermis sandwiches reduced the maximum flux by approximately 73% compared with that obtained after using epidermal membrane (theoretical reduction expected was 75%). These results again suggest that the shunt route plays a negligible role in the skin delivery of estradiol from saturated aqueous solution in-vitro and also indicate the validity of the experimental design with respect to the use of equation 2.

Possible role of shunts in skin delivery of estradiol from lipid vesicles

The SC/epidermis sandwich was used to assess formulations selected from previously examined deformable and traditional liposomes. The preparations included sodium cholate-containing (D1) and Span-containing (D2) deformable vesicles. From the traditional formulations, the pure soybean PC liposomes (T1) and the DPPC and cholesterol liposomes (T4) were selected. Thus, representative examples of deformable vesicles, non-rigid and rigid traditional liposomes were used. The study used the large diffusion cells (diffusional area of 0.95 cm^2) and used a low-dose design (open application). For individual formulations, the SC/epidermis sandwich and the epidermis were obtained from the same skin donors. The flux profiles of estradiol obtained after application of liposomes to SC/epidermis sandwich or epidermis are shown in Figure 4. The permeation parameters are presented in Table 3.

The flux profiles of estradiol obtained from liposomes penetrating through epidermal membranes revealed typical finite dose profiles, where the flux increased with time to a maximum, after which it decreased. The last portion of the profile often tended towards a constant value or at least did not continue falling (these values were always above zero). This flux profile was obtained with all liposomes (Figure 4). These profiles indicate that there is initial liposomal delivery (producing the maximum), followed by delivery that is presumably limited by drug release from liposomes (the last portion of the profiles).

Using the SC/epidermis sandwich instead of the single epidermal membrane reduced the value of the maximum and prolonged the time required for the flux to reach the maximum values. The profiles revealed a flux increasing with time to reach a maximum value and the flux then tended to stay around this level. The peak of maximum flux was therefore very flat. Again, the profiles were very similar with all formulations. These flux profiles may indicate that some of the liposomes deposited on the skin surface, from where drug was released, before permeating through the membranes. However, it should be noted that the flux values obtained (through SC/epidermis sandwiches) were over the range of 18.5–41.3 ng cm^{-2} h^{-1} (Table 3), which was higher than that obtained from a saturated solution permeating through SC/epidermis sandwiches (5.15 ng cm^{-2} h^{-1}; Table 2), indicating that it is not only the free drug permeating after being released on the skin surface. There may be a possible penetration enhancing effect of liposome components, but this is unlikely to fully explain the improved delivery, as demonstrated previously (El Maghraby et al 1999). Another possibility could be that liposomes penetrated some distance into the sandwiches, from where they release the drug for further penetration through the rest of the membrane. On this basis, the distance travelled by the free drug through the membrane would be reduced after liposome deposition in the skin, with the result that the flux values obtained through the sandwiches would be higher for liposomes compared with the drug flux from saturated solution.

As described earlier, the maximum flux (J_{max}) of estradiol delivered from liposomes through the SC/epidermis sandwich membrane was theoretically expected to be reduced by 75% of that through a simple epidermal membrane. Interestingly, for the traditional liposomes (pure soybean PC vesicles and DPPC and cholesterol vesicles), the SC/epidermis sandwiches reduced J_{max} by 70 and 75%, respectively, compared with that through epidermal membranes obtained from the same skin donor. These results, which closely match the theoretical expectation, thus show that follicular delivery is not the main route for estradiol delivery through human skin from liposomes.

Similar results were found with the deformable liposomes. Transdermal flux from sodium cholate-containing vesicles through the sandwich was reduced by 72% compared with the single epidermal membrane from the same donor, and the reduction for the Span-containing deformable vesicles was 64% through the double membrane compared with the single epidermal membrane from the same donor. Taking into account that we neglected the barrier contribution of the nucleated epi-

