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Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen

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Deformable liposomes and ethosomes were investigated as carriers for skin delivery of ketotifen (KT) in terms of vesicle size, entrapment efficiency, stability, *in vitro* permeation and skin deposition properties. Phosphatidylcholine (PC) from soybean lecithin was used in the preparation of all vesicles. Sodium cholate, sodium deoxycholate and Tween® 80 were investigated as edge activators in preparation of KT deformable liposomes. KT ethosomes were prepared in two PC concentrations, 2% and 4.25% w/v, in 30% v/v ethanol. KT deformable liposomes showed improved entrapment efficiency over KT ethosomes. KT deformable liposomes with Tween® 80 as an edge activator were more stable upon storage at 5 ± 1 °C than those prepared using sodium cholate or sodium deoxycholate and were more stable than KT ethosomes. *In vitro* permeation and skin deposition studies employed only deformable liposomes with Tween® 80 as an edge activator and ethosomes with 4.25% w/v PC concentration. Both of them improved skin delivery of KT over controls and over traditional liposomes, with greater improvement of KT skin deposition than KT skin permeation, hence are more useful for dermal than for transdermal delivery of KT.

1. Introduction

Recently, it became evident that classic liposomes are of little or no value as carriers for transdermal drug delivery as they do not deeply penetrate skin, but rather remain confined to upper layers of the stratum corneum (Touitou et al. 2000) and intact liposomes were reported to be unable to penetrate into granular layers of the epidermis (Kirjavainen et al. 1999).

Intensive research led to the development of a new type of lipid vesicles called deformable liposomes (Transfersomes®) that were reported to penetrate intact skin carrying therapeutic concentrations of drugs, but only when applied under non-occluded conditions (Cevc and Blume 1992). In these vesicles, edge activators are incorporated into the vesicular membrane (El Maghraby et al. 2000a). An edge activator is often a single chain surfactant that destabilizes lipid bilayers of vesicles and increases deformability of the bilayers (Honeywell-Nguyen and Bouwstra 2005). Sodium cholate, Span®80, Tween®80 and dipotassium glycyrrhizinate were employed as edge activators (Cevc 1996; El Maghraby et al. 1999, 2000a, 2000b; Trotta et al. 2004). Several studies have reported that deformable liposomes were able to improve *in vitro* skin delivery of various drugs (El Maghraby et al. 1999, 2001a; Trotta et al. 2002; Boinpally et al. 2003; Trotta et al. 2004) and to penetrate intact skin *in vivo* transferring therapeutic amounts of drugs (Cevc and Blume 2001, 2003, 2004) with efficiency comparable with subcutaneous administration (Cevc et al. 1995; Paul et al. 1995; Cevc et al. 1998; Cevc 2003).

Ethosomes are other novel lipid carriers, recently developed by Touitou et al., showing enhanced skin delivery. The ethosomal system is composed of phospholipid, ethanol and water (Touitou et al. 2000). Ethosomes were reported to improve skin delivery of various drugs (Dayan and Touitou 2000; Touitou et al. 2000; Ainbinder and Touitou 2005; Paolino et al. 2005).

The aim of the present comparative study was to investigate the characteristics of deformable liposomes and ethosomes in terms of vesicle size, entrapment efficiency, stability, *in vitro* permeation and skin deposition properties, using ketotifen fumarate (KT) as a model drug. KT is also a good candidate for a transdermal delivery system (Nakamura et al. 1996; Chiang et al. 1998). Several studies were carried out to examine and enhance *in vitro* transdermal absorption of KT and to develop suitable transdermal delivery systems for the drug (Lee et al. 1994; Nakamura et al. 1996; Kobayashi et al. 1997; Chiang et al. 1998; Inoue et al. 2000; Kitagawa and Ikarashi 2003).

Details of each formulation are summarized in Table 1.

