Original Article

IMPROVED SKIN PERMEABILITY OF DL-α-TOCOPHEROL IN TOPICAL MACRO EMULSIONS

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ABSTRACT

Objective: To investigate the effectiveness of liposomal *dl*-α-tocopherol in a topical delivery system.

Methods: A Franz diffusion cell was used for in vitro permeation studies using an excised pig ear or a cellophane membrane. The membrane permeability of *dl*-α-tocopherol was evaluated with three formulations: 1. *dl*-α-tocopherol incorporated macro emulsion, 2. *dl*-α-tocopherol encapsulated liposome and 3. Liposomal *dl*-α-tocopherolin corporated macro emulsion, each consisting of 0.05%(w/v)*dl*-α-tocopherol. The receiver solution was phosphate buffered saline (PBS). Permeability studies were carried out for 5 and 24 hours with the pig ear and cellophane membrane, respectively.

Results: Liposomes in macro emulsion showed the highest permeability compared to other formulations. Average membrane permeability values (Kp) for formulations 1, 2 and 3 with the pig ear were 5.8×10^{-6} cm min^{-1}, 9.3×10^{-6} cm min^{-1} and 3.7×10^{-3}cm min^{-1}, respectively. The average permeability values for the three formulations 1, 2 and 3 with the cellophane membrane were 1.3×10^{-6} cm min^{-1}, 1.6×10^{-6} cm min^{-1} and 2.6×10^{-6} cm min^{-1}, respectively.

Conclusion: These results suggest that incorporation of liposomal *dl*-α-tocopherol in macro emulsions is more effective than mere addition of *dl*-α-tocopherol in emulsions,for topical delivery of vitamin E.

Keywords: *Dl*-α-tocopherol, Skin permeability, Macro emulsion, Liposomes.

INTRODUCTION

Topical drug delivery has become a major focus in the pharmaceutical industry, mainly, because topical application produces local effects rather than systemic effects, thereby decreasing or eliminating toxic effects associated with drugs[1,2]. The stratum corneum, the outermost layer of the skin, is the principal barrier for drug permeation through the skin. Thus, topically applied drugs are formulated either to enhance the partitioning into stratum corneum by altering the drug-vehicle interaction or to modify its structure; the latter is designed to decrease its resistivity towards the drug. One commonly utilized method of delivering drugs topically is incorporating the drug in an emulsion which usually alters the structure of the stratum corneum to allow drug penetration through the skin [3, 4, 5]. In the work described, *dl*-α-tocopherol, (vitamin E), was incorporated in an oil-in-water macro emulsion with a view to improving topical delivery of this compound.

dl-α-Tocopherol is an excellent antioxidant and contributes to neutralize the oxidative stress produced by oxygen and other free radicals [6].Recent developments in medicine point to the involvement of free radicals in many human diseases. Tissue damage due to reaction of oxygen free radicals with DNA strands and other biomolecules leads to carcinogenesis [7], inflammation processes [8], cardiovascular disease [9], rheumatoid arthritis, neurodegenerative disease [10], and the ageing process [11]. Antioxidants prevent undesirable oxidation processes by reacting with free radicals, chelating free catalytic metals and also by acting as O_2 scavengers [12].In addition to functioning as the major antioxidant which protects the lipidic component of cells from deleterious oxidation processes, *dl*-α-tocopherol plays important roles in other biological processes such as maintenance of cell integrity, anti-inflammatory effects, deoxyribonucleic acid (DNA) synthesis and stimulation of immune response [13]. Its role in cosmetics is based on the function of *dl*-α-tocopherol as a skin protectant from lipid peroxidation induced by UVA and UVB. It has been shown that topical supplementation with *dl*-α-tocopherol results in

considerable reduction of UVA induced free radical formation. When human skin is exposed to UVA and UVB radiation, a depletion of *dl*-α-tocopherol in the stratum corneum occurs either by direct absorption of UVB radiation by *dl*-α-tocopherol or by reacting with free radicals generated through the reaction of photosensitizers with other reactive species [14]. Due to, mainly, the photoprotective and skin barrier stabilizing properties of *dl*-α-tocopherol, different approaches have been used to enhance the skin permeation of *dl*-α-tocopherol in pharmaceutical and cosmeceutical products.

Among the numerous strategies adopted in the pharmaceutical and cosmetic industries for topical delivery of bioactive agents, liposomes, composed of an aqueous central core and one or more outer lipid bilayers [15], have been exceptional due to the numerous functions they execute simultaneously. Firstly, the lipophilic molecules such as *dl*-α-tocopherol are incorporated to the lipid bilayer of liposomes and are solubilized more effectively in aqueous environments [16]. Secondly, the incorporation within the liposome confers a protective role to the incorporated molecule [17]. Thirdly, liposomes may act as a drug reservoir that facilitates slow-release of those drugs [18]. Most importantly, although the mechanism of action has not been fully understood, liposomes have demonstrated the ability to improve topical delivery of numerous drugs and bioactive agents. For instance, Gupta and co-workers showed that the skin permeation of fluconazole increased when a gel containing liposomal drug was administered topically [19]. Thus, it is postulated that *dl*-α-tocopherol carrying liposomes could facilitate its permeability, probably through improved interaction with the lipid bilayers of the stratum corneum.

Since both emulsions and liposomes facilitate topical delivery of bioactive agents, combination of these strategies may further improve topical delivery of those molecules. Therefore, the aim of this study was to investigate the ability of liposomal *dl*-α-tocopherolin emulsions to improve topical delivery. Since most cosmetic formulations are macro emulsions, this study investigated

the role of liposomes in enhancing the permeability of *dl*-α-tocopherol in macro emulsions.

MATERIALS AND METHODS

Materials

dl-α-Tocopherol(98 %), phosphatidylcholine (∼ 60 %, TLC) (PC), cholesterol (99 %) (CH) and Tween 80 were from Sigma-Aldrich; Olive oil, sodium chloride, potassium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate were of analytical grade. Ethanol, methanol, hexane, and chloroform were distilled before use. Adult pig ear (2 hours old) was obtained from a local slaughter house while cellophane membrane was purchased from a local supplier.

Preparation of the pig skin for in vitro permeation studies

The pig ear was sectioned into two halves and separated from cartilage. The fat layer was removed with care to expose the epidermis using a scalpel and forceps. Finally, a circular portion (diameter of 2.0 (± 0.1) cm) was cut and inserted into PBS solution to equilibrate until used.

Preparation of dl-α-tocopherol incorporated formulations

formulation 1

A macro emulsion was prepared by mixing distilled water (7.253±0.001) g, Tween 80 (3.488±0.001) g and olive oil (1.240±0.001) g at room temperature. A solution of 1 mg ml^{-1} vitamin E in methