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Human skin sandwich for assessing shunt route penetration during passive and iontophoretic drug and liposome delivery

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Abstract

This work explored the role of skin appendages (shunt route) in passive and iontophoretic drug and liposome penetration. The technique used an epidermis and stratum corneum sandwich from the same skin donor with the additional stratum corneum forming the top layer of the sandwich. Penetration was monitored during occluded passive and iontophoretic (0.5 mA cm^{-2}) delivery of mannitol and estradiol solutions, and ultradeformable liposomes containing estradiol. The shunt route had a significant role during passive penetration of mannitol (hydrophilic compound), but was negligible during penetration of estradiol (lipophilic drug) and liposomes. In iontophoresis, the shunt route significantly contributed to the overall flux of all preparations, being highest for mannitol. However, shunts were not the only pathway for iontophoretic drug delivery and evidence was observed for the creation of new aqueous pathways via disorganization of the intercellular lipid domain of stratum corneum. The skin sandwich technique should prove valuable for general studies on routes of skin penetration.

Introduction

The concept of drug delivery through the skin is now a practical reality and patients readily accept transdermal patches. However, the natural barrier properties of the stratum corneum, the outermost layer of the skin, limit the passive transdermal delivery of many drugs. There has been a continuous quest for strategies to facilitate drug penetration across the stratum corneum barrier. Iontophoresis is one such approach for facilitating transdermal drug delivery by applying a potential difference across the membrane (Singh & Maibach 1996).

Although originally intended for charged compounds, iontophoresis can also facilitate the delivery of uncharged compounds by the process of electroosmosis, which is the bulk volume flow in the direction of positive ion transport (Pikal & Shah 1990a, b). During iontophoresis, the greatest concentration of ionised species is expected to move into damaged regions of the skin and along the sweat glands and hair follicles, as the diffusional resistance of the skin is lowest in these regions. There is much evidence supporting the idea that the appendageal pathway is the main route for such drug delivery. For example, the use of pilocarpine in the diagnosis of cystic fibrosis suggests that current travels down the sweat ducts. A dot-like pattern was also observed over the sweat gland openings following iontophoresis of charged dyes in human skin in-vivo (Abramson & Gorin 1940). Cullander & Guy (1991) have used a vibrating probe electrode (which can rapidly detect currents on the skin surface) to measure current flow through hairless mouse skin and found that the largest currents occurred in the vicinity of residual hair.

Besides the theory of an appendageal pathway for iontophoretic delivery, a nonappendageal pathway has also been suggested which probably implies that current is capable of temporarily disrupting the highly organized structure of stratum corneum (Jadoul et al 1996; Pikal 2001). An early study showed that epidermal alterations secondary to lidocaine (lignocaine) iontophoresis in porcine skin developed uniformly over the skin surface rather than at focal points, implying that transport took place over

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the skin surface as a whole (Monteiro-Riviere 1990). Additionally, iontophoretic transport of penadolol and calcitonin through both guinea-pig skin and human skin equivalent were similar. Since living skin equivalent contains no appendages, this suggests that an appendageal pathway is not necessary for iontophoretic delivery of drugs (Hager et al 1994).

This work aimed to delineate further the role of skin appendages (shunts) on iontophoretic drug transport. A recent technique using a sandwich of stratum corneum and epidermal membrane was adopted. It was assumed that the top stratum corneum membrane would essentially block all available shunts in the lower epidermal membrane. Consequently, any drug penetration through such a sandwich was expected to be via pathways other than the shunt route (El Maghraby et al 2001). In this study, we measured in-vitro occluded passive and iontophoretic (0.5 mA cm^{-2}) skin delivery of mannitol (hydrophilic), estradiol (lipophilic) and ultradeformable liposomes through human stratum corneum/epidermis sandwiches, and compared it with that of epidermis alone.

Materials and Methods

Materials

Estradiol (2,4,6,7-^{3}H(N)) was obtained from DuPont NEN Life Science Products. D-[1-^{14}C]mannitol, soybean phosphatidylcholine (purity 99%), 17β-estradiol (98%), sodium cholate, mannitol, trypsin (from bovine pancreas), sodium hydrogen carbonate, HEPES (*N*-2-hydroxy-ethylpiperazine-*N*-ethanosulfonic acid) and tetrabutylammonium hydroxide were purchased from Sigma Chemical Company, St Louis, MO. Silver wire (99%) was bought from Aldrich Chemical Company, Inc., USA. Scintillation fluid (Optiphase HiSafe 3) was obtained from LKB Scintillation Products Ltd, UK. Chemicals were used without further purification. Water was deionized, double distilled.

Preparation of skin membranes

Midline abdominal post-mortem skin samples from 14 donors (5 male), average age 69.3 $\pm$ 6.41 years, were used. Epidermal membranes were prepared by a heat separation technique (Kligman & Christophers 1963). Subsequent to removal of the excess fat and adipose tissue, samples were immersed in a water bath at 60°C for 45 s. Epidermal membranes were then easily teased off the underlying dermis and floated on 0.002% w/v sodium azide solution.

To prepare stratum corneum membranes, epidermal membranes were floated (epidermal side down) on an aqueous solution containing 0.0001% w/v trypsin and 0.5% w/v sodium hydrogen carbonate for 12 h at 37°C. The thin stratum corneum membranes were picked up on filter paper and digested cells were washed off with water. The membranes were then floated on water for 2 h to remove the remaining digested cells and residual trypsin. Membranes were dried overnight on stainless-steel wire mesh at ambient conditions, rinsed with cold acetone for 10 s to remove any surface contamination (such as fat), and stored in a desiccator. Membranes were hydrated by floating, with stratum corneum side uppermost, on 0.002% w/v aqueous sodium azide solution for at least 24 h. Then, they were equilibrated for a further 12 h on the diffusion cells under occlusion with the same solution. This solution was removed before applying the donor preparation.

Donor preparations

Mannitol and estradiol

In attempting to probe the role of shunt route during passive and iontophoretic penetration, it was advantageous to use penetrants of different hydrophilicities. Consequently, the donors have been chosen to represent a wide range of water solubilities. Neutral mannitol is a highly soluble compound with a log octanol/water partition coefficient of -2.47 (Barry & Bennett 1987). Its water solubility ranges from 179 to 223 mg mL^{-1} at 25–37°C (Wade & Weller 1994). Estradiol, a poorly soluble neutral compound, has a log partition coefficient of 2.29 (Williams et al 1992), and water solubility of 0.003 mg mL^{-1} at 25°C (Salole 1986) and 0.0036 mg mL^{-1} at 32°C (El Maghraby 2000). Additionally, a complex structure of highly insoluble negatively charged lipid vesicles containing estradiol as a model drug was used (see below). Such a selection enabled monitoring of the role of the shunt route in iontophoresis of both hydrophilic and lipophilic drugs, which are assumed to have different penetration pathways during their passive penetration. Mannitol aqueous solution (5 mM spiked with ^{14}C-mannitol to give a concentration of 5 μCi mL^{-1}) was prepared in HEPES buffer adjusted to pH 7.2–7.4 with tetrabutylammonium hydroxide. Saturated aqueous estradiol solution was formulated in the same buffer (1 mg mL^{-1}) with ^{3}H-labelled estradiol to give a final concentration of 25 μCi mL^{-1}, with excess radiolabelled crystals to ensure saturation of the solution during the penetration studies.

Liposomes

Ultradeformable vesicles (Transfersomes), as previously optimised by El Maghraby et al (2000) and containing sodium cholate as edge activator, were used. The lipid vesicles consisted of phosphatidylcholine–sodium cholate (86:14% w/w), and contained estradiol as a marker. Vesicles were prepared by bath sonication and homogenised by manual extrusion. Both lipid and edge activator were dissolved in ethanol, then tritium-labelled estradiol sufficient to produce 1 mg mL^{-1} (25 μCi mL^{-1}) in the final preparation was added. Ethanol was removed under a stream of nitrogen at room temperature. The remaining ethanol traces were removed under vacuum overnight. The deposited film was then hydrated with 7% v/v ethanol in HEPES buffer, pH 7.2–7.4, by vortexing for 30 min. The vesicles obtained were swollen for 2 h at room temperature before bath sonication for another 30 min (New 1990). The multilamellar vesicles were manually extruded through a stack of two polycarbonate membranes (0.2 and 0.1 μm) to produce a 5% w/v unilamellar liposomal suspension.

Characterisation of liposomes

Liposomes prepared using cold drug were characterised for their particle size and zeta potential using a Zeta master S particle electrophoresis and particle size analyser (Malvern Instrument Ltd, Malvern, UK).

Penetration studies

To investigate the role of shunt route in the skin delivery of compounds, the technique of using a stratum corneum/epidermis sandwich, designed by El Maghraby et al (2001), was used. The study monitored the delivery of compounds through human epidermal membrane compared to that through a sandwich of stratum corneum and epidermis, where the additional stratum corneum formed the top layer. To minimise interindividual variability, each sandwich was prepared using stratum corneum and epidermal membranes from the same skin sample. Knowing that skin appendages represent only about 0.1% of the total skin surface area (Scheuplein 1967; Illel & Schaefer 1991), there was a negligible chance that the orifices of these appendages in the two membranes would superimpose. It was therefore assumed that the top layer of stratum corneum would block most of the shunts available in the bottom membrane.

The use of a stratum corneum/epidermis sandwich increased the thickness of the membrane. Earlier work by Williams & Barry (1991) found no significant difference between the resistance of the stratum corneum and epidermis (stratum corneum plus nucleated epidermis) to estradiol permeation. Thus, it could be concluded that the presence of nucleated epidermis can be neglected and considered simply as a mechanical support, as it did not add significantly to the resistance of the membrane. Hence, when using the stratum corneum/epidermis sandwich, for calculation we simply double the membrane thickness (resistance) compared with epidermal membrane, when shunt route passage is negligible. The mathematical treatment of this technique was previously described by El Maghraby et al (2001). Briefly, steady-state passive drug permeation is governed by equation 1:

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

where J is the flux, D is the diffusion coefficient, P is the partition coefficient of the diffusant between the membrane and the bathing solution or vehicle, C is the donor concentration and h is the membrane thickness (Barry 1983). The lag time (L) is given by equation 2:

$$L = h^2/6D \quad (2)$$

Based on the above equations, doubling the membrane thickness is expected to reduce the steady-state flux by one half and prolong the lag time by 4 fold, compared with epidermal membrane, provided that shunt route penetration plays no part in the permeation process. Consequently, any further reduction in flux or prolongation of the lag time may indicate that the shunt route does have an important role and should be taken into consideration during drug delivery.

Passive penetration

It was desirable to validate the stratum corneum/epidermis composite technique and compare the results with theoretical expectations. An automated diffusion apparatus developed and validated by Akhter et al (1984) was used. Each cell consisted of a donor chamber that provided an effective diffusion area of 0.126 cm^2, and a continuous flow-through receptor compartment. The membranes (epidermis or stratum corneum/epidermis sandwich) were mounted with stratum corneum side uppermost and maintained at 32°C overnight to equilibrate under occlusion with 0.002% w/v sodium azide solution. The solution was then removed from the donor compartment, and 200 μL donor added (mannitol solution, estradiol solution or 5% w/v ultradeformable liposomes suspension). Heated degassed receptor fluid (0.002% w/v sodium azide solution) was continuously flushed through the receptor compartment from a peristaltic pump at a rate of 1 mL h^{-1} replacing the receptor fluid, maintaining essentially sink conditions. The receptor fluid was collected in scintillation vials, for 18 h for estradiol solution and ultradeformable liposomal suspension. However, for mannitol as a highly hydrophilic compound that was predicted to penetrate the skin through the shunt route to a significant extent, the passive penetration protocol consisted of two parts. In the first early stage, 1-mL samples were collected every 30 min for 9 h to assess mainly shunt penetration. Thereafter, samples were collected every 3 h for 81 h and 141 h for epidermis and stratum corneum/epidermis sandwiches, respectively, to assess bulk steady-state penetration. Collected samples were mixed with scintillation fluid before counting using a Packard Tri-Carb scintillation counter.

Iontophoretic penetration

Iontophoretic studies employed glass diffusion cells especially adapted for iontophoresis, with a diffusional area of 0.126 cm^2. Skin membranes (epidermis or stratum corneum/epidermis sandwich) were mounted with stratum corneum side uppermost and equilibrated at 32°C overnight, with buffer solution on both sides of the membrane. Donor chambers were dried and 150 μL of each donor preparation was applied. Anodic (neutral mannitol and estradiol) and cathodic (negative ultradeformable vesicles) iontophoresis of 0.5 mA cm^{-2} constant current was applied for 5 h, using Ag/AgCl reversible electrodes. Receptor solution was continuously agitated using magnetic stirrers, samples were taken hourly and drug concentrations were determined by radioactive counting. Student's *t*-test was used for statistical assessments.

Results and Discussion

Characterisation of liposomes

The small unilamellar vesicles had a Z average mean diameter of 126±15 nm (n = 3). This was as expected as the vesicles were homogenised through 200- and 100-nm polycarbonate membranes, a method that is known to produce vesicles of defined diameters around that of the

smallest pore through which they are extruded (Olson et al 1979). The vesicles had a zeta potential of -20 ± 1.3 mV, indicating the effect of the cholate anion.

Passive drug penetration through epidermis and stratum corneum/epidermis sandwich

In general, a penetrant has three potential routes of entry to the subepidermal tissue; hair follicles with their associated sebaceous glands, sweat ducts or across the continuous stratum corneum between these appendages (transepidermal). Because the skin appendages (hair follicles and sweat glands) penetrate the skin barrier, they bypass the bulk epidermis and are referred to as the shunt pathway. Penetration through these shunts should not always be ignored during the early period of the diffusion process, even when the ultimate steady-state flux through this pathway is small. As sweat ducts are very narrow slits that may swell shut during full hydration of skin, their role during in-vitro drug penetration is usually neglected.

The other pathway is the transepidermal route, that theoretically involves drug penetration through the highly keratinised cells (transcellular) and through the lipid domain between these cells (intercellular pathway). The intercellular pathway is widely believed to be the principal route, and the major barrier to most drug penetration.

The specific role of hair follicles in percutaneous drug 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). Some authors used hairless rodents to probe follicular delivery (Du Plessis et al 1992). However, these animals form underdeveloped follicles and so their skin cannot exclusively represent the transepidermal pathway (Lauer et al 1996). Consequently, the use of a stratum corneum/epidermis sandwich provides a practical means for the in-vitro evaluation of the role of shunts in percutaneous drug penetration through human skin, assuming blocking of the shunts by the top stratum corneum layer.

The passive penetration of compounds with a range of hydrophilicities through stratum corneum/epidermis sandwiches was studied. The results were compared with those obtained through the epidermis in respect of the expected theoretical values of 50% reduction in flux (i.e. sandwich/epidermis flux ratio of 0.5) and 4-fold increase in lag time, if the shunt route does not contribute to the penetration process. Penetration profiles for compounds through epidermis and sandwiches were obtained by plotting the cumulative amounts versus time (Figures 1 and 2). Fluxes were calculated from the regression slopes fitted to the linear parts of each penetration profile (Table 1).