2. Investigations, results and discussion

2.1. Physical characterization of vesicles

Table 2 shows the mean vesicle size of different formulations. Deformable liposomes with Tween®80 as an edge activator were found to have significantly ($P < 0.001$) lower mean vesicle size (108.6 ± 6.4 nm) than that of traditional liposomes (209.2 ± 5.5 nm) prepared by the same

Table 1: Formulation code and composition[a]

Code	Type	Composition	PC concentration (% w/v)
DL-SC	Deformable liposomes	PC-Sodium cholate; 86 : 14 (w/w) in 7% Ethanol (Hydration medium)	4.25
DL-SDC	Deformable liposomes	PC-Sodium deoxycholate; 86 : 14 (w/w) in 7% Ethanol (Hydration medium)	4.25
DL-T80	Deformable liposomes	PC-Tween®80; 84.5 : 15.5 (w/w) in 7% Ethanol (Hydration medium)	4.25
ES-2	Ethosomes	PC in 30% Ethanol	2.00
ES-4	Ethosomes	PC in 30% Ethanol	4.25
TL	Traditional liposomes (Membrane-stabilized)	PC-Cholesterol; 1 : 1 (molar ratio) in distilled water (Hydration medium)	4.25

[a] Ketotifen fumarate (KT) concentration in all formulations was $0.51 \pm 0.02\%$ w/v

Table 2: Entrapment efficiency and vesicle size of different formulations over time[a]

	Entrapment efficiency			Mean vesicle size (nm)	
Day from preparation	1	30	60	1	60
DL-SC	67.62 ± 4.94	79.29 ± 3.45	80.96 ± 0.37	223.0 ± 2.4	3309.3 ± 182.8
DL-SDC	68.78 ± 0.45	81.60 ± 0.07	83.16 ± 1.52	227.2 ± 8.7	3126.8 ± 409.0
DL-T80	74.51 ± 0.86	85.84 ± 2.29	87.58 ± 0.65	108.6 ± 6.4	127.1 ± 14.4
ES-2	32.27 ± 2.73	31.03 ± 1.27	11.22 ± 1.80	185.9 ± 22.3	94.6 ± 1.5
ES-4	43.98 ± 0.96	42.55 ± 2.02	28.83 ± 0.49	91.2 ± 3.7	94.7 ± 3.5
TL	24.18 ± 2.00			209.2 ± 5.5	

[a] Data are means ± s.e.m.

method. Inclusion of Tween®80, a surfactant with high affinity to interact with lipid bilayers (El Maghraby et al. 2000a), in addition to 7% v/v ethanol used (deformable liposomes), instead of cholesterol (traditional liposomes), could explain this reduction in vesicle size. However, when sodium cholate and sodium deoxycholate were used as edge activators, produced vesicles showed a mean vesicle size (223 ± 2 and 227 ± 8 nm, respectively) that was not significantly different ($P > 0.05$) from that of traditional liposomes. The reported lower affinity of sodium cholate to interact with lipid bilayers relative to Tween®80 (El Maghraby et al. 2000a) might give an explanation for these findings.

Ethosomes having the same PC concentration had a size of 91.2 ± 3.7 nm which was more than 50% lower than traditional liposomes, although the method of preparation of traditional liposomes involved sonication as a size reduction step, while preparation of ethosomes involved no size reduction step. One reported characteristic of ethosomes is their small size relative to liposomes, when both are obtained by preparation methods not involving any size reduction steps (Dayan and Touitou 2000). This reduction in vesicle size could be explained as a result of incorporation of high ethanol concentration. Ethanol confers a surface negative net charge to the liposome which causes the size of vesicle to decrease (Touitou et al. 2000; Lopez-Pinto et al. 2005). The size of ethosomal vesicles was reported to increase with decreasing ethanol concentration in the ethanol concentration range of 20–45% (Touitou et al. 2000). In the present work, the effect of PC concentration on KT ethosomal vesicle size was also studied. Decreasing PC concentration from 4.25% w/v to 2% w/v resulted in about 2-fold increase in vesicle size of ethosomes (Table 2). The presence of KT in the current study, a weakly basic drug with $pK_a = 8.5$ (Inoue et al. 2000), might have affected ethosomal vesicle surface negative charge, with subsequent increase in vesicle size in case of ethosomal vesicles with higher KT–PC ratio (2% w/v PC ethosomes).

Results of entrapment efficiency determination are shown in Table 2. KT entrapment efficiency was significantly higher in deformable liposomes (67.62 ± 4.94 and $74.51 \pm 0.86\%$ for deformable liposomes with sodium cholate, sodium deoxycholate and Tween®80 as edge activators, respectively) than in traditional liposomes ($24.18 \pm 2.00\%$). The incorporation of anionic edge activators is expected to produce negatively charged vesicles. Tween®80 was also reported to produce vesicles with more negative zeta potential than those produced by sodium cholate and sodium deoxycholate, although Tween®80 is a non-ionic edge activator whereas the other two bile salts are anionic edge activators (Lee et al. 2005). This may be due to the fact that commonly used Tweens typically contain impurities originating from industrial manufacturing process or chemical degradation, which renders such polysorbate molecules electrically charged (Simoes et al. 2005). This may give an explanation for the observed higher entrapment efficiency of deformable liposomes based on a possible ionic interaction between the cationic drug and the negatively charged vesicular lipid bilayers components.

KT ethosomes with 4.25% w/v PC concentration showed also significantly higher entrapment efficiency ($43.98 \pm 0.96\%$) than traditional liposomes. The high entrapment efficiency of ethosomes, relative to traditional liposomes, may be explained by the presence of ethanol in ethosomes, which allows for better solubility of KT. Decreasing PC concentration from 4.25% w/v to 2% w/v resulted in about 27% reduction in entrapment efficiency. Both KT deformable liposomes (prepared using different edge activators) showed greater entrapment efficiencies than those of KT ethosomes.

2.2. Vesicle stability

Stability of different types of vesicles was determined by assessing the extent of leakage and the size of vesicles over time. Table 2 shows the change in the entrapment efficiency and the size of different types of vesicles over time.

Regarding deformable liposomes with Tween®80 as an edge activator, only slight and non-significant ($P > 0.05$) increase in mean vesicle size was observed after storage for 60 days with no observed leakage. However, when sodium cholate or sodium deoxycholate was used as an edge activator, more than 10-fold increase in vesicle size was observed after storage for 60 days, indicating aggregation of vesicles, and no leakage was observed.

Ethosomes with 4.25% w/v PC concentration were stable after 60 days storage in terms of vesicle size only. However, no reduction in entrapment efficiency was observed after 30 days of storage, and significant reduction in entrapment efficiency (34% of initially entrapped drug leaked) was observed after 60 days of storage (Table 2). The effect of PC concentration on stability of ethosomes was also studied. Ethosomes with 2% w/v PC concentration were not stable after storage for 60 days in terms of both vesicle size and extent of leakage. Significant (49%) reduction in vesicle size was observed after storage for 60 days. Similar to ethosomes with 4.25% w/v PC concentration, no reduction in entrapment efficiency was observed after 30 days of storage and significant reduction in entrapment efficiency (65% of initially entrapped drug leaked) was observed after 60 days of storage (Table 2). A possible explanation of the current study findings is that high ethanol concentration, in addition to presence of KT, a drug with surface-active properties (Brockman et al. 2000; Brockman et al. 2003; Graff et al. 2004), might have destabilized or disrupted ethosomal lipid membranes over time, so that they became more leaky to entrapped drug. This explanation is strengthened by results showing that instability was more pronounced in vesicles with higher KT-PC or higher ethanol-PC ratio (2% w/v PC ethosomes).

These findings suggested that Tween®80 as an edge activator for KT deformable liposomes resulted in more stable vesicles than those produced with bile salts. KT deformable liposomes with Tween®80 as an edge activator were also more stable than KT ethosomes. These findings led to the choice of KT deformable liposomes prepared using Tween®80 and KT ethosomes with 4.25% w/v PC concentration for carrying out *in vitro* permeation and skin deposition studies.

2.3. *In vitro permeation and skin deposition*

Non-occluded application of deformable liposomes 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 and Blume 1992). The *in vitro* study design used in the present study was similar to that described by El Maghraby et al. (1999; 2000a).