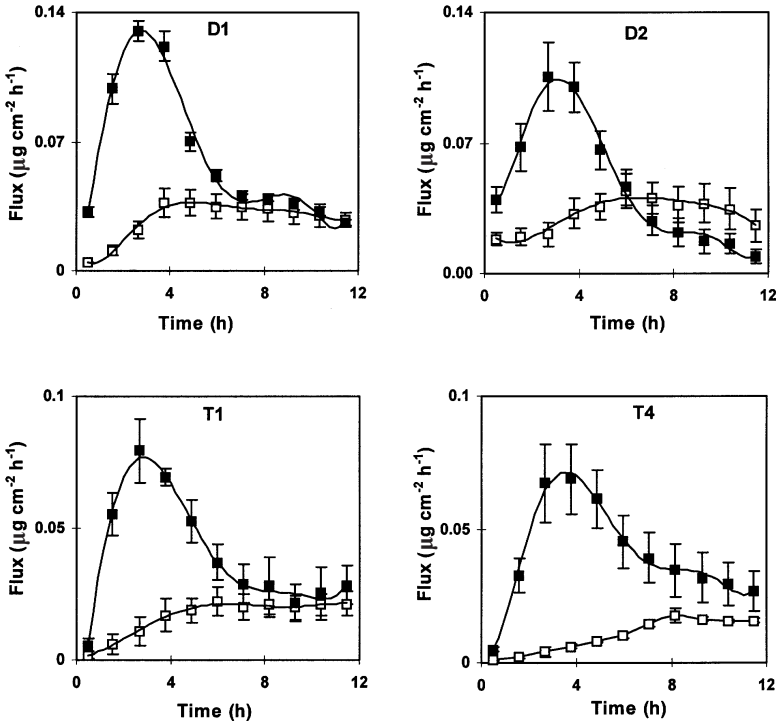

Figure 4 The flux profiles of estradiol obtained after open application of sodium cholate-containing vesicles (D1), Span-containing vesicles (D2), soybean PC liposomes (T1) or DPPC and cholesterol liposomes (T4) to SC/epidermis sandwiches (□) or epidermis (■).

Table 3 The permeation parameter (J_{max}) of estradiol obtained after open application of sodium cholate-containing (D1), Span-containing (D2), pure soybean PC (T1) or DPPC and cholesterol (T4) liposomes to SC/epidermis sandwiches or epidermis.

Formulation	J_{max} (ng cm^{-2} h^{-1})		Ratio (sandwich/ epidermis)
	Sandwich	Epidermis	
Sodium cholate vesicles (D1)	37.2 (7.2, 5)	134 (6.1, 6)	0.278
Span 80 vesicles (D2)	41.3 (9.5, 4)	116 (13, 4)	0.356
PC vesicles (T1)	23.2 (4.9, 4)	77.0 (13, 4)	0.301
DPPC and cholesterol (T4)	18.5 (1.6, 4)	73.4 (10, 4)	0.252

Values in parentheses are the s.e.m. and number of replicates, respectively.

dermis, the above results show that the shunt pathway can produce at most only a very minor effect on estradiol delivery from deformable vesicles through human skin.

Topical application of carboxyfluorescein in liposomes (PC, cholesterol, phosphatidylserine) produced significantly higher accumulation in the pilosebaceous unit compared with non-liposomal formulations (Lieb et al 1992). Egg PC-based liposomes produced greater skin deposition of γ-interferon compared with aqueous solution and the greatest effect was found in hamster skin (high follicular density) compared with human and mouse skin. It was thus concluded that the follicular pathway is a feasible route for liposomal delivery of hydrophilic molecules (Du Plessis et al 1992). Non-ionic liposomal formulation of glyceryl dilaurate, cholesterol and polyoxyethylene-10-stearyl either facilitated the deposition of both hydrophilic (α-interferon) and lipophilic (ciclosporin) drugs into the pilosebaceous unit via the follicular route (Niemiec et al 1995). Substantial amounts of isotretinoin were delivered to the sebaceous glands via the follicular route, with an ethanolic gel being as efficient as liposomal or mixed micellar gel (Tschan et al 1997). Egg PC liposomes localized the anti-androgenic RU 58841 in sebaceous glands (Bernard et al 1997).

From these literature reports, it could be concluded that the follicular pathway may play a role in liposomal skin delivery of drug. However, our present study revealed a reduction in the drug flux after using SC/epidermis sandwiches compared with epidermis. The reduction was close to the theoretical expectations calcu-

lated assuming that the SC/epidermis sandwich has a thickness double that of the epidermis (i.e. neglecting the presence of the nucleated epidermis). This indicates an absence of any significant role for the shunt route in skin delivery of estradiol from liposomes. However, in our work we were not concerned with examining localization of liposomes in the hair follicles (i.e. targeting to the hair follicle). We have thus only looked at the possible significance of the shunt route for penetration of estradiol through the epidermis.

Conclusions

Non-occlusive application was found to be better than occlusive application for both deformable and traditional liposomes with respect to in-vitro skin delivery of estradiol. This may imply a role for skin hydration in liposomal delivery of estradiol.

Although it was reported that shunts such as follicles and glands can play an important role for poorly penetrating aggregates such as traditional liposomes (Cevc et al 1996), our data indicated that the shunt route could have only a minor role in estradiol skin delivery from deformable and traditional liposomes. Our study also revealed a relatively similar behaviour for both deformable and traditional liposomes.

The superiority of liposomes over saturated aqueous solution in estradiol skin delivery and the absence of any role for shunt routes suggest the possibility for vesicles penetrating skin. This conclusion can be further supported considering previous mechanistic studies, indicating that penetration enhancement is not the main mechanism and revealing improved drug uptake from liposomes compared with saturated aqueous solution (El Maghraby et al 1999).

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