J. Pharm. Pharmacol. 1999, 51: 1123–1134
Received March 17, 1999
Accepted May 25, 1999

Skin Delivery of Oestradiol from Deformable and Traditional Liposomes: Mechanistic Studies

GAMAL M. M. EL MAGHRABY, ADRIAN C. WILLIAMS AND BRIAN W. BARRY

Drug Delivery Group, Postgraduate Studies in Pharmaceutical Technology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK

Abstract

Deformable vesicles and traditional liposomes were compared as delivery systems for oestradiol to elucidate possible mechanisms of drug delivery through human skin. Accordingly, epidermal permeation of oestradiol from optimized deformable vesicles and traditional liposome formulations was studied under low dose non-occluded conditions. Five mechanisms were investigated. A free drug mechanism compared low-dose permeation through skin with drug release determined after separation of the free drug. Penetration enhancement was researched by studying skin pretreatment with empty vesicles. Improved drug uptake by skin was monitored by dipping stratum corneum into different formulations for 10 min and determining drug uptake. The possibility that intact vesicles permeate through the epidermis was tested by comparing permeation from 136-nm vesicles with that from >500-nm vesicles, assuming that penetration depends on vesicle size. The possibility that different entrapment efficiencies in alternative formulations could be responsible for the difference in delivery was also evaluated.

Lipid vesicles improved the skin delivery of oestradiol compared with delivery from an aqueous control. Maximum flux (J_{max}) was increased 14- to 17-fold by use of deformable vesicles and 8·2- to 9·8-fold by use of traditional liposomes. Deformable vesicles were thus superior to traditional liposomes. Drug release was negligible over the period during which skin flux was maximum. Pretreatment with empty vesicles resulted in an enhancement ratio of 4·3 for pure phosphatidylcholine (PC) vesicles but the enhancement ratio ranged from only 0·8 to 2·4 for other formulations. Vesicles increased drug uptake into the stratum corneum 23- to 29-fold. Relative flux values obtained from small and large vesicles were similar. No correlation was found between entrapment efficiency and skin delivery.

The results showed no evidence of a free drug mechanism, but revealed a possible penetration-enhancing effect for pure PC vesicles, although this was not the only mechanism operating. The positive uptake suggested that lipid vesicles increased drug partitioning into the skin. The data provided no evidence for in-vitro liposome penetration through skin as distinct from vesicle penetration into the stratum corneum.

The barrier nature of the skin makes it difficult for most drugs to permeate this membrane (Barry 1983). The strategy of using lipid vesicles to overcome this difficulty is controversial. Most reports in this field describe the localizing effect of liposomes i.e. deposition in the epidermis, dermis and appendages (Mezei & Gulasekharam 1980, 1982; Touitou et al 1994; Bernard et al 1995; Coderch et al 1996; Fresta & Puglisi 1996). A transdermal effect (penetration into the systemic circulation and body organs) has occasionally been recorded (Mezei 1992). Recently a special type of highly deformable lipid vesicle (a transfersome) has been reported to penetrate intact skin if applied non-occlusively in-vivo (Cevc & Blume 1992; Cevc et al 1996). In addition, alternative mechanisms for skin delivery of drugs from liposomal systems have been considered (Ganesan et al 1984; Cevc & Blume 1992; Hofland et al 1995; Cevc et al 1996).

Correspondence: B. W. Barry, Drug Delivery Group, Postgraduate Studies in Pharmaceutical Technology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK. Preliminary data were presented at the Sixth International Conference on Perspectives in Percutaneous Penetration, Leiden, The Netherlands, September 1998.

Accordingly, the aim of this study was to investigate in-vitro skin delivery of a model lipophilic drug (oestradiol) from optimized deformable vesicles (El Maghraby et al 1998) and from traditional liposome formulations. The project was then extended to study possible mechanisms by which different lipid vesicles could improve skin delivery of oestradiol. Five potential mechanisms of action were investigated. First, a free drug mechanism whereby the drug is released from the vesicles and then independently permeates the skin; this was researched by comparing low-dose permeation through skin with drug release determined after separation of the free drug. Second, penetration enhancement was tested by studying skin pretreatment with empty vesicles. Third, improved drug uptake by skin was monitored by dipping stratum corneum into different formulations for 10 min and determining drug uptake. Fourth, the possibility that intact vesicles permeate the tissue was probed by comparing permeation from 136-nm vesicles with that from >500-nm vesicles, assuming that permeation depends on vesicle size. The selected size-range was based on the reported characteristics of deformable vesicles, which are supposed to be able to permeate pores one tenth their own diameters; thus, deformable vesicles of sizes up to 200–300 nm can permeate intact skin (Cevc et al 1995). Accordingly, the selected sizes encompassed small vesicles (136 nm) in the range believed to permeate intact skin and larger vesicles (>500 nm) outside this range, which should therefore not traverse intact skin. In addition to these mechanisms, the possibility that different entrapment efficiencies of formulations could be responsible for any variation in performance was also evaluated by comparing skin delivery with entrapment efficiency.

The preparations included deformable vesicles and traditional liposomes. Details of each formulation are summarized in Table 1. Deformable vesicles included three optimized formulations (El Maghraby et al 1998) which contained phosphatidylcholine (PC) as the main component together with edge activators which provide the deformability of these vesicles (Cevc et al 1995). These edge activators included sodium cholate, reported by Cevc et al (1995), and two other surfactants, Span and Tween 80, used at optimum concentrations. Traditional liposome formulations included the prototype pure PC liposomes (non-rigid, i.e. with low membrane transition temperature T_m) and PC mixed with cholesterol, a membrane stabilizer. In addition two rigid formulations were used (pure dipalmitoylphosphatidylcholine (DPPC) and DPPC with cholesterol). Thus the systems selected included a variety of non-rigid, deformable, membrane-stabilized and rigid liposomes.

Materials and Methods

Materials

Oestradiol, [2,4,6,7-^{3}H(N)] was obtained from NEN Life Science Products. Phosphatidylcholine (PC) from soybeans (purity 99%), 17β-oestradiol (purity 98%), dipalmitoylphosphatidylcholine (DPPC), sodium cholate, sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80), cholesterol and Sephadex G50 medium were from Sigma (St Louis, MO). Scintillation fluid, Opti-Phase Hisafe 3 was obtained from LKB Scintillation Products (UK). Soluene 350 (tissue solubilizer) was obtained from Packard (Meriden, USA). All chemicals were used without further purification. Water was double-distilled.

Preparation of liposomes

Large multilamellar vesicles (LMLV) were prepared by mechanical shaking. Briefly, the lipid mixtures were dissolved in ethanol or in 2 : 1 chloroform–ethanol, depending on lipid solubility—thus cho-

Table 1. Formulation codes, compositions, entrapment efficiencies and vesicle sizes (large multilamellar vesicles) expressed as Z averages

Code[a]	Composition	Type	Entrapment efficiency (%)	Z-average (nm)
D1	Phosphatidylcholine–sodium cholate; 86 : 14 (w/w)	Deformable	95 ± 1·2	577 ± 13
D2	Phosphatidylcholine–Span; 86·7 : 13·3 (w/w)	Deformable	94 ± 0·30	566 ± 49
D3	Phosphatidylcholine–Tween; 84·5 : 15·5 (w/w)	Deformable	93 ± 0·70	557 ± 29
T1	Pure phosphatidylcholine	Non rigid	99 ± 0·58	777 ± 41
T2	Phosphatidylcholine–cholesterol; 1 : 1 (molar ratio)	Membrane-stabilized	74 ± 1·5	929 ± 37
T3	Pure dipalmitoylphosphatidylcholine	Rigid	96 ± 1·0	1038 ± 58
T4	Dipalmitoylphosphatidylcholine–cholesterol; 2 : 1 (molar ratio)	Rigid	91 ± 1·5	1214 ± 57

Data are means ± s.e.m. (n = 3). [a]D = deformable, T = traditional.

lesterol- or DPPC-containing formulations were dissolved in chloroform–ethanol. Radiolabelled oestradiol was added to furnish 1 mg mL^{-1} (25 μCi mL^{-1}) in the final preparations. The organic solvent was removed by rotary evaporation above the lipid transition temperature—room temperature (deformable and pure PC vesicles) or 50°C (cholesterol- or DPPC-containing formulations). Final traces of solvent were removed under vacuum, overnight. The deposited lipid films were hydrated either with 7% v/v ethanol in distilled water (deformable vesicles) or with distilled water (traditional formulations) by rotation at 60 rev min^{-1} for 1 h at the corresponding temperatures; the resulting vesicles were swollen for 2 h at room temperature.

To prepare smaller vesicles, LMLV were bath-sonicated at room temperature or 50°C for 30 min using a B12 FTZ bath sonicator (New 1990). The sonicated vesicles were homogenized by manual extrusion 10 times through a sandwich of 200- and 100-nm polycarbonate membranes. The final lipid concentration in all suspensions was 5% w/v.

Determination of entrapment efficiency

The entrapment efficiency was determined, after separation of the non-entrapped drug, by use of the mini-column centrifugation method (Fry et al 1978; New 1990). Sephadex G50 (10 g) was swollen in distilled water at room temperature, with occasional shaking, for at least 5 h, after which the gel was stored at 4°C. To prepare the mini-column, Whatman GF/B filter pads were placed at the bottoms of the barrels of 2·5 mL syringes which were filled with the gel. Excess water was removed by centrifugation (WIFUG, Bradford, UK; laboratory centrifuge) at 3000 rev min^{-1} for 3 min, and 500 μL liposome suspension was applied dropwise to the centre of the column, followed by centrifugation as before and collection of vesicles. Distilled water (625 μL) was added to the mini-column and centrifugation was repeated. Liposomes (depending on their type and size) can be recovered from the first or the first and second stages of centrifugation (New 1990). Here, two stages were necessary to recover the vesicles. No free drug remained (tested by its absence in the centrifugate after application of saturated drug solution instead of the vesicles). When a saturated drug solution was used instead of the liposome suspensions, all the drug remained bound to the gel. This confirmed that there would be no free drug present after recovering the vesicles. The amount of drug entrapped in the vesicles was then determined by liquid-scintillation counting (Tri Carb Packard counter) after correction for the dilution factor, and the amount of drug entrapped was calculated.

Determination of drug release

Liposome suspensions were incubated at 32°C (to correlate with skin permeation experiments) and 500-μL samples were taken at different times and the free drug was separated by mini-column centrifugation as above. The amount of drug released was then calculated indirectly from the amount of drug entrapped taking the amount entrapped at zero time as the initial amount (100% entrapped and 0% released).

Determination of vesicle size

Samples were prepared in distilled water filtered through 0·2-μm membranes and diluted with filtered saline before sizing by photon-correlation spectroscopy (Zetasizer, Malvern Instruments, Malvern, UK).

Preparation of human skin membranes

Mid-line abdominal Caucasian skin samples obtained post-mortem were from 39 donors, 31 female, average age 71·1 ± 9·8 years. The 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 then 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 samples, the epidermal membranes were floated overnight, stratum corneum side up, on an aqueous solution of trypsin (0·0001% w/v) and sodium bicarbonate (0·5% w/v) maintained at 37°C. To remove digested matter, membranes were squeezed between two filter papers, placed on the filter paper, with nucleated tissue side uppermost, and any remaining digested material was removed by washing with water and gentle swabbing. The stratum corneum was then floated on aqueous sodium azide (0·002% w/v) for 1 h to ensure removal of any remaining digested matter. The membranes were dried on a mesh screen for 24 h and rinsed with cold acetone for 10 s before storage in a desiccator.

A hydration protocol maintained the reported in-vivo transepidermal hydration gradient (Warner et al 1988) which has been proposed as generating the driving force for transfersome skin penetration (Cevc & Blume 1992). The membranes were thus

floated, with the stratum corneum side up, on 0·002% aqueous sodium azide and the upper surface was left open to the atmosphere for 24 h (open hydration protocol).

Permeation experiments

In-vitro permeation studies used an automated diffusion apparatus with cells having flow-through receptor compartments with 0·126 cm^2 areas (Akhter et al 1984). Sink conditions were maintained by pumping heated degassed receptor solution (0·002% w/v aqueous sodium azide) through the receptor compartments at 2 mL h^{-1}. Epidermal membranes were mounted, with the stratum corneum side up, and equilibrated for 12 h with receptor fluid flowing beneath the membranes and the donor compartments dry and open to the atmosphere (to continue the open hydration protocol). The system maintained the skin surface at 32°C.

Permeation studies involved a low-dose design (20 μL dose, open application) and were performed in two stages. The first regime used aqueous sodium azide as the receptor medium for 12 h at the end of which the donor compartments were washed five times with warm (45°C) receptor fluid. The second stage employed 50% v/v ethanol in sodium azide as the receptor solution for a further 12 h. This design enabled calculation of permeation parameters from the first stage. It also gave a good measure of skin deposition as determined by using 50% ethanolic receptor which will diffuse through the skin, disrupting any liposome structure and extracting the deposited drug from the skin. This second step was performed after removing the donor for two reasons—to avoid the effect of ethanol back-diffusion to the surface, and thus possible extraction of drug from the donor compartment, and to ensure that the possible effect of 50% ethanol on membrane permeability would not alter the results. Saturated aqueous oestradiol was used as control.

In addition to measuring the permeation of oestradiol from different vesicles, the effect of skin pretreatment with empty vesicles was studied to investigate any penetration-enhancing effect of liposome components. This involved an open application of 20 μL empty vesicles for 12 h, then washing and studying the permeation of oestradiol from saturated aqueous solution through the treated membranes using the same experimental design as above. The control cells were treated with 20 μL water.

Uptake studies

Stratum corneum membranes were cut into 13-mm diameter discs, weighed dry, and hydrated for 48 h on sodium azide solution (Megrab et al 1995). To study the effect of pretreatment with empty liposomes on drug uptake into the stratum corneum, the hydrated membranes were soaked in 0·5-mL empty liposome suspensions for 12 h. The control was soaked in distilled water. The membranes were dipped into a filtered saturated drug solution for 10 min (Megrab et al 1995), dried on tissue paper, and the amount of drug present in the membrane was determined by scintillation counting. This involved solubilizing the membranes in tissue solubilizer (Soluene 350, 1 mL) for 2 h then neutralizing with glacial acetic acid (0·1 mL), adding scintillation fluid (5 mL), and leaving for at least 16 h for chemiluminescence to subside before liquid-scintillation counting.

To study the effect of liposomal encapsulation of oestradiol on its uptake, the membranes were dipped in liposome suspensions (0·5 mL) for 10 min. Membranes were similarly dipped into filtered saturated drug solution as a control. As described above, the membranes were dried and solubilized before liquid-scintillation counting. The amount of drug taken up by the stratum corneum membranes was then calculated by use of equation 1:

$$\text{Uptake} = (\text{CPM} \times C_0)/(\text{CPM}_0 \times \text{W}) \qquad (1)$$

where CPM denotes the counts min^{-1} of the drug in the membrane, C_0 is the amount of drug in 1 mL drug solution or liposome suspension, CPM_0 denotes the counts min^{-1} in 1 mL of the corresponding solution and W is weight of the corresponding dry membrane disc. Uptake ratios (UR) were the ratios of the test values to the control values. The Student *t*-test was used to test for significance.

Results and Discussion

Entrapment efficiency

The entrapment efficiency is expressed as the percentage entrapment of the initial drug added (Table 1). The data revealed high entrapment efficiencies, as expected, for a lipophilic drug such as oestradiol. This efficiency depended on the concentration of phospholipid; incorporation of cholesterol or surfactant reduced it. These results indicated that the amount of drug added was sufficient to saturate the lipids and thus maintain equal (maximum) thermodynamic activity in all formulations. Thus the

maximum entrapment efficiency obtained was 99% for pure PC liposomes. Accordingly, at least 1% of the initial drug added remained free. This amount of free drug (at least $10\,\mu g\,mL^{-1}$) is above the saturation solubility of oestradiol in water.

Drug release

The in-vitro release of a lipophilic steroid, progesterone, from DPPC liposomes was reported to be negligible over 60 h (Ganesan et al 1984). In the current study at 32°C we monitored the release of our lipophilic model drug, oestradiol, from liposomes of different lipid composition. Figure 1 shows that release depended on the lipid composition of the vesicles. A similar pattern of release was obtained from all formulations—a stable period during which negligible release occurred and after which release could be detected. The duration of this stable period again depended on vesicle composition.

Starting with traditional liposomes, drug release from pure PC vesicles, the prototype of non-rigid traditional liposomes (T1) revealed the general trend discussed above with 6% (approx.) of drug liberated after 48 h. The release pattern started with a stable period after which release began at a relatively high rate and then tailed off (Figure 1A). Incorporation of cholesterol, a membrane stabilizer (T2), reduced the amount of drug released but did not inhibit release completely (Figure 1B) . The release pattern and the action of cholesterol indicated that drug release from T1 could be partially because of liposome breakdown, promoted by temperature (32°C—but see below for lack of change in vesicle size in 48 h) and partially because of drug diffusing from the vesicles.

For the prototype of rigid traditional liposomes, pure DPPC vesicles (T3), the stable time was longer than for non-rigid vesicles and only 3% (approx.) oestradiol was released after 48 h (Figure 1C). This indicated better drug retention in rigid vesicles at 32°C, which is below the transition temperature of DPPC (41·4°C). Incorporation of cholesterol (Figure 1D) again improved drug retention but did not inhibit drug release completely.

For deformable vesicles which contained different edge activators (surfactants) with PC, the release patterns (Figure 1E–G) revealed characteristic sigmoidal shapes. This pattern indicated release as a result of the vesicles being more leaky because of incorporation of these edge activators and possible lipid autoxidation with the characteristic stages—initiation, propagation and termination. Cholate and Span had greater effects than Tween.

To summarize, oestradiol release from lipid vesicles depends on lipid composition, with better retention by rigid phospholipid liposomes (DPPC). Incorporation of surfactants increased the amount of drug released. In contrast, cholesterol reduced the amount of drug released with time.

In a trial attempting to correlate drug release with changes in vesicle size (used as a tool to enable further understanding of the mechanism of drug release), three formulations, pure PC (T1), PC–cholesterol (T2) and cholate-containing (D1) vesicles were selected and vesicle size was monitored with time at 32°C. The data (Figure 2) revealed significant ($P < 0{\cdot}05$) changes in vesicle diameters only after 48 h and again cholesterol reduced this change. These results indicated that vesicle size does not affect drug release in the first 48 h.

Vesicle size

Expressing vesicle size as a Z average, the mean sizes of the small unilamellar vesicles ranged from 127 to 146 nm with no significant difference ($P > 0{\cdot}05$) between different liposomal formulations. This result is expected for vesicles prepared by sonication and homogenized by tenfold manual extrusion through polycarbonate membranes. For large multilamellar vesicles (LMLV), however, the diameter depended on the lipid composition of the formulations (Table 1). Cholesterol resulted in a trend of somewhat increased vesicle size (pure PC compared with PC–cholesterol and DPPC compared with DPPC–cholesterol), a finding in good agreement with work on MLV vesicles analysed using the same technique (Panico et al 1993). Surfactants significantly ($P < 0{\cdot}05$) reduced vesicle size (cholate-, Span 80- and Tween 80-containing vesicles compared with pure PC liposomes).

Skin delivery of oestradiol—deformable vesicles compared with traditional liposomes

Non-occluded application of deformable vesicles (transfersomes) has been recommended for optimum delivery through skin in-vivo. This procedure produces the transepidermal hydration gradient which their proponents believe is the driving force for vesicle transfer through skin (Cevc & Blume 1992). Accordingly, a low-dose design (20 μL open application) was used by us in two stages (see section Permeation experiments). Data analysis entailed plotting curves of the cumulative amount permeating against time. Rates were calculated from the slopes of the line between each two

adjacent points. Values plotted at corresponding mid-time points produced the rate plots. The individual rate plots were fitted to a polynomial and the equations of the best fits were used to calculate the maximum flux (J_{max}) and the time of J_{max} (T_{max}). The total amount permeating in the second phase was taken as a measure of skin deposition (El Maghraby et al 1998). Saturated aqueous oestradiol (maximum thermodynamic activity) was used as control.

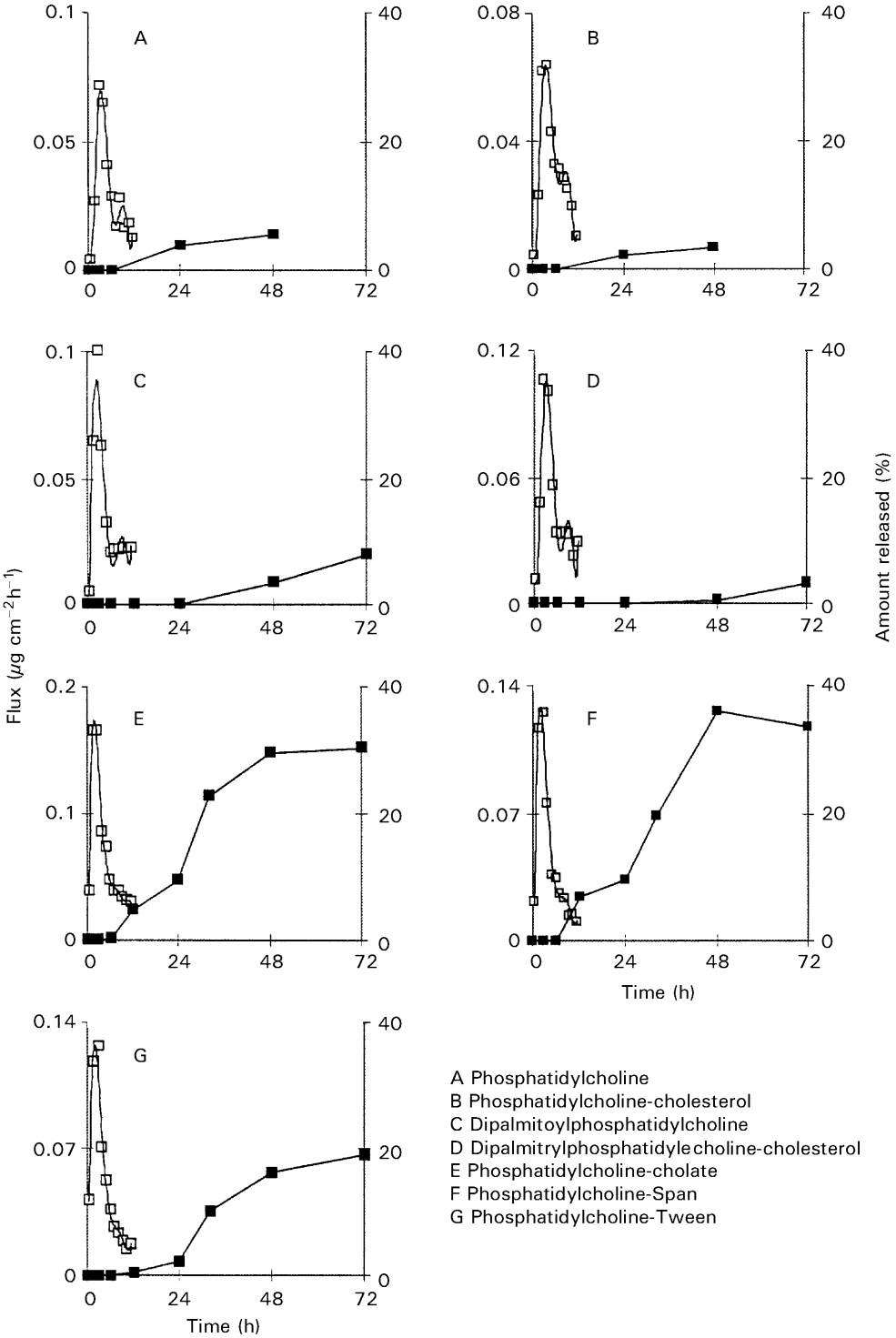

Figure 1. Examples of oestradiol release (■) from traditional (A–D) and deformable (E–G) liposomes, studied at 32°C and plotted in comparison with the transepidermal flux (□) of oestradiol from the same vesicles (using the automatic diffusion apparatus, low-dose open application).

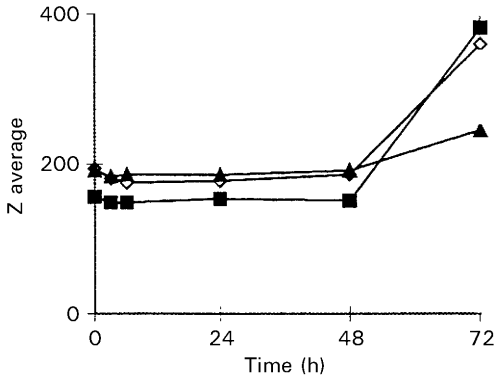

Figure 2. Change of vesicle size with time at 32°C for pure phosphatidylcholine vesicles (□), phosphatidylcholine–cholesterol liposomes (▲) and cholate-containing deformable vesicles (■).

J_{max} for oestradiol permeating from saturated aqueous solution (Tables 2 and 3) varied from 5·84 to 11·5 ng $cm^{-2} h^{-1}$, in good agreement with the values reported after steady-state studies (Megrab et al 1995).

Deformable vesicles and traditional liposomes were both studied. J_{max}, T_{max} and skin deposition values from the test formulations and values of their corresponding controls are summarized in Table 2; the acceptable s.e.m. values show that reproducibility was good. When different formulations were compared, relative values (test/own control) were used to minimize the effects of skin variability. These relative values are also presented in Table 2.

Deformable vesicles containing sodium cholate have been reported to penetrate intact skin in-vivo and to transfer therapeutic amounts of drugs with an efficiency comparable with subcutaneous administration (Cevc et al 1995; Paul et al 1995). However, most publications concerning traditional liposomes with variable lipid composition reported only a localizing effect where liposomal delivery produced more drug in the skin strata and reduced systemic absorption (Mezei & Gulasekharam 1980, 1982; Touitou et al 1994; Bernard et al 1995; Coderch et al 1996; Fresta & Puglisi 1996). Few studies recorded transdermal delivery whereby liposomal input improved the systemic effect (Artman et al 1990a, b; Mezei 1992).

In our study, deformable vesicles (cholate-, Span 80- and Tween 80-containing vesicles, D1–D3) significantly improved skin delivery of oestradiol (Table 2). They increased J_{max} 17-, 17- and 14-fold and increased skin deposition 9·4-, 9·2- and 11-fold compared with controls. They reduced T_{max} by 16, 12 and 11% compared with controls. Although these reductions were not significant ($P > 0.05$) there was a clear trend with T_{max} always decreasing for 36 diffusion cells. The results show that relative

Table 2. In-vitro transepidermal maximum flux, its time, and skin deposition (values and ratios relative to controls) of oestradiol from deformable and traditional small unilamellar liposomes after open application of 20 μL.

Formulation[a]	n	Maximum flux (ng $cm^{-2} h^{-1}$)	Relative maximum flux	Time of maximum flux (h)	Relative time of maximum flux	Deposition (ng $cm^{-2} \times 10^{-3}$)	Relative deposition
Cholate (D1)	17	171 ± 17***	17	1·88 ± 0·13	0·84	1·80 ± 0·23***	9·4
Control	17	9·88 ± 1·1		2·25 ± 0·13		0·191 ± 0·025	
Span (D2)	10	161 ± 33**	17	1·96 ± 0·11	0·88	1·22 ± 0·25**	9·2
Control	10	9·26 ± 1·6		2·22 ± 0·19		0·133 ± 0·016	
Tween (D3)	9	128 ± 12***	14	1·98 ± 0·16	0·89	1·39 ± 0·28**	11
Control	10	9·26 ± 1·6		2·22 ± 0·19		0·133 ± 0·016	
Pure phosphatidylcholine (T1)	9	98·8 ± 18**	8·6	2·41 ± 0·16	1·0	1·55 ± 0·33**	7·2
Control	12	11·50 ± 1·3		2·42 ± 0·20		0·217 ± 0·015	
Phosphatidylcholine–cholesterol (T2)	12	74·10 ± 16**	8·2	2·55 ± 0·19	1·1	1·29 ± 0·20***	8·4
Control	10	9·04 ± 1·5		2·30 ± 0·11		0·155 ± 0·014	
Pure dipalmitoylphosphatidylcholine (T3)	10	109 ± 21**	9·8	2·95 ± 0·16	1·2	1·96 ± 0·52**	10
Control	11	11·10 ± 1·6		2·37 ± 0·14		0·189 ± 0·017	
Dipalmitoylphosphatidylcholine–cholesterol (T4)	9	83·8 ± 15**	8·2	2·61 ± 0·18	1·1	1·860 ± 0·40**	9·9
Control	9	10·2 ± 1·7		2·38 ± 0·17		0·188 ± 0·032	

Data are means ± s.e.m. (n is the number of replicates). **$P < 0.01$, ***$P < 0.001$ compared with control (saturated aqueous solutions). [a]D = deformable, T = traditional.

Table 3. Effect of pretreatment of skin with empty liposomes on the in-vitro transepidermal maximum flux, its time, and skin deposition of oestradiol delivered from saturated aqueous solution.

Formulation[a]	n	Maximum flux ($ng\,cm^{-2}\,h^{-1}$)	Relative maximum flux	Time of maximum flux (h)	Relative time of maximum flux	Deposition ($ng\,cm^{-2} \times 10^{-3}$)	Relative deposition
Cholate (D1)	5	9·22 ± 6·7	1·6	1·95 ± 0·061	0·97	84·4 ± 4·3	0·52
Span (D2)	5	11·3 ± 4·0	1·9	1·88 ± 0·18	0·94	307 ± 85	1·9
Tween (D3)	5	14·1 ± 3·1	2·4	1·64 ± 0·031	0·82	171 ± 34	1·1
Pure phosphatidylcholine (T1)	4	25·4 ± 9·4	4·3	1·90 ± 0·34	0·95	378 ± 150	2·3
Phosphatidylcholine–cholesterol (T2)	3	9·69 ± 2·0	1·7	1·89 ± 0·28	0·95	88·6 ± 8·6	0·54
Pure dipalmitoylphosphatidylcholine (T3)	5	5·85 ± 0·47	0·80	2·70 ± 0·10	0·97	329 ± 170	1·1
Dipalmitoylphosphatidylcholine–cholesterol (T4)	7	10·7 ± 1·1	1·5	2·26 ± 0·22	0·81	208 ± 38	0·68
Control	5	5·84 ± 1·7		2·00 ± 0·17		163 ± 61	
Control[b]	7	7·35 ± 1·6		2·80 ± 0·15		308 ± 56	

Values and ratios are relative to controls; controls were pretreated with water; [b]control for T3 and T4. Data are means ± s.e.m. (n is the number of replicates). [a]D = deformable, T = traditional.

skin deposition was not directly comparable with relative J_{max}. When considered together with the reduced T_{max}, this could indicate that deformable vesicles improved the permeation of oestradiol through the skin more than the partitioning of the compound into the skin—if there were similar influences on permeation and partitioning we should obtain similar relative values for J_{max} and skin deposition.

Traditional liposomes (pure PC, PC–cholesterol, DPPC and DPPC–cholesterol, T1–T4) also significantly improved skin delivery of oestradiol. They increased J_{max} 8·6-, 8·2-, 9·8- and 8·2-fold and increased skin deposition 7·2-, 8·4-, 10·3- and 9·9-fold compared with controls. The relative T_{max} values were 1·0, 1·1, 1·2 and 1·1 compared with the controls showing that traditional liposomes had no effect on the time of maximum flux. These results reveal comparable values for J_{max} and skin deposition which, together with the small effect on T_{max}, indicates improved drug partitioning into the skin.

Comparison of the individual formulations (Table 2) revealed no significant ($P > 0·05$) differences between different deformable formulations (D1–D3). Similarly there were no significant differences ($P > 0·05$) between traditional formulations (T1–T4). On the other hand, deformable vesicles were better than traditional liposomes in delivering oestradiol through human skin in-vitro, although this difference did not reflect the reported highly effective action of deformable vesicles applied in-vivo. Also only low percentages of the applied dose (1·3–2%) permeated through and deposited into skin, much lower than the values reported in-vivo (at least 50%; Cevc & Blume 1992; Cevc et al 1995, 1996; Paul et al 1995). However, our overall results are indicative of improved transepidermal delivery of oestradiol from both deformable and traditional vesicles compared with delivery from saturated aqueous solution. This might indicate possible improvements in the pharmacodynamic properties of the drug when delivered from liposomes.

Mechanism(s) of improved in-vitro skin delivery of oestradiol from deformable vesicles and traditional liposomes

After obtaining these results we investigated the mechanisms of action of these formulations as skin-delivery systems. Reported mechanisms include a free drug process in which drug is released from liposomes before diffusing through skin, with drug release as the rate-limiting step; direct transfer of drug from the liposome to the skin; or a combination of both depending on the physicochemical properties of the drug (Ganesan et al 1984).

To investigate the free drug mechanism, the transepidermal flux plots of each individual formulation were compared with in-vitro drug release from the corresponding formulation (examples are given in Figure 1). Drug release was studied with aqueous dispersions of liposomes but skin permeation experiments involved open application, in which the donor water-volume decreases with evaporation time. Because it is believed that reduction of the volume of external water in liposome suspensions reduces drug release, the corre-

lation between drug release and skin permeation can be considered a rigorous test for the free drug mechanism. The results indicate that the peak flux of oestradiol through the skin occurred at a time during which drug release was negligible. This strongly suggests that a free drug mechanism does not operate for any of the formulations, in agreement with the reported mechanism for the lipophilic steroid progesterone in DPPC liposomes (T3) (Ganesan et al 1984).

Ultrastructural changes in the stratum corneum have been reported after skin was treated with liposomes containing high concentrations of phosphatidylcholine (Hofland et al 1995). In addition, depending on the lipid composition, liposomes have been recorded as interacting with stratum corneum components, destabilizing the lipid matrix without penetration of intact liposomes (Kirjavainen et al 1996). These actions suggest a possible penetration-enhancing effect of liposome components (Barry 1987). Accordingly, the effect of skin pretreatment with empty lipid vesicles on epidermal permeability to oestradiol delivered from saturated aqueous solution was studied using the same low-dose design. The results are summarized in Table 3. The data show the experiments to be highly reproducible, as indicated by the acceptable s.e.m. values. As the study involved different runs, for comparison the enhancement ratios (ER) were also calculated from J_{max} values of the treated skin and the control. Apart from pure PC vesicles, all formulations had a marginal effect on epidermal permeability to oestradiol in respect of J_{max}, T_{max} and skin deposition, suggesting the absence of a penetration-enhancing effect. The enhancement ratios were 1·6, 1·9 and 2·4 for deformable vesicles containing cholate, Span 80 and Tween 80 and 1·7, 0·8 and 1·5 for traditional PC–cholesterol, DPPC and DPPC–cholesterol formulations. Although the enhancement ratio (ER) of 4·3 obtained for pure traditional PC liposomes implies that PC could have a penetration-enhancing effect, the relative J_{max} (8·6) obtained after application of oestradiol encapsulated in the same formulation (Table 2) was significantly greater, indicating that vesicular delivery is superior to pretreatment even for the formulation giving the highest penetration-enhancing effect. It can be concluded that for maximum effect oestradiol should be encapsulated in, or applied simultaneously with, the phospholipids. Du Plessis et al (1994) came to a similar conclusion; our study did not enable us to assess the necessity for the phospholipid to be in the form of vesicles.

Workers using hydrocortisone considered it possible that the phospholipid component of liposomes could enter the skin rapidly and because hydrocortisone was dissolved in it, the steroid could ''follow the fate of phospholipid'' (Kim et al 1997). Fast penetration of liposomal lecithin into the skin of man has also been reported (Wohlrab et al 1989). Accordingly, an uptake study was designed in which stratum corneum membranes were dipped into the test formulation or aqueous solution for a short time (10 min). Both the effect of pretreatment with empty vesicles and the uptake from medicated vesicles were studied. The uptake values and ratios are summarized in Table 4. Pretreatment with empty vesicles had no significant effect on drug uptake from aqueous solution. On the other hand the uptake from medicated vesicles was significantly higher than that from the control (Table 4). The uptake ratios (UR) for vesicles and solution ranged from 23 to 29 with no significant differences ($P > 0{\cdot}05$) between individual formulations. Comparing UR for each formulation (Table 4) with the corresponding relative J_{max} values (in the range 8·2 to 17, Table 2), UR was greater, which could indicate that the uptake from vesicles is faster than that from a saturated solution. The processes of adhesion on to the skin surface, fusion or mixing

Table 4. Uptake (values and ratios relative to control) of oestradiol into stratum corneum from aqueous solution (10 min dipping) after pretreatment with different empty lipid vesicles and uptake from the corresponding medicated vesicles without pretreatment.

Formulation[a]	Uptake after pretreatment ($\mu g\,g^{-1}$)	Uptake ratio	Uptake from vesicles ($\mu g\,g^{-1} \times 10^{-3}$)	Uptake ratio
Cholate (D1)	30·0 ± 6·4	0·80	1·18 ± 0·20*	23
Span (D2)	37·4 ± 2·5	1·0	1·14 ± 0·23*	23
Tween (D3)	42·7 ± 2·0	1·2	1·19 ± 0·21*	24
Pure phosphatidylcholine (T1)	57·0 ± 15	1·5	1·22 ± 0·14**	24
Phosphatidylcholine–cholesterol (T2)	51·6 ± 17	1·4	1·44 ± 0·076***	29
Pure dipalmitoylphosphatidylcholine (T3)	ND	ND	1·19 ± 0·092**	24
Dipalmitoylphosphatidylcholine–cholesterol (T4)	ND	ND	1·25 ± 0·072***	25
Control	37·1 ± 2·0		0·0502 ± 0·0061	

Data are means ± s.e.m. (n = 3–4). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ compared with control. [a]D = deformable, T = traditional.

Table 5. In-vitro transepidermal maximum flux, its time, and skin deposition of oestradiol from deformable and traditional large multilamellar vesicles after 20 μL open application.

Formulation[a]	n	Maximum flux ($ng\,cm^{-2}\,h^{-1}$)	Relative maximum flux	Time of maximum flux (h)	Relative time of maximum flux	Deposition ($ng\,cm^{-2} \times 10^{-3}$)	Relative deposition
Cholate (D1)	6	151 ± 6·7***	15	1·96 ± 0·18	0·90	1·94 ± 0·081***	9·1
Control	6	9·84 ± 1·1		2·19 ± 0·14		0·213 ± 0·022	
Span (D2)	6	166 ± 40*	16	2·02 ± 0·22	0·91	1·82 ± 0·61*	14
Control	5	10·3 ± 2·6		2·21 ± 0·23		0·133 ± 0·024	
Tween (D3)	3	142 ± 27*	14	1·99 ± 0·38	0·90	1·79 ± 0·47	14
Control	5	10·3 ± 2·6		2·21 ± 0·23		0·133 ± 0·024	
Pure phosphatidyl-choline (T1)	12	88·9 ± 15***	9·0	2·40 ± 0·16	0·99	1·11 ± 0·13***	6·8
Control	10	9·90 ± 1·3		2·42 ± 0·19		0·165 ± 0·022	
Phos-phatidylcholine–cholesterol (T2)	8	67·4 ± 15**	6·5	2·73 ± 0·34	1·2	1·71 ± 0·26***	8·2
Control	8	10·4 ± 1·5		2·28 ± 0·12		0·209 ± 0·019	
Pure dipalmitoyl-phosphatidyl-choline (T3)	11	91·5 ± 16***	8·2	2·80 ± 0·18	1·2	1·77 ± 0·34***	9·4
Control	11	11·1 ± 1·6		2·37 ± 0·14		0·189 ± 0·017	
Dipalmitoy-lphosphatidyl-choline–cholesterol (T4)	10	93·9 ± 21**	9·0	2·70 ± 0·17	1·1	2·00 ± 0·44**	11
Control (saturated aqueous solution)	10	10·4 ± 1·7		2·40 ± 0·18		0·173 ± 0·028	

Values and ratios are relative to controls (saturated aqueous solutions). Data are means ± s.e.m. (n is the number of replicates) *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with from control. [a]D = deformable, T = traditional.

with the lipid matrix of the stratum corneum, have been suggested for liposome lipids (Kirjavainen et al 1996). The uptake results support these suggestions. Correlation of the superiority of deformable vesicles over traditional liposomes in increasing transepidermal flux with no significant difference between URs for short contact times suggests that deformable vesicles either improved the diffusion or penetrated further into the epidermis, which enabled more efficient drug clearance.

Accordingly, the epidermal permeation of oestradiol from large multilamellar vesicles (LMLV; at least 557 nm in diameter, Table 1) was studied and compared with that obtained from smaller vesicles (SULV; mean size 136 nm) in an attempt to investigate the possibility that intact vesicles penetrate the skin, assuming that this penetration is a function of the vesicle size. The SULV are less than the maximum size reported to penetrate skin and the minimum size of the LMLV is above the maximum size which can penetrate skin (Cevc et al 1995). Data obtained after low-dose non-occluded application of LMLV are summarized in Table 5.

Skin delivery of oestradiol from LMLV (Table 5) was significantly higher for individual formulations than for the corresponding control. Deformable vesicles were superior to traditional formulations with regard to J_{max}, a similar trend to that obtained for SULV—see above. To evaluate penetration of vesicles through the epidermis, maximum flux was considered. Comparison of the relative J_{max} values obtained from SULV (Table 2) with those obtained from LMLV (Table 5), on the basis of the assumption that permeation would be a function of vesicle diameter, revealed no significant differences between LMLV, a finding which suggests that intact vesicles do not permeate epidermal membrane from man in-vitro. These results are contrary to data reported for deformable vesicles in-vivo for which vesicle penetration through intact skin was proposed as the mechanism whereby drug delivery was improved (Cevc & Blume 1992). However, it should be noted that we do not yet know what happens to vesicle size on drying after open application.

To test the simple concept that different entrapment efficiencies of the formulations could be responsible for variable drug-delivery capabilities, the ranking order of entrapment efficiency of the formulations was compared with the rank orders of relative J_{max} and relative skin deposition obtained from liposomes. For entrapment efficiency (Table 1), the ranking order was vesicles containing pure PC (T1) > DPPC (T3) > cholate (D1) > Span (D2) > Tween (D3) > DPPC–cholesterol (T4) > PC–

cholesterol (T2) whereas for relative J_{max} (Table 2), it was D1 = D2 > D3 > T3 > T1 > T2 = T4 and the ranking order for the relative skin deposition was D3 > T3 > T4 > D1 > D2 > T2 > T1. No correlations were found among these rank orders. Interestingly, the two extremes in entrapment efficiency (pure PC and PC–cholesterol vesicles, T1, T2) were adjacent in the ranking order of delivery (relative J_{max} and skin deposition). Thus, variation in entrapment efficiency is not a factor responsible for different delivering capabilities of the liposomes.

Conclusions

Lipid vesicles improved in-vitro skin delivery of the lipophilic drug, oestradiol, compared with saturated aqueous solution; deformable vesicles were superior to traditional liposomes. The difference between the two types of vesicle did not reflect the reported highly effective action of deformable vesicles in-vivo. A free-drug transfer mechanism did not operate. A penetration-enhancing process was possible for pure PC vesicles although it was not the only mechanism accounting for the increased drug delivery. Liposome adhesion, fusion or even penetration into the stratum corneum were possible with potentially deeper penetration of deformable vesicles compared with traditional liposomes. However, there was no evidence to support the hypothesis that intact vesicles permeate through human epidermal membrane in-vitro.