Table 3 shows cumulative amounts of KT permeated and skin deposited after 24 h, from different formulations and from controls. Traditional liposomes did not show significant change in KT skin permeation relative to aqueous KT solution. However, traditional liposomes significantly improved skin deposited KT (1.8-fold increase) after 24 h over aqueous control. These results suggest that traditional liposomes improved delivery of KT, possibly to upper layers of skin only. This is in accordance with most recent publications concerning traditional liposomes reporting that classic liposomes are of little or no value as carriers for transdermal drug delivery because they do not deeply penetrate skin, but rather remain confined to upper layers of the stratum corneum showing only a localizing effect

Table 3: Cumulative KT permeated and skin deposited after 24 h from different formulations and from controls [a, b]

	Drug permeated (μg)	Drug deposited in skin (μg)
DL-T80 (n = 5)	49.43 ± 3.23 (6.59 ± 0.43)	247.85 ± 17.30 (33.05 ± 2.31)
ES-4 (n = 5)	35.78 ± 1.13 (4.77 ± 0.15)	370.20 ± 13.58 (49.36 ± 1.81)
TL (n = 5)	29.70 ± 2.18 (3.96 ± 0.29)	212.93 ± 7.58 (28.39 ± 1.01)
Solution in 30% ethanol (n = 3)	25.20 ± 0.98 (3.36 ± 0.13)	59.78 ± 12.45 (7.97 ± 1.66)
Aqueous solution (n = 3)	30.38 ± 1.65 (4.05 ± 0.22)	113.33 ± 22.80 (15.11 ± 3.04)

[a] Data are means ± s.e.m.
[b] Values in parentheses are percentages of total amount of KT in the applied dose

with more drug in skin strata (Kirjavainen et al. 1999; Touitou et al. 2000; El Maghraby et al. 2001a; Williams 2003).

Deformable liposomes (DL-T80) significantly improved ($P < 0.01$) cumulative KT permeated and skin deposited after 24 h, over aqueous control, with 1.6-fold and 2.2-fold increase, respectively. Cumulative KT that permeated skin after 24 h was also significantly higher (1.7-fold) from deformable liposomes (DL-T80) than from traditional liposomes. However, only slight (1.2-fold) and non-significant ($P > 0.05$) improvement of skin deposition after 24 h, over traditional liposomes, was observed. An important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles which enables them alone to squeeze between cells in the stratum corneum, despite large average vesicle size (Cevc et al. 2002). Thus, they can trespass intact skin spontaneously, under the influence of the naturally occurring, transcutaneous hydration gradient (Cevc and Blume 2001), without permanent disintegration (Cevc et al. 2002).

Although deformable liposomes, in the current study, significantly improved *in vitro* KT skin permeation over aqueous control and traditional liposomes, cumulative KT that permeated skin after 24 h did not exceed 7% of total KT in the applied dose. These results could not reflect the reported highly effective action of deformable liposomes applied *in vivo* (Cevc et al. 1995; Paul et al. 1995; Cevc et al. 1998; Cevc and Blume 2001; Cevc 2003). Several other studies have also reported that, although deformable liposomes were able to greatly improve *in vitro* skin permeation and deposition of various drugs, indicating improved transdermal delivery (El Maghraby et al. 1999; Guo et al. 2000; Trotta et al. 2004), transport rates were usually much lower than exceptional high transport rates reported *in vivo*. Two recent studies showed also that deformable liposomes improved only skin deposition of dipotassium glycyrrhizinate (Trotta et al. 2002) and 5-fluorouracil (El Maghraby et al. 2001a), hence were only useful for dermal delivery of these drugs. Therefore, we could suggest that when transdermal delivery is targeted, highly deformable carriers need to be designed and tested on a case by case basis. The role of deformable liposomes in improving dermal and transdermal delivery of drugs might vary widely according to nature and physico-chemical properties of the drug itself.

Ethosomes (ES-4) significantly improved cumulative KT permeated ($P < 0.01$) and skin deposited ($P < 0.0001$)

after 24 h, over KT solution (0.5% w/v) in 30% v/v ethanol, with 1.4-fold and 6.2-fold increase, respectively. Ethosomes (ES-4) also significantly improved KT skin deposition after 24 h over traditional liposomes (1.7-fold increase, $P < 0.01$) and over aqueous control (3.3-fold increase, $P < 0.001$). Ethanol is a well known permeation enhancer (Williams 2003). However, in the current work, KT solution in 30% v/v ethanol in distilled water produced slight, non-significant, reduction in cumulative KT permeated and skin deposited after 24 h relative to aqueous solution (Table 3). This suggests that the permeation enhancer effect of ethanol is not an operating mechanism in the observed improvement of skin deposition produced by ethosomes. Although the exact process of drug delivery by ethosomes remains a matter of speculation (Dayan and Touitou 2000), most likely, a combination of processes contribute to the enhancing effect (Touitou et al. 2000). Some kind of synergistic mechanism was suggested between ethanol, vesicles and skin lipids (Touitou et al. 2000). Ethanol may provide vesicles with soft flexible characteristics which allow them to more easily penetrate into deeper layers of skin. It was also proposed that phospholipid vesicles with ethanol may penetrate into skin and influence the bilayer structure of the stratum corneum (Kirjavainen et al. 1999) and this may lead to enhancement of drug penetration.

Our overall results showed that both deformable liposomes (DL-T80) and ethosomes (ES-4) significantly improved KT skin delivery. Both types of vesicles showed greater improvement of KT skin deposition than improvement of KT skin permeation, hence are more useful for dermal than for transdermal delivery of KT.

3. Experimental

3.1. Materials

Lipoid S 100 (phosphatidylcholine (PC) from soybean lecithin), containing not less than 94% PC (95.8% in the batch used in the present study), was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Polyoxyethylene sorbitan monooleate (Tween®80) was from ADWIC, El-Nasr Pharmaceutical Chemicals Co. (Abu Zaabal, Egypt). Sodium cholate and sodium deoxycholate were kindly supplied by New Zealand Pharmaceuticals Limited (Palmerston North, New Zealand). Ketotifen fumarate (KT) (Xiamen Mchem Pharma (Group) Ltd., China) was a kind gift from Rameda Co. for Pharmaceutical Industries and Diagnostic Reagents (Sixth of October City, Egypt). All other chemicals were of analytical grade and used as received.

3.2. Preparation of traditional and deformable liposomes

Traditional and deformable liposomes were prepared by the conventional mechanical dispersion method. Briefly, lipid mixtures (in addition to the edge activator in case of deformable liposomes) were dissolved either in 2 : 1 chloroform-ethanol (traditional liposomes) or in ethanol (deformable liposomes). The drug was added to furnish the desired concentration in final preparations (Table 1). The organic solvent was removed by rotary evaporation (Rotavapor, Buchi, Germany) above the lipid transition temperature (50 °C for traditional liposomes and 43 °C for deformable liposomes). Final traces of solvent were removed under vacuum, overnight. Deposited lipid films were hydrated either with distilled water (traditional liposomes) or with 7% v/v ethanol in distilled water (deformable liposomes) by rotation at 100 rpm for 30 minutes at corresponding temperatures (50 °C for traditional liposomes and 43 °C for deformable liposomes). Resulting vesicles were allowed to swell for 2 h at room temperature. Liposomal suspensions were then sonicated for 20 min.

3.3. Preparation of ethosomes

Ethosomes were prepared as described previously (Dayan and Touitou 2000; Touitou et al. 2000). Briefly, Lipoid S 100 and the drug (Table 1) were dissolved in ethanol. Distilled water was added slowly in a fine stream at constant rate in a well-sealed container with constant mixing at 700 rpm. Mixing was continued for additional 5 min. The system was kept at 30 °C throughout the preparation.

3.4. Physical characterization of vesicles

3.4.1. Vesicle size and size distribution

Vesicle size and size distribution of different formulations were determined using the particle size analyzer CILAS 1064 (Cilas, USA), which works on a laser diffraction principle. Filtered distilled water was used as dilution medium.