Mannitol was selected partly to test the validity of the stratum corneum/epidermis sandwich method, as it is a very polar compound ($\log P = -2.47$) that should penetrate skin essentially through the shunt pathway, at short times. Therefore, the passive penetration protocol was designed to enable us to follow mannitol penetration through the shunt route during the early period of the experiment. The experiment was also extended long enough for mannitol to reach its overall steady-state penetration.

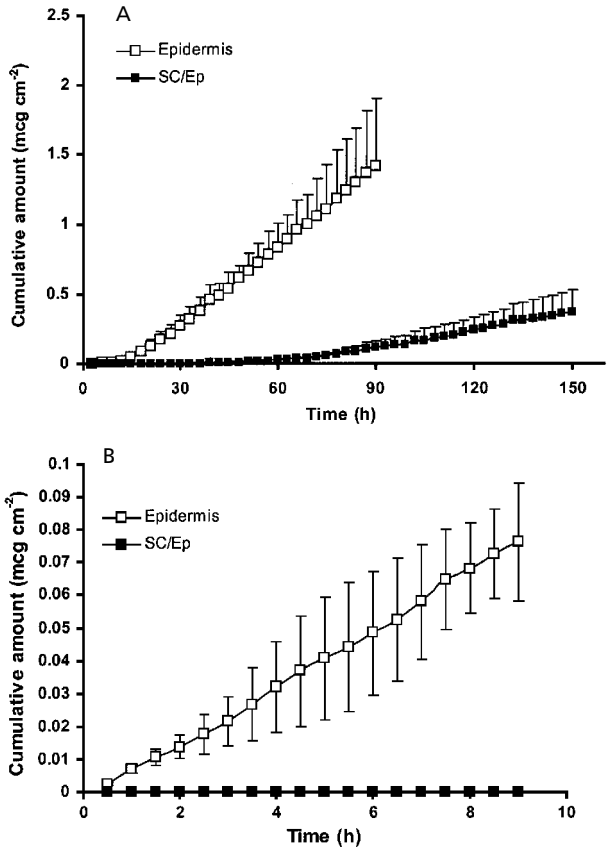

Figure 1 Passive penetration profiles of aqueous mannitol solution through human epidermis and stratum corneum/epidermis (SC/Ep) sandwiches (A), along with the early stage penetration profiles (B). Standard error bars if not shown are embedded within the symbols.

The overall penetration profile of mannitol through epidermis (Figure 1A) showed an initial rapid linear period of drug penetration, followed by a curved region until overall steady state was reached.

With the stratum corneum/epidermis sandwich, doubling the membrane thickness should quadruple the lag time relative to transepidermal permeation, so the passive penetration of mannitol through the sandwich was studied for a longer time (150 h). The penetration profile (Figure 1A) showed that there was a long period when no drug came through the sandwich; radioactive counts were not significantly above the background activity ($P > 0.05$, using Student's t-test), so the amount penetrated was considered to be zero. As the experiment proceeded, mannitol started to be detected in the receptor solution, indicating slow penetration, until steady state was reached.

To examine further the role of shunt route in mannitol penetration, the early stage (short time) of mannitol penetration is magnified in Figure 1B. With epidermis, mannitol was detected in the receptor solution from the first sampling point. Such rapid drug penetration is most probably due to drug coming through shunts. The shunt pathway resulted in a considerable amount of mannitol penetrating through the epidermis with a flux derived from the

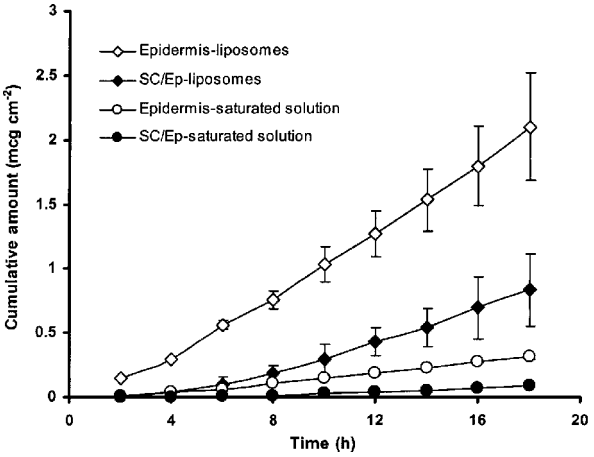

Figure 2 Passive penetration profiles of estradiol from saturated aqueous solution and ultradeformable liposome suspension through human epidermis and stratum corneum/epidermis (SC/Ep) sandwiches. Standard error bars if not shown are embedded within the symbols.

slope of the graph of 8.81 ng cm^{-2} h^{-1} (shunt route flux). The lag time was 0.34 h, which is considered to be relatively long for such a quick pathway (Table 1). This may be explained by the mannitol structure with its 6 hydroxyl groups that tend to form hydrogen bonds with the polar groups within these pores. This interaction may prolong the apparent lag time until these binding sites saturate with mannitol molecules.

For the stratum corneum/epidermis sandwich, no mannitol came through the sandwich during this initial time, indicating complete blocking of all lower shunts in the epidermis; flux was zero (Table 1). These data confirm the validity of the sandwich model as essentially blocking shunt route in the lower epidermal membrane. Mathematical modelling confirmed that there was a negligible contribution from lateral diffusion between the two skin layers (unpublished data). We therefore concluded that not only was the shunt route an important pathway for mannitol penetration through epidermis at early times, but that the skin sandwich technique allowed us to isolate the pathway from the transepidermal route.

