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Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration☆

Gamal M.M. El Maghraby, Adrian C. Williams, Brian W. Barry *

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

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Abstract

The aims of this study were to refine ultradeformable liposomes for oestradiol skin delivery and to evaluate Span 80 and Tween 80 as edge activators compared with sodium cholate. Vesicles containing phosphatidylcholine (PC) mixed with edge activators and oestradiol were prepared. Entrapment efficiency and vesicle size were determined. Interactions between activators and vesicles were investigated using differential scanning calorimetry. Transepidermal permeation of oestradiol from vesicles was studied compared to saturated aqueous control in vitro. The maximum flux (J_{max}) and its time (T_{max}) were calculated from the flux curves and skin deposition was assessed. The compositions of refined formulations were predicted, liposomes prepared, and tested against control. Entrapment efficiency depended on PC concentration with some contribution from sodium cholate and Tween 80. Vesicle sizes ranged from 124 to 135 nm. Edge activators interacted with lipid bilayers and disrupted packing. The refined edge activator concentrations in PC vesicles were 14.0, 13.3 and 15.5% w/w for sodium cholate, Span 80 and Tween 80, respectively; they increased J_{max} by 18, 16 and 15-fold and skin deposition by 8, 7 and 8-fold compared with control. Ultradeformable vesicles thus improved skin delivery of oestradiol compared to control and Span 80 and Tween 80 were equivalent to sodium cholate as edge-activators.

Keywords: Skin delivery; Oestradiol; Vesicles-ultradeformable; Liposomes-ultradeformable

1. Introduction

Human skin provides an excellent barrier in two directions, controlling loss of water and body constituents and preventing entry of noxious substance from the external environment. The percutaneous route for drug administration has many advantages over other pathways including avoiding hepatic first pass effects, delivering drugs continuously, having fewer side effects and improving patient compliance (Barry, 1983; Weissinger, 1993). However, the barrier nature of the skin inhibits the penetration of most drugs. The strategy of using lipid vesicles to overcome this difficulty is gaining interest, but it remains contro-

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* Corresponding author. Tel.: +44-1274-234760; fax: +44-1274-234769.

E-mail address: b.w.barry@bradford.ac.uk (B.W. Barry)

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versial. Most relevant reports cite the localising effect of liposomes (Mezei and Gulasekharam, 1980, 1982; Touitou et al., 1994; Bernard et al., 1995; Coderch et al., 1996; Fresta and Puglisi, 1996), with transport processes reported in few cases depending on the formulation (Mezei, 1992). Additionally, a novel type of highly deformable lipid vesicle (transfersome) has been recorded to penetrate intact skin if applied non-occlusively in vivo (Cevc and Blume, 1992; Cevc et al., 1996). Transfersomes have been defined as specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. Accordingly, transfersomes resemble lipid vesicles (liposomes) in morphology but not in function (Planas et al., 1992). These authors suggest that transfersomes can respond to external stresses by rapid shape transformations requiring low energy. This high deformability allows them to deliver drugs across barriers, including skin (Cevc et al., 1995). To prepare these vesicles, so called edge activators were incorporated into the vesicular membrane; surfactants were suggested as examples of such edge activators (Cevc et al., 1993), and sodium cholate or sodium deoxycholate has been used for this purpose (Planas et al., 1992; Cevc et al., 1995, 1997; Paul et al., 1995).

The aims of the present study were to refine the formulation of ultradeformable vesicles for in vitro skin delivery of a model lipophilic drug, oestradiol, and to evaluate Span 80 and Tween 80 as edge activators compared to sodium cholate. Span 80 and Tween 80 were selected as they are pharmaceutically acceptable, typical non-ionic surfactants. The effect of different concentrations of surfactants in the vesicles on skin delivery was studied. The entrapment efficiencies, vesicle sizes, edge activator-vesicle interactions and the possibility of tritium exchange were also determined.

2. Materials and methods

2.1. Materials

Oestradiol, [2,4,6,7-^{3}H(N)] was obtained from NEN Life Science Products. Phosphatidylcholine (PC) from soybeans (purity 99%), dipalmitoylphosphatidylcholine (DPPC) (99%), 17β-oestradiol (98%), sodium cholate, sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80) and Sephadex G50 medium were purchased from Sigma Chemical Company, St Louis, MO. Water was double distilled.

2.2. Preparation of lipid vesicles

Lipid vesicles containing PC mixed with 0, 10, 16, 20 and 30% w/w sodium cholate, Span 80 or Tween 80 were prepared by the sonication method (New, 1990) using a B12 FTZ bath sonicator. The lipid components were dissolved in ethanol and a radiolabelled solution of oestradiol in ethanol sufficient to produce a drug concentration of 1 mg ml^{-1} (25 μCi ml^{-1}) in the final preparation was added. The organic solvent was evaporated under a stream of nitrogen; solvent traces were removed by maintaining the deposited lipid films under vacuum overnight. The films were hydrated with 7% v/v ethanol in distilled water by vortexing for 15 min. The hydrated vesicles were swollen for 2 h at room temperature and then bath sonicated for 30 min. The sonicated vesicles were homogenised by ten times manual extrusion through sandwiches of 200 and 100 nm polycarbonate membranes. Final lipid concentration was 5% w/v.

2.3. Entrapment efficiency

This was determined after separation of the non-entrapped drug using 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, then stored at 4°C. To prepare the mini-columns, Whatman GF/B filter pads were inserted in the bottom of the barrels of 2.5 cm^3 syringes which were then filled with gel. Excess water was centrifuged off at 3000 rpm for 3 min using a WIFUG Lab centrifuge (WIFUG, Bradford, UK), and 500 μl of liposomes was added dropwise to the centre of the column, followed by centrifugation as before. To the mini-

column, 625 μl of distilled water was added and centrifugation 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. 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 determined by liquid scintillation counting after correction for dilution.

2.4. Vesicle size

Diameters were determined using photon correlation spectroscopy employing a Zetasizer (Malvern Instruments, Malvern, UK). Samples used distilled water filtered through 0.2 μm membranes to minimise interference from particulate matter. Vesicles were suitably diluted with filtered solution of 1 mM sodium chloride before sizing.

2.5. Interaction between surfactants and lipid vesicles

High sensitivity differential scanning calorimetry (HSDSC) with a Microcal MCS differential scanning calorimeter (Microcal Amherst, MA) were used to investigate possible interactions between surfactants and liposomes. DPPC was used as a model phospholipid; vesicles of lipid concentration 5 mg ml^{-1} in distilled water were prepared as before by the bath sonication method but they were dried, and hydrated at 45°C (above the transition temperature of DPPC). Liposomes were either pure DPPC or contained the surfactants at the refined concentrations. Degassed sample and reference (water) were loaded into the corresponding cell using a special syringe with a long needle which was introduced to just above the bottom of the DSC cell. Firm and rapid injection of the last 100–200 μl removed any trapped air bubbles. Both reference and sample cells were filled completely to maintain equal volumes and similar amounts of lipid from run to run. The samples and reference were then heated from 20–60°C at 1°C min^{-1}. A baseline was run before each determination by loading the reference solution (water) in both the sample and reference cells; this was subtracted from individual results on data analysis performed with Origin DSC data analysis software.

2.6. Preparation of epidermal membrane

Human caucasian epidermal cadaver membranes were used. Mid-line abdominal samples obtained post-mortem were flattened and stored in vacuum-sealed double polythene bags at −20°C (Harrison et al., 1984). Skin samples were from 15 donors of whom 12 were female, and had a mean age of 69.2 ± 6.7 years. The membranes were prepared by a heat separation technique (Kligman and Christophers, 1963); fat and connective tissue were removed, the skin was then soaked for 45 s in a water bath at 60°C after which the epidermis was gently teased off the underlying dermis.

A hydration protocol was designed to maintain an in vivo transepidermal hydration gradient (Warner et al., 1988) which has been suggested as generating the driving force for vesicular skin penetration (Cevc and Blume, 1992). The membranes were thus floated with the stratum corneum side up on 0.002% w/v aqueous sodium azide and the upper surface was left open to the atmosphere (open hydration protocol).

2.7. Permeation experiments

In vitro permeation studies employed an automated diffusion apparatus (Akhter et al., 1984). Each diffusion cell had a flow-through receptor compartment and a diffusional area of 0.126 cm^2. Sink conditions were maintained by pumping degassed receptor solution (0.002% w/v aqueous sodium azide) at 2 ml h^{-1} through the receptor compartments. Epidermal membranes were mounted with the stratum corneum side up before equilibration for 12 h with receptor flowing and the donor compartment dry and open to the

atmosphere so as to continue the open hydration protocol. Skin surface was at 32°C.

As non-occlusive application was recommended for optimum transdermal drug delivery with highly deformable vesicles (Cevc and Blume, 1992), the permeation studies involved a low dose design (20 μl open application). They were performed in two stages. The first 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 receptor fluid (45°C). This washing protocol was verified and was found to remove more than 99% of the applied dose at zero time. The permeation parameters were calculated from this stage. The second stage used 50% v/v ethanol in sodium azide as the receptor solution for a further 12 h. During this stage ethanolic receptor will diffuse into skin, disrupting the vesicular structure of any liposomes which may have penetrated and deposited in the tissue and thus release both liposome bound and free oestradiol for collection by the receptor. As 50% ethanol used as a receptor can reduce slightly the barrier nature of the stratum corneum, the second stage was performed after removal of the donor to avoid any excess permeation due to the ethanol enhancing effect. The second stage can thus provide a useful measure for the amount of drug deposited in the stratum corneum and nucleate epidermis after 12 h delivery. This quantity is referred to as 'skin deposition'.

Assays were by liquid scintillation counting using the scintillation cocktail, OptiPhase 'HiSafe' 3, Fisher Chemicals, UK and a Tri Carb® Packard counter.

2.8. Investigation of tritium exchange with water

When using liquid scintillation counting with tritium labelling it is important to ensure that the obtained results are accurate and not falsely elevated due to tritium exchange with water. Accordingly, the transepidermal permeation of oestradiol from sodium cholate-containing lipid vesicles was studied in an additional experiment. The receptor fluid samples were divided into halves. One was dried under vacuum so that any tritium exchanged with water would evaporate, and then the residual was reconstituted with receptor fluid to the initial volume. Both halves were separately mixed with the same amount of scintillation cocktail before counting. The permeation data calculated from dried and non dried halves were compared.

2.9. Permeation data analysis

Cumulative amounts versus time plots were used to calculate the rate at the mid time points to produce the flux plots using a Visual Basic Microsoft Excel computer program. The flux data of the first stage of permeation (using aqueous receptor) were fitted by polynomial (see, e.g. Fig. 1). Polynomials best fitted the data compared with other available models (linear, logarithmic, exponential or moving average) as judged simply by correlation coefficients. A computer program based on differentiating equations of the best fit and solving the differentiated equations by the bisection method determined the time at which maximum flux was achieved (T_{max}). This T_{max} was then used to calculate the maximum flux (J_{max}) from the original equations.

The total amount permeated in the second stage (using ethanolic receptor) was taken as a measure for 'skin deposition'.

When comparing different runs, values relative to saturated aqueous controls were used to minimise the effect of inherent variations between different samples of skin.

The Student's t-test was used as a test for significance.

3. Results and discussion

3.1. Entrapment efficiency

Entrapment efficiency is the percentage of the initial drug incorporated into liposomes. The maximum entrapment efficiency obtained was 99% for pure PC liposomes (0 concentration of surfactants) which demonstrates that the amount of drug added was sufficient to saturate all formulations and thus maintain equal maximum ther-

modynamic activities. This is because at least 1% of the initial drug added remained free. This amount of free drug (at least 10 μg ml^{-1}) is above the saturation solubility of oestradiol in water. The effects of surfactant and phospholipid concentrations in the lipid components of vesicles on the entrapment efficiency of the lipophilic model drug, oestradiol, are shown in Fig. 2.

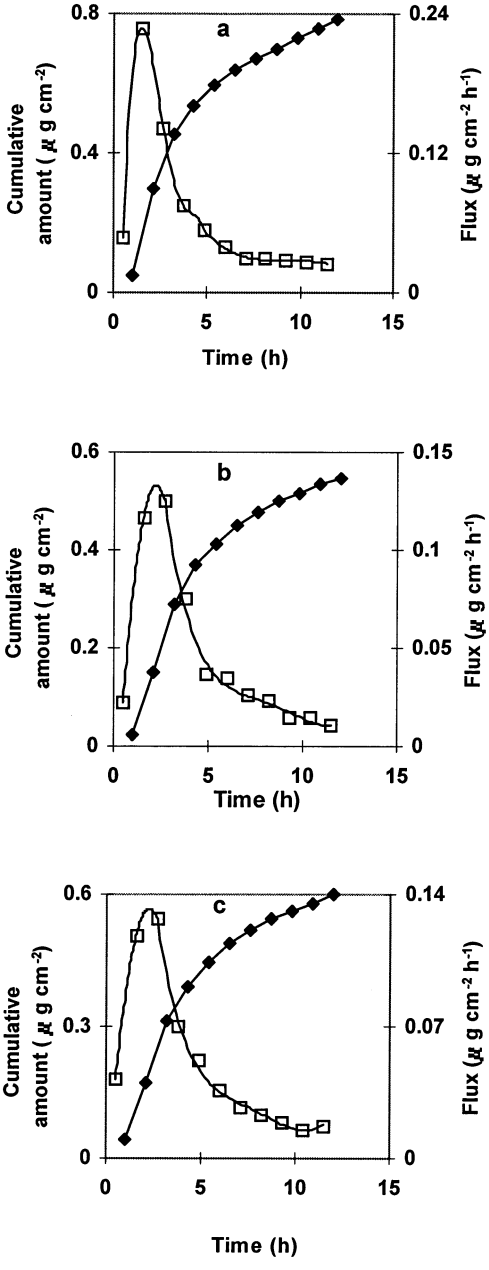

Fig. 1. Examples of cumulative amounts (◆) and transepidermal fluxes (□) of oestradiol from liposomes containing sodium cholate (a), Span 80 (b), and Tween 80 (c) in vitro (low dose study).

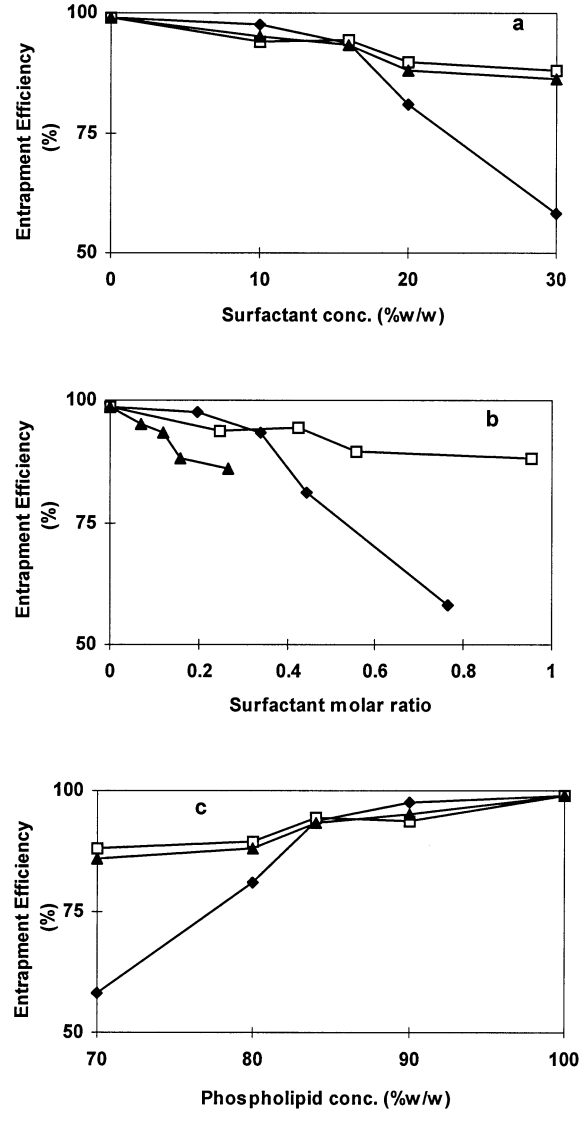

Fig. 2. Effect of surfactants (a and b) and phospholipid (c) concentrations in the lipid components of liposomes on the entrapment efficiency of a lipophilic model drug, oestradiol; (◆) cholate, (□) Span 80 and (▲) Tween 80. Error bars were generally within the size of the symbol and are thus not shown.

The efficiency decreased with increasing surfactant concentration (Fig. 2a–b) and thus increased with increasing PC concentration (Fig. 2c). Relating the entrapment efficiency to the surfactant concentrations in the vesicles expressed as % w/w (surfactant/lipid) (Fig. 2a), only high concentrations of sodium cholate (20 and 30% w/w) produced a clear effect. However, expressing surfactant concentrations as molar ratio (Fig. 2b) (RMM of Tween 80 calculated assuming the total number of ethylene oxide in each molecule to be 20), the reduction in entrapment efficiency depended on surfactant type. Span 80 produced a relatively small effect compared with Tween 80 whose effect was bigger even at low molar ratios. However, sodium cholate produced a greater effect only at high levels (molar ratios of 0.45 and 0.76). The sodium cholate effect may be related to the fact that the cholate has a steroidal structure similar to oestradiol and may thus displace some of it from the bilayer. This displacement involves competition between species and thus becomes noticeable at high concentrations of sodium cholate. An alternative explanation for the effect of sodium cholate could be due to the possible coexistence of vesicles and mixed micelles at high cholate concentrations (Almog et al., 1986), with the consequence of lower drug entrapment in mixed micelles. Relating the entrapment efficiency to PC concentration (Fig. 2c), the results revealed some dependence on PC concentration. However, it is difficult to consider the effect of any one variable (surfactant or PC concentration) in the absence of the others. Thus, entrapment efficiency of the lipophilic model drug, oestradiol, depends on PC concentration with a contribution from surfactants depending on their type and concentration.

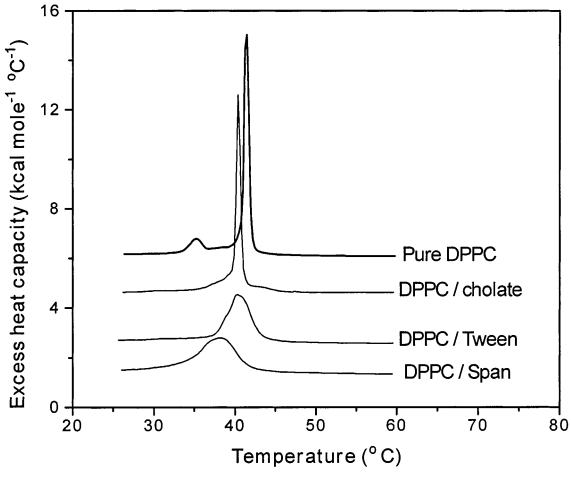

Fig. 3. Examples of high sensitivity differential scanning calorimetric traces of pure DPPC vesicles and DPPC vesicles containing surfactants at the refined concentration (surfactant/lipid); 14% w/w sodium cholate, 13.3% w/w Span 80 and 15.5% w/w Tween 80.

3.2. Vesicle size

Liposome sizes were measured for refined formulations (see Section 3.5); results are expressed as Z average $\pm$ SD ($n = 4$–5). The mean Z average values were 127 ± 7.8, 136 ± 10 and 133 ± 25 nm for vesicles containing sodium cholate, Span 80 and Tween 80, respectively. There were no significant differences ($P > 0.05$) between liposomes containing different surfactants. These results correlate with the method of preparation where the vesicles were homogenised by extrusion through a sandwich of 200 and 100 nm membranes.

3.3. Interaction between surfactants and lipid vesicles

Differential scanning calorimetry has been used to study the phase behaviour of liposomes and to locate and investigate interactions between drugs, proteins or penetration enhancers with liposomes (Papahadjopoulos et al., 1975; Rolland et al., 1991; Kim et al., 1993; Lo and Rahman, 1995). Accordingly we used HSDSC to evaluate the interactions between edge activators (surfactants) and liposomes. Although the skin permeation studies used PC as the phospholipid component of deformable vesicles, DPPC was selected for HSDSC studies. This choice was based on the fact that DPPC is one of the most widely used models for studying interactions with lipid bilayers and its transition temperature (T_m) can be measured easily as it has a narrow main endothermic peak (Montenegro et al., 1996).

Fig. 3 shows HSDSC traces of pure DPPC vesicles and DPPC vesicles containing surfactants at refined concentrations (see Section 3.5). Values presented are the mean of three replicates ± SD. The HSDSC scan of pure DPPC vesicles showed a typical profile with a pretransition peak at 35.36 ± 0.28°C and the main transition peak at 41.44 ± 0.02°C in agreement with published data (Mabrey-Gaud, 1981; Rolland et al., 1991; Lo and Rahman, 1995). No post-transition peaks were detected by this technique compared with those recorded by FT-Raman spectroscopy (Lawson et al., 1998).

Incorporation of edge activators (surfactants) into vesicles abolished the pretransition peak and significantly reduced the main T_m. They produced T_m values of 40.65 ± 0.26, 38.18 ± 0.10 and 40.58 ± 0.27°C for sodium cholate, Span 80 and Tween 80 containing vesicles, respectively. The temperature ranges at half peak height were 0.723 ± 0.003, 4.64 ± 0.13 and 1.69 ± 1.2°C compared with pure DPPC vesicles (0.725 ± 0.004). The difference was significant for Span 80 and can be considered as a trend for Tween 80 because it was recorded in each single experiment. Thus Span 80 and Tween 80 broaden the main transition peak (i.e. reduced the coopertivity of the transition) which reflects the presence of a system having different degrees of disruption in the packing characteristics. The enthalpy of the main transition was 6.4 ± 0.96, 6.9 ± 0.14 and 7.3 ± 1.3 kcal mol^{-1} compared with 7.5 ± 0.58 for pure DPPC liposomes.

Pure DPPC liposomes undergo distinct structural changes at the phase transition temperature. Below the pretransition temperature the bilayer lipids are in the highly ordered gel state with the hydrocarbon chains in an all-trans configuration (tilted one dimensional arrangement). At the pretransition temperature lipids change from the tilted one dimensional arrangements to two dimensional arrangements with periodic undulations. Above T_m, *trans*/*gauche* rotational isomerisation along the chains laterally expands and decreases the thickness of the bilayer and the system reverts to one dimensional arrangements. Thus the lipids become more fluid (Janiak et al., 1976; Rolland et al., 1991). The tilted one dimensional arrangement (below pretransition) is favourable. This is because the head groups of the phospholipids are relatively bulky taking more space compared with the hydrocarbon chains. The hydrocarbon chains thus tilt relative to the plane of the membrane to fill the extra space created by the headgroups (New, 1990). Applying these considerations to the HSDSC trace of pure DPPC liposomes, incorporation of edge activators abolishes the pretransition peak. This means that the surfactants interact with the lipid vesicles and change the conformation so that the highly ordered, tilted, one dimensional arrangement no longer exists, possibly because surfactants fitted with their lipophilic parts between the hydrocarbon chains, compensating for the bulkiness of the head groups. In addition, and depending on the degree of perturbation, the main T_m falls which can indicate that the surfactants perturb the packing characteristics and thus fluidise the lipid bilayer.

To explain the differences in the extent of interaction of different surfactants with the lipid bilayers, the molar ratio of individual surfactants in the vesicles may be considered. These were 1:3.6, 1:3.1 and 1:9.2 (surfactant:lipid) for sodium cholate, Span 80 and Tween 80, respectively. Span 80, which was used at the highest molar ratio, produced the greatest effect but the differences between effects produced by sodium cholate and Tween 80 were limited although there was a considerable difference in the molar ratios. Also, comparison between Span 80 and each of sodium cholate or Tween 80 revealed that molar ratio is not the only factor operating. Accordingly, the HLB (hydrophilic/lipophilic balance), which gives a measure of the physicochemical properties of surfactants in terms of their affinity for, or solubility in, water or lipids, was also considered. HLB values are 4.3 and 15 for Span 80 and Tween 80, respectively (Attwood and Florence, 1983). Although the HLB is an arbitrary scale from 0 to 20 originally derived for non-ionic surfactants, it can also be applied to ionic surfactants (Attwood and Florence, 1983). Accordingly, we calculated the HLB for sodium cholate to be 16.7. Based on these HLB values, the affinity for lipids is expected to be in the order of Span

Table 1
Maximum flux (J_{max}), its time (T_{max}) and skin deposition of oestradiol from lipid vesicles containing surfactants[a]

Surfactant concentration (% w/w)	J_{max} (ng cm^{-2} h^{-1})	T_{max} (h)	Skin deposition (ng cm^{-2}) $\times 10^{-3}$
Cholate			
10	48.5 (5.7, 6)	2.37 (0.10, 6)	1.30 (0.20, 6)
16	167 (8.1, 5)	1.74 (0.19, 5)	1.59 (0.26, 5)
20	44.9 (8.8, 6)	2.42 (0.19, 6)	0.780 (0.20, 6)
30	52.7 (13, 5)	2.46 (0.27, 5)	0.690 (0.11, 6)
Control	4.61 (0.88, 5)	2.54 (0.14, 5)	0.137 (0.010, 6)
Control[b]	11.5 (1.9, 6)	2.19 (0.12, 6)	0.187 (0.038, 6)
Span 80			
0	51.3 (14, 3)	2.48 (0.12, 3)	0.741 (0.085, 3)
10	71.8 (21, 6)	1.95 (0.21, 6)	0.839 (0.18, 6)
16	78.3 (21, 5)	2.07 (0.16, 5)	0.919 (0.28, 5)
20	51.1 (8.2, 5)	2.49 (0.42, 5)	1.12 (0.22, 5)
30	55.7 (14, 5)	2.34 (0.17, 5)	0.912 (0.25, 5)
Control	6.63 (1.5, 6)	2.51 (0.061, 6)	0.180 (0.046, 6)
Tween 80			
0	65.1 (23, 5)	2.23 (0.35, 5)	0.756 (0.12, 5)
10	80.0 (13,4)	2.01 (0.22, 4)	0.911 (0.27,4)
16	102 (31,5)	1.85 (0.19, 5)	0.806 (0.11, 5)
20	76.1 (23, 5)	1.91 (0.20, 5)	0.894 (0.18,5)
30	85.8 (27, 6)	1.91 (0.21, 6)	0.668 (0.23,6)
Control	6.66 (2.5,3)	2.15 (0.33, 3)	0.184 (0.030,3)

[a] Control is saturated aqueous solution, values between brackets are SEM and n, respectively.
[b] is the control for 16% cholate.

80 > Tween 80 > sodium cholate. Thus at similar molar ratios the extent of surfactant interaction with lipid bilayers should be in the same order of lipid affinity so that Span 80 should produce the greatest effect. This correlates well with the results obtained when considered together with the molar ratios of individual surfactants used.

3.4. Effect of concentration of edge activators on epidermal permeation of oestradiol in vitro

Three surfactants (sodium cholate, Span 80 and Tween 80) were studied, with saturated aqueous oestradiol (maximum thermodynamic activity) as control. The permeation parameters and skin deposition are summarised in Table 1. Fig. 4 presents J_{max} and skin deposition data (relative to control) versus surfactant concentration. The average maximum transepidermal flux (J_{max}) of oestradiol from saturated aqueous solution was 7.7 ± 0.8 ng cm^{-2} h^{-1} ($n = 27$) which is close to the published values (Megrab et al., 1995). The data show good reproducibility as indicated by the acceptable SEM values (Table 1).

With respect to drug delivery from vesicles (Table 1), J_{max} first increased with increasing surfactant concentration, then decreased. In contrast, T_{max} decreased with surfactant concentration and then increased. Thus the T_{max} trends were the reverse of J_{max} profiles, a common phenomenon seen with all three surfactants. These results suggested that too low or too high concentrations of edge activators (surfactants) are not beneficial in vesicular delivery of oestradiol through skin and also indicated that the possible penetration enhancing effect of surfactants is not mainly responsible for improved oestradiol skin delivery from deformable vesicles; these findings agree with published data (Cevc et al., 1993). A possible explanation for lower drug delivery at high surfactant concentrations may be that surfactant at high concentrations decreased the entrapment efficiency and disrupted the lipid membrane so that it became more leaky to the entrapped drug. This

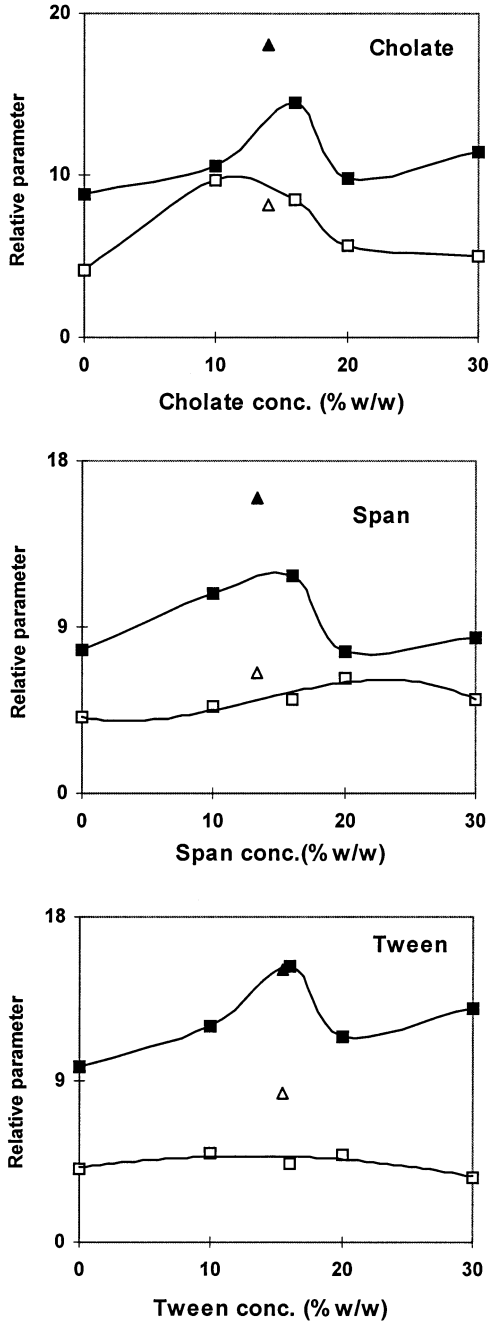

Fig. 4. Effect of different concentrations of edge activators in lipid vesicles on the skin delivery of oestradiol, where: (■) is relative maximum flux; and (□) is relative skin deposition (relative to saturated aqueous control); (▲) is the relative maximum flux; and (△) is relative skin deposition for refined formulations (see text). Note: high concentrations may form mixed micelles (Almog et al., 1986 see text).

will in turn reduce the delivery especially if we consider the possible carrier function of these deformable vesicles. Another possible explanation for the obtained J_{max} profile with surfactant concentration particularly in case of sodium cholate-containing vesicles is that at high concentrations vesicles and mixed micelles coexist (20%) with only mixed micelles at 30% (Almog et al., 1986). At 30% concentration, the Tween system was noticeably less turbid, suggesting similar loss of liposome structure. These mixed micelles are believed to be less effective as skin delivery systems compared with ultradeformable vesicles (Cevc et al., 1993, 1996).

For sodium cholate, increasing concentration produced a profile for skin deposition similar to that of the flux but the concentration corresponding to the highest skin deposition was less than that corresponding to the highest flux (Fig. 4). For Span 80 and Tween 80, however, increasing concentration provided a relatively flat profile for skin deposition. This indicates that the relative skin deposition is not comparable with the relative maximum flux.

The overall results suggested that there may be an optimum concentration of surfactant in lipid vesicles for maximum skin delivery of oestradiol. Therefore, we could fine-tune our formulations to move closer to this theoretical optimum. This procedure is dealt with below.

3.5. Determination and evaluation of refined concentration of surfactants in liposomes for skin delivery of oestradiol

To determine the concentrations of different surfactants required to formulate these refined highly deformable liposomes, the relative J_{max} (relative to control) was considered. This consideration was based on the fact that ultradeformable vesicles aim to enhance transdermal delivery. Fig. 4, showing the relative transepidermal flux and relative skin deposition of oestradiol as a function of surfactant concentration in the liposomes, was used to estimate the edge activator amounts corresponding to maximum drug flux for each formulation. Ideally, a known equation which relates flux to surfactant concentration

Table 2
Effect of surfactants at refined concentration in lipid vesicles on skin delivery of oestradiol in vitro[a]

Surfactant	J_{max} (ng cm^{-2} h^{-1})	T_{max} (h)	Skin deposition (ng cm^{-2}) $\times 10^{-3}$
Sodium cholate	145 (37, 6)	1.78 (0.21, 6)	1.08 (0.12, 6)
Span 80	135 (22, 5)	1.86 (0.13, 5)	0.868 (0.21, 5)
Tween 80	124 (19, 5)	1.96 (0.25, 5)	1.11 (0.17, 5)
Control	8.24 (2.0, 5)	2.23 (0.33, 5)	0.133 (0.024, 5)

[a] Values between brackets are SEM and n, respectively.

would be used for the refinement procedure. However, no such equation exists particularly for the complex situation of a low dose liposome formulation drying out on human skin and the drug penetrating the stratum corneum. Accordingly, we fitted the relative flux data by polynomials and treated the equations of the best fits as reported in section Section 2.9. The surfactant concentrations in the refined formulations were assessed to be 14.0, 13.3 and 15.5% w/w for sodium cholate, Span 80 and Tween 80, respectively.

The calculated refined concentrations of surfactants were then used to prepare further lipid vesicles. The epidermal permeation of oestradiol from these liposomes was studied simultaneously in one run with a saturated aqueous solution of oestradiol as the control. J_{max}, T_{max} and skin deposition were calculated (Table 2). Surfactant-containing vesicles significantly improved the epidermal delivery of oestradiol compared to the aqueous control. The maximum flux increased by 18, 16 and 15-fold for vesicles containing sodium cholate, Span 80 and Tween 80 compared with control. The skin deposition also increased by 8, 7 and 8-fold compared with control. These relative flux and deposition values are plotted in Fig. 4 as separate data points. The increases in the relative flux values over those of the vesicles containing 16% surfactant confirms the validity of this vesicle refinement approach for cholate and Span 80. Values were indistinguishable for the Tween 80 liposomes, which is expected as concentrations are similar (15.5 and 16%). In addition, T_{max} was reduced by 20, 17 and 12% for sodium cholate, Span 80 and Tween 80 formulations, respectively. The relative increase in skin deposition was approximately half that for maximum flux. These results, together with the reduced values of T_{max}, may indicate that this type of lipid vesicle can influence penetration through skin more than partitioning into it. This is because if there are similar influences on both penetration and partitioning, we expect to obtain similar relative values for J_{max} and deposition. But the question remains — do the positive results arise from free drug permeation or actual vesicle penetration? Further mechanistic investigations are necessary before we can reach a decision.

There were no significant differences between vesicles containing different surfactants in terms of J_{max}, T_{max} or skin deposition. Thus, the efficiencies of Span 80 and Tween 80 incorporated into liposomes were comparable to that of sodium cholate when used to deliver oestradiol through skin in vitro.

3.6. Investigation of tritium exchange with water

The high relative transepidermal flux of oestradiol obtained from different liposomes indicated that a check should be made for tritium exchange with water. Accordingly, the potential for such exchange was monitored by evaporating half of the receptor samples and counting after reconstitution to the initial volumes with receptor fluid. The flux of oestradiol was calculated and compared with that obtained for the other half samples counted directly without evaporation. Sodium cholate containing vesicles, which showed the shortest T_{max}, were selected as these would be the most likely candidates for tritium exchange. The flux data of oestradiol calculated for both evaporated and non-evaporated samples (Fig. 5)

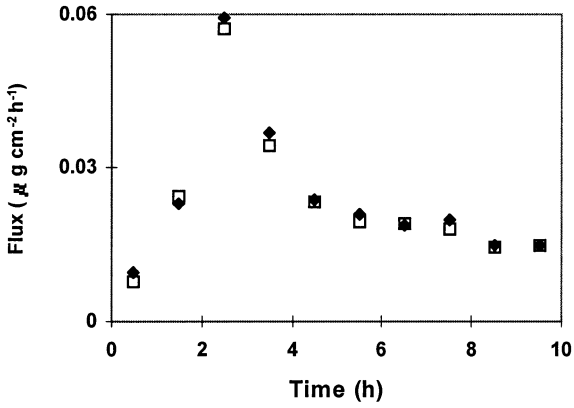

Fig. 5. Flux profile of oestradiol permeating through human epidermal membrane in vitro obtained after application of sodium cholate-containing liposomes; receptor samples counted directly (□) and after drying and reconstitution (◆). Error bars omitted for clarity.

were essentially identical, thus excluding significant tritium exchange and confirming the reliability of the analytical data.

3.7. General conclusion

Ultradeformable liposomes significantly improve the in vitro skin delivery of oestradiol compared to saturated aqueous solution (maximum thermodynamic activity) when applied non-occlusively. There is a specific concentration for each surfactant for incorporation into lipid vesicles. Span 80 and Tween 80 are as effective as sodium cholate as edge activators in PC vesicles.

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