3.4.2. Determination of vesicle entrapment efficiency

Entrapment efficiency of KT by different types of vesicles was measured by the ultracentrifugation method (Heeremans et al. 1995). Different formulations were kept overnight at 5 °C and centrifuged in 3K 30 refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Germany) at 23 000 rpm, at 6 °C for 2 h. KT was assayed by HPLC both in the supernatant and in the sediment. Sediment lysis was performed by addition of methanol before assay. Entrapment efficiency of KT was calculated from the relationship $(C/T) \times 100$, where T is the total amount of KT detected both in the supernatant and the sediment, and C is the amount of KT detected only in the sediment. Experiments were run in at least triplicate.

3.5. Vesicle stability

Stability of different types of vesicles was determined by assessing the extent of leakage and the size of vesicles over time. Vesicles were stored in sealed tubes refrigerated at 5 ± 1 °C. For assessment of the extent of leakage, sampling was performed at intervals of 30 and 60 days and the entrapment efficiency after storage of the sample was calculated as described previously. Vesicle size was only reassessed at the end of the 60 days.

3.6. In vitro permeation and skin deposition studies

Rabbit pinna skin from 1.5–2 kg male albino rabbits (University of Alexandria, Egypt) was used. The pinna skin, including epidermis and dermis, was taken from the inner side of the ear, after sacrificing the animals, by cutting along the tip of the ear and peeling skin from underlying cartilage (Corbo et al. 1990). A preliminary wash of skin was done with normal saline followed by drying between two filter papers. Skin was used directly without storage. All animals were treated in accordance with the institutional laboratory animal care approved ethical guidelines.

Experiments were run in Franz diffusion cells having a receptor compartment volume of 8 ml. Experiments were performed in two stages similar to that described by El Maghraby et al. (1999; 2000a). The first stage was used in determination of drug permeating the skin. This stage used pH 7.4 isotonic phosphate buffer containing 0.11% w/v formaldehyde as preservative as the receptor medium. Skin membranes were mounted, with the stratum corneum side up and the donor compartment dry and open to atmosphere, and floated on receiver solution for 24 h for equilibration and pre-hydration (open hydration protocol) (El Maghraby et al. 1999), in order to maintain a transepidermal hydration gradient (Warner et al. 1988) which has been proposed as generating the driving force for skin penetration of deformable liposomes (Cevc and Blume 1992). Receiver content was then replaced by a fresh medium. Test formulations (150 µl open application) were applied to skin surface, which had an available diffusion area of 3.14 cm^2, and left to dry. Aqueous KT solution (0.5%w/v) and KT solution (0.5% w/v) in 30% v/v ethanol in distilled water were used as controls. Three ml samples of the receptor were removed at appropriate intervals for HPLC assay and immediately replaced with fresh medium. At the end of this stage (24 h), the donor compartment and the skin surface were washed 5 times with warm (45 °C) receptor medium.

The second stage was employed to determine skin deposited drug. The receptor content was completely removed and replaced by 50% v/v ethanol in distilled water and kept for a further 12 h followed by HPLC assay. This receiver solution (50% v/v ethanol in distilled water) was suggested to diffuse through skin, disrupting any liposome structure and extracting deposited drug from skin, thus giving a measure of skin deposition (El Maghraby et al. 1999, 2000a, 2001a). The receptor medium was kept at 37 ± 1 °C throughout experiments in order to maintain skin surface at 32 °C.

3.7. HPLC assay

The concentration of KT was determined by HPLC. HPLC method used was a previously developed method (Elsayed 2006) with slight modifications. HPLC System was Perkin Elmer Series 200 equipped with Series 200 LC pump, Series 200 autosampler, Series 200 UV/Vis detector, Series 600 interface and TotalChrom Navigator 6.2.0.0 Computerized Chromatography Analysis Software. Separation was carried out on Spheri-5, RP-8 column (Perkin Elmer) with a particle size of 5 µm. The mobile phase was a mixture of methanol and triethylamine acetate buffer (pH 3.5; 0.035 M) (70: 30, v/v) at a flow rate of 1.5 ml/min. UV detection was performed at a wavelength of 297 nm. The method was validated for selectivity, linearity, accuracy and precision.

3.8. Statistical analysis

All reported data are means ± s.e.m. Statistical significance was checked by Student's t-test and considered to be granted at $P < 0.05$ unless otherwise indicated.

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