As penetration through epidermis proceeded, mannitol also partitioned into and penetrated through the intercellular pathway, thus eventually adding to the total flux which reached steady state (Figure 1A). Thus, the steady-state flux (Table 1) represented the sum of the two penetration pathways: a shunt route that provided a rapidly observed flux through a small area and a transepidermal pathway that slowly yielded a greater flux through a large diffusional area of the skin, the intact stratum corneum (although the cross-sectional area of the intercellular matrix is much smaller than the apparent skin surface). The lag time (16 h) was relatively long and could be explained again by mannitol's polarity and its binding with the polar head groups of molecules in the skin.

For the stratum corneum/epidermis sandwich, as the experiment continued, significant amounts of mannitol were detected in the receptor solution and its penetration increased with time until a steady-state penetration was achieved (Figure 1A). The steady-state flux was 4.35 ng cm^{-2} h^{-1}, with a lag time that was 4-fold higher than that through epidermis (Table 1), agreeing with equation 2. However, the flux ratio shown in Table 1 (0.23) needs to be adjusted as explained below.

The results of the early stages of drug penetration provided good evidence for essentially complete blocking of the shunts in the bottom epidermal membrane by the top layer of stratum corneum (i.e. complete obstruction of mannitol's shunt route penetration for the stratum corneum/epidermis sandwich (Figure 1B)). Therefore, to delineate the efficiency of the stratum corneum/epidermis sandwich in blocking the shunt pathway, mannitol flux through epidermis was adjusted to evaluate the true transepidermal flux (i.e. excluding shunt route flux). This was then compared with flux through the sandwich. This adjustment was made by subtracting the shunt route flux (measured from Figure 1B) from the final steady-state flux (measured from the linear part of the overall penetration profile in Figure 1A) to give the true transepidermal flux (10.1 ng cm^{-2} h^{-1}; Table 1). This adjusted transepidermal

Table 1 Passive steady-state penetration fluxes (F) and lag times (L) of aqueous solutions of mannitol and estradiol and ultradeformable liposome suspension through human epidermis and stratum corneum/epidermis sandwich, with sandwich/epidermis flux and lag time ratios.

Penetrant	Epidermis		Stratum corneum/epidermis sandwich		F ratio[a] (F_2/F_1)	L ratio[b] (L_2/L_1)
	F_1 (ng cm^{-2} h^{-1})	L_1 (h)	F_2 (ng cm^{-2} h^{-1})	L_2 (h)		
Mannitol-early time	8.81±3.02 (3)	0.34±0.03 (3)	0.00	N.D.[c]	0.00	N.D.[c]
Mannitol-long time	18.9±4.80 (3)	16±3.4 (3)	4.35±1.33 (3)	64±5.2 (3)	(0.23)[d]	4.0
Mannitol-adjusted	10.1[e]	16	4.35±1.33 (3)	64±5.2 (3)	0.43	4.0
Estradiol	20.1±3.04 (8)	2.5±0.62 (8)	9.33±1.51 (6)	8.2±1.3 (6)	0.46	3.3
Liposomes (estradiol)	122±11.6 (7)	1.3±0.40 (7)	65.2±8.70 (5)	5.4±0.85 (5)	0.53	4.1

Values are means±s.e.m.; number of replicates are given in brackets. [a]Should be 0.5 if shunt route has no role in drug penetration, or 0.0 if shunts are the only pathway. [b]Should be 4 if shunt route has no role, or non-detectable if shunts are the only penetration pathway. [c]Not determined as no material penetrated. [d]To be adjusted in the next line. [e]Calculated by subtracting the shunt route flux from the steady-state flux.

flux is the flux due to penetration of mannitol through the bulk of the skin. From the value of the adjusted flux, we can conclude that even during steady-state permeation, mannitol penetration through the shunt route still makes a significant contribution to the overall flux. In theory, we should also adjust the lag time value. Such a lag time observed for multiple pathways is a function of the fractional areas, partition coefficients and diffusion coefficients of the individual routes (Barry 1983). However, because of the short time for the epidermal shunt route, and using an acceptable accuracy of two significant figures for lag times, we can approximate the lag time for transepidermal delivery to 16 h.

The sandwich/adjusted epidermis flux and lag time ratios were then calculated as 0.43 and 4, respectively (Table 1). These agree with the theoretically expected values, bearing in mind the variability inherent in skin penetration experiments.

In general, the results of mannitol passive penetration through epidermis and stratum corneum/epidermis sandwich showed that the shunt route may play a significant role in the overall transdermal penetration of mannitol. Moreover, the technique of using a stratum corneum/epidermis composite essentially blocks the shunt route in the bottom epidermal layer as the flux and lag time ratios correlated well with the theoretically expected values, assuming doubling of the membrane thickness.

For estradiol in saturated aqueous solution (a lipophilic penetrant), the passive penetration data across epidermis and stratum corneum/epidermis sandwiches are shown in Figure 2. The experiment was conducted for 18 h only, as lag times were expected to be relatively short based on the drug's lipophilic nature and the work of El Maghraby (2000). Penetration through epidermis gave a steady-state flux of 20.1 ng cm^{-2} h^{-1} with a lag time of 2.5 h. Such values are comparable with those in the literature (Megrab et al 1995; Essa et al 2002).

When using the stratum corneum/epidermis sandwich, the flux reduced to 46% compared with that through epidermis (see Table 1). Furthermore, the lag time increased by about 3.3 fold. For a lipophilic compound such as estradiol (log P = 2.30), the predominant penetration mechanism is expected to be through partitioning and then diffusion through the intercellular lipid domain of the stratum corneum. The sandwich/epidermis flux ratio of 0.46 is close to the expected ratio of 0.5, assuming doubling of the membrane thickness. Likewise, the lag time ratio is within 80% of the theoretical value. These data confirmed that estradiol passively penetrates mainly through the intercellular pathway and that the shunt route plays a minimal role, in agreement with El Maghraby et al (2001). The validity of the skin sandwich method was once again confirmed.

For ultradeformable liposomes (also known as Transfersomes), authors have recommended an open application protocol to obtain maximum skin delivery, as it was reported that the main driving force for such vesicle penetration is the transepidermal hydration gradient that is inhibited by occlusion (Cevc & Blume 1992). Transdermal delivery of estradiol from cholate-containing ultradeformable liposomes using the recommended non-occluded (open) application has been previously described in detail (El Maghraby et al 1999). In addition, the same group studied the role of the shunt route in the non-occluded passive penetration of estradiol from cholate- and Span 80-containing ultradeformable liposomes and traditional liposomes, using their novel technique of a stratum corneum/epidermis sandwich (El Maghraby et al 2001). They found that the shunt route, at most, marginally contributed to their passive penetration. However, simple iontophoretic studies needed a suitable volume of donor in which an electrode can be immersed, and we were also interested in comparing the iontophoretic penetration with passive delivery. Thus, we used an occlusion protocol for passive penetration of ultradeformable liposomes. The transepidermal penetration conducted for 18 h (Figure 2) showed a typical profile with a flux of 122 ng cm^{-2} h^{-1} with a lag time of 1.3 h (Table 1), in reasonable agreement with previous work (Essa et al 2002). With stratum corneum/epidermis sandwiches the flux reduced to 53% of that through epidermis, while the lag time was prolonged by 4 fold relative to that across epidermis.

It was reported that liposome of phospholipids such as phosphatidylcholine, with a low value of gel–liquid crystalline phase transition temperature, penetrate stratum corneum, mix with and disturb the rigid bilayer structure of the stratum corneum lipids, leading to increased drug penetration (Blume et al 1993; Hofland et al 1995; Kirjavainen et al 1999). Moreover, it was also claimed that egg phosphatidylcholine fluidises the stratum corneum lipid bilayers in-vitro (Kirjavainen et al 2000). Recently, it was reported that elastic vesicles could be detected intact deep in the stratum corneum (Honeywell-Nguyen et al 2002). These vesicles were mainly localised in channel-like regions that were not identified by the authors as appendages. Thus, it was expected that the main pathway for such lipid vesicles would be through the tortuous spaces between the horny cells of the stratum corneum. Although it was reported that the shunt route could play an important role for the poorly penetrating aggregates such as liposomes (Cevc et al 1996), our sandwich/epidermis flux ratio of 0.53 and 4-fold increase in lag time indicate that shunts have only a minor role in the occluded passive skin penetration from ultradeformable liposomes.

Therefore, it can be concluded that shunt route has a minimal role in the overall passive penetration of lipophilic compounds, as the reduction in flux was close to theoretical expectations calculated assuming that the stratum corneum/epidermis sandwich has a thickness double that of the epidermis (neglecting the nucleated epidermis). However, for drugs more lipophilic than estradiol, the resistance of the nucleated epidermis may, of course, also have to be taken into account.

Possible role of shunt route during iontophoretic drug penetration

Iontophoresis enhances transdermal drug delivery by three different mechanisms. Firstly, the drug ion–electric field interaction, in which the electric field imposes a repulsion