References

Akhter, S. A., Bennett, S. L., Waller, I. L., Barry, B. W. (1984) An automated diffusion apparatus for studying skin penetration. Int. J. Pharm. 21: 17–26

Artman, C., Roding, J., Ghyczy, M., Pratzel, H. G. (1990a) Liposomes from soya phospholipids as percutaneous drug carriers: Qualitative in vivo investigations with antibody-loaded liposomes. Arzneim. Forsch. 40: 1363–1365

Artman, C., Roding, J., Ghyczy, M., Pratzel, H. G. (1990b) Liposomes from soya phospholipids as percutaneous drug carriers: Quantitative in vivo investigations with radioactivity. Arzneim. Forsch. 40: 1365–1368

Barry, B. W. (1983) Dermatological Formulations: Percutaneous Absorption, Marcel Dekker, New York

Barry, B. W. (1987) Mode of action of penetration enhancers in human skin. J. Contr. Rel. 6: 85–97

Bernard, E., Dubois, J. L., Wepierre, J. (1995) Percutaneous absorption of new antiandrogen included in liposomes or in solution. Int. J. Pharm. 126: 235–243

Cevc, G., Blume, G. (1992) Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradient and hydration force. Biochim. Biophys. Acta 1104: 226–232

Cevc, G., Schätzlein, A., Blume, G. (1995) Transdermal drug carriers: basic properties, optimization and transfer efficiency in case of epicutaneously applied peptides. J. Contr. Rel. 36: 3–16

Cevc, G., Blume, G., Schäzlein, A., Gebauer, D., Paul, A. (1996) The skin: a pathway for systemic treatment with patches and lipid-based agent carriers. Adv. Drug Del. Rev. 18: 349–378

Coderch, L., Oliva, M., Pons, M., De La Maza, A., Manich, A. M., Parra, J. L. (1996) Percutaneous penetration of liposomes using the tape stripping technique. Int. J. Pharm. 139: 197–203

Du Plessis, J., Weiner, N., Muller, D. G. (1994) The influence of in vivo treatment of skin with liposomes on topical absorption of hydrophobic drug in vitro. Int. J. Pharm. 103: R1–R5

El Maghraby, G. M. M., Williams, A. C., Barry, B. W. (1998) Optimization of deformable vesicles for epidermal delivery of oestradiol. J. Pharm. Pharmacol. 50 (Suppl.): 146

Fresta, M., Puglisi, G. (1996) Application of liposomes as potential cutaneous drug delivery systems. In vitro and in vivo investigation with radioactivity labelled vesicles. J. Drug Target. 4: 95–101

Fry, D. W., White, J. C., Goldman, I. D. (1978) Rapid separation of low molecular weight solutes from liposomes without dilution. J. Anal. Biochem. 90: 809–815

Ganesan, M. G., Weiner, N. D., Flynn, G. L., Ho, N. F. H. (1984) Influence of liposomal drug entrapment on percutaneous absorption. Int. J. Pharm. 20: 139–154

Harrison, S. M., Barry, B. W., Dugard, P. H. (1984) Effect of freezing on human skin permeability. J. Pharm. Pharmacol. 36: 261–262

Hofland, H. E. J., Bouwstra, J. A., Bodde, H. E., Spies, F., Junginger, H. E. (1995) Interaction between liposomes and human stratum corneum in vitro: freeze fracture electron microscopical visualization and small-angle X-ray scattering studies. Br. J. Dermatol. 132: 853–866

Kim, M. K., Chung, S. J., Lee, M. H., Cho, A. R., Shim, C. K. (1997) Targeted and sustained delivery of hydrocortisone to normal and stratum corneum-removed skin without enhanced skin absorption using a liposome gel. J. Contr. Rel. 46: 243–251

Kirjavainen, M., Urtti, A., Jaaskelainen, I., Suhonen, T. M., Paronen, P., Koskela, R. V., Kiesvaara, J., Monkkonen, J. (1996) Interaction of liposomes with human skin in vitro—the influence of lipid composition and structure. Biochim. Biophys. Acta 1304: 179–189

Kligman, A. M., Christophers, E. (1963) Preparation of isolated sheets of human stratum corneum. Arch. Dermatol. 88: 70–73

Megrab, N. A., Williams, A. C., Barry, B. W. (1995) Oestradiol permeation through human skin and silastic membrane: effect of propylene glycol and supersaturation. J. Contr. Rel. 36: 277–294

Mezei, M. (1992) Biodisposition of liposome-encapsulated active ingredients applied on the skin. In: Falco, O. B., Korting, H. C., Maibach, H. I. (eds) Liposome Dermatics, Springer, Berlin, pp 206–214

Mezei, M., Gulasekharam, V. (1980) Liposomes: a selective drug delivery system for the topical route of administration. I. Lotion dosage form. Life Sci. 26: 1473–1477

Mezei, M., Gulasekharam, V. (1982) Liposomes: a selective drug delivery system for the topical route of administration. Gel dosage form. J. Pharm. Pharmacol. 34: 473–474

New, R. R. C. (1990) Liposomes: A Practical Approach, Oxford University Press, Oxford

Panico, A. M., Pignatello, R., Mazzone, S., Cardile, V., Bindoni, M., Cordopatri, F., Villari, A., Micali, N., Mazzone, G. (1993) Lipid vesicles loaded with thymopentin: characterization and in vitro activity on tumoral cells. Int. J. Pharm. 98: 19–28

Paul, A., Cevc, G., Bachhawat, B. K. (1995) Transdermal immunization with large proteins by means of ultradeformable drug carriers. Eur. J. Immunol. 25: 3521–3524

Touitou, E., Schaffer, F. L., Dayan, N., Alhaique, F., Riccieri, F. (1994) Modulation of caffeine delivery by carrier design: liposomes versus permeation enhancers. Int. J. Pharm. 103: 131–136

Warner, R. R., Myers, M. C., Taylor, D. A. (1988) Electron probe analysis of human skin: determination of the water concentration profile. J. Invest. Dermatol. 90: 218–224

Wohlrab, W., Lachmann, U., Lasch, J. (1989) Penetration of lecithin from hydrocortisone-containing liposomes into human skin. Dermatol. Mon. Schr. 175: 344–377