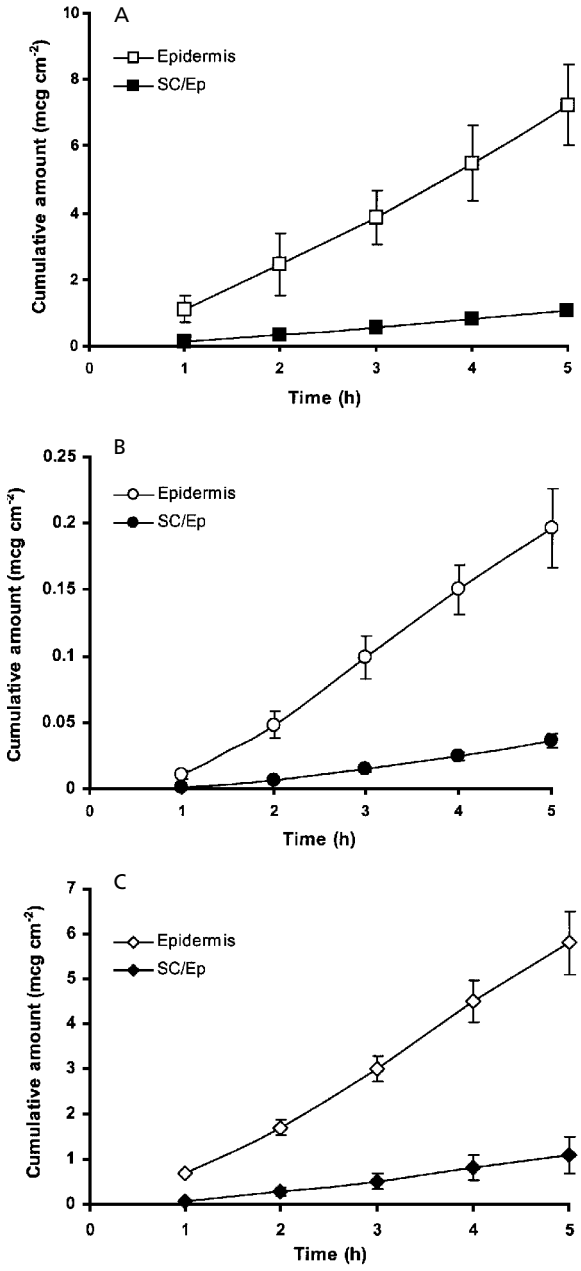

Figure 3 Iontophoretic penetration profiles of mannitol (A), saturated estradiol solution (B) and ultradeformable liposome suspension containing estradiol (C) through human epidermis and stratum corneum/epidermis (SC/Ep) sandwiches; 0.5 mA cm^{-2} constant current. Standard error bars if not shown are embedded within the symbols.

force on ions of the same charge, that adds to and often dominates the diffusion force or concentration gradient. This additional force then drives the ions through the membrane more efficiently than in pure diffusion or passive transdermal drug delivery. Secondarily, the electroosmotic flow that is the bulk fluid flow that occurs when a voltage difference is imposed across a charged membrane and is always in the same direction as the flow of counter ions.

Table 2 Iontophoretic steady-state fluxes of aqueous solutions of mannitol and estradiol and ultradeformable liposome suspension through human epidermis and stratum corneum/epidermis sandwich, along with flux ratios.

Penetrant	Flux (ng cm^{-2} h^{-1})		Flux ratio[a] (sandwich/ epidermis)
	Epidermis	Sandwich	
Mannitol	1672±54.31 (5)	252.0±9.140 (4)	0.15
Estradiol	48.40±12.30 (4)	10.60±2.785 (3)	0.22
Liposomes (estradiol)	1218±171.0 (4)	339.0±67.60 (4)	0.28

Values are means±s.e.m.; number of replicates are given in brackets.
[a]Should be 0.5 if shunt route has no role in drug penetration, or 0.0 if shunts are the only pathway.

Since human skin is negatively charged above about pH 4, counter ions are positive ions and electroosmotic flow occurs from anode to cathode carrying ions or neutral species with the solvent stream. The main pathway for electroosmotic flow is through skin appendages and aqueous pores of the skin. Thirdly, the flow of current may increase the permeability of the skin by possibly fluidising the intercellular lipid of the stratum corneum (Pikal 2001). Therefore, for neutral compounds the predominant mechanism for their iontophoretic penetration will be the electroosmotic flow through shunts, with possibly some contribution from modified skin permeability. For charged compounds the three mechanisms are encountered, with the electroosmotic flow either assisting or hindering drug transport, depending on the charge type.

As the results of passive drug penetration through stratum corneum/epidermis sandwich indicated blocking of the lower shunt pathway by the added stratum corneum layer, the same technique was used to evaluate the role of shunt route during iontophoretic delivery. The same three model penetrants were used, so as to elucidate any potential relation between the nature of the penetrant and the possible iontophoretic pathways. As the great majority of skin iontophoresis studies used constant current instead of constant voltage, and to compare the results with our previous work, constant current was also used. It is often preferable to use 0.5 mA cm^{-2} current density as a maximum density for clinical relevance (Banga 1998), so this current density was used in our study.

As electroosmotic flow is considered as the main driving force for iontophoretic penetration of neutral compounds, both mannitol and estradiol were driven under the anode. Being negatively charged, ultradeformable liposomes were iontophoresed under the cathode. The cumulative amount versus time plots through epidermis and stratum corneum/epidermis sandwiches are shown in Figure 3. Fluxes were calculated from slopes of the linear parts of each plot (Table 2). As iontophoresis should rapidly increase skin drug penetration within minutes, lag times will not be considered in this part of work.

From the shape of all plots we see that there is a relatively

slow build up to a straight line, shown as a slight curving of each plot at early times. This suggests that the membrane barrier properties initially decreased as current was constantly applied. Fluxes were therefore determined from the final time points of each plot at which steady drug penetration was achieved.

For the highly polar mannitol (Figure 3A), anodic iontophoresis through epidermis markedly enhanced penetration; the iontophoretic flux was about 90-fold higher than that of passive epidermal flux, in reasonable agreement with Kim et al (1993). This improvement in penetration under current application most probably arises from electroosmotic flow of the solvent through the aqueous shunt pathways of the skin. Therefore, blocking of these shunts by addition of a top layer of stratum corneum should markedly inhibit penetration. However, the results through stratum corneum/epidermis sandwiches showed that in spite of a large reduction in the flux, a considerable amount of mannitol came through the sandwich (Table 2); the sandwich/epidermis flux ratio was 0.15, instead of 0.0 assuming blocking of all shunts. As a highly polar compound, mannitol was expected to penetrate mainly through aqueous pathways. The achieved flux ratio suggested that the applied current formed additional aqueous pores within the bulk of the skin, through which mannitol molecules were carried by the solvent via electroosmosis. It is known that most of the applied current traverses the skin through the least resistant skin region (appendages) that had been blocked in the stratum corneum/epidermis sandwich. However, as constant current was applied, so the membrane resistance changed, most probably by forming new aqueous pathways. The shapes of the iontophoretic penetration profiles for all penetrants provided good evidence for such changes in skin as more pores were formed (see Figure 3). This may explain why the sandwich/epidermis flux ratio did not reduce to near zero, as might be expected from a simple unchanging skin sandwich model.

For estradiol in saturated aqueous solution, the iontophoretic penetration profiles through epidermis and stratum corneum/epidermis sandwiches are shown in Figure 3B. Iontophoresis improved estradiol penetration through the epidermis compared with passive input (Table 2), where the iontophoretic transepidermal flux was 2.4-fold higher than the passive one. When using stratum corneum/epidermis sandwich, the iontophoretic flux reduced, yielding a sandwich/epidermis flux ratio of 0.22 due to eradication of electroosmotic flow through the shunt route. However, the flux ratio was higher than that of mannitol, indicating that estradiol penetrated through the sandwich to a greater extent. This may be because of the lipophilic nature of estradiol that would favour the intercellular lipid pathway, as shown by the results of passive penetration. So in addition to drug movement along with the bulk solvent stream through the electrically created artificial pores, estradiol also penetrated through the intercellular lipid domain of stratum corneum that had become more disorganised by current application.

With ultradeformable liposomes, passive penetration studies indicated a minimal role for the shunt pathway and implied that the intercellular route was the major portal of entry for these vesicles. The iontophoretic delivery of estradiol-containing ultradeformable liposomes in deionised water was previously investigated and the results reflected an enhancement of the encapsulated estradiol penetration relative to passive delivery (Essa et al 2002). It was thus suggested that such vesicles, with their specific flexible properties, could penetrate at least partly through the skin under iontophoresis. In this study, we investigated iontophoretic delivery of the same vesicles through epidermis and stratum corneum/epidermis sandwich to discover whether the shunt route has any role in enhanced delivery of such complex structures. As the net charge on the liposomal surface was negative, cathodic iontophoresis was employed. The cumulative amount versus time plots of ultradeformable vesicles through epidermal membranes and sandwiches are shown in Figure 3C; steady-state fluxes are in Table 2. Comparison of iontophoretic flux through epidermis to passive transepidermal flux showed about a 10-fold enhancement, although the flux was against electroosmosis. As mentioned above, the phospholipid component of vesicles can fuse with and temporary fluidise the intercellular lipid domain of stratum corneum. Additionally, the applied current can partially disorganise the same area of stratum corneum (Jadoul et al 1996). Therefore, iontophoresis-improved penetration could be explained by the synergistic fluidising effect of both phospholipids and current on the intercellular pathway in the bulk of skin (Kirjavainen et al 2000). Therefore, it was expected that when using stratum corneum/epidermis sandwich the iontophoretic flux would be reduced to the theoretically expected value (i.e. 0.5), if shunts have no role in liposomal penetration. Unexpectedly, this ratio went down to 0.28 after using the sandwich (Table 2), indicating a significant participation of the shunt route in the iontophoretic delivery of the vesicles through epidermis. As the lipid vesicles contained surfactant as an edge activator, it was proposed that they could respond to external stress by transient concentration of surfactant molecules at the site of the highest stress (Cevc et al 1998). In our studies, the applied current of the same polarity as the liposomes forced them to move towards the skin by repulsion, and could be considered as an external stress that allows the vesicle to respond by deformation. Thus, we may predict that such vesicles may become deformable enough to squeeze within the appendages. Hence, this leads us to conclude that despite the lipophilic nature of these vesicles and their relatively large size, the shunt route through skin appendages may possibly provide a significant contribution to the overall iontophoretic delivery of ultradeformable liposomes through skin.

Conclusions

Occluded passive penetration studies using a human skin sandwich technique indicated that the shunt route played a significant role in the early and steady-state flux of the hydrophilic compound, mannitol. However, its contribution to the overall passive penetration of a lipophilic compound (estradiol) and lipid vesicles was minor; the

transepidermal pathway through the intercellular lipid domain of the intact stratum corneum is suggested as the main pathway. During iontophoresis, the shunt route has an important role in penetration, the degree of which depends on the nature of the penetrant, being higher for a polar compound. Ultradeformable liposomes, regardless of their lipophilic nature and relatively large size, can penetrate through shunts under iontophoresis, aided by their deformable properties. However, anatomical shunts are not the only possible iontophoretic pathway and the theory of artificial pore formation due to disorganisation of the intercellular lipid lamellae of the barrier is also supported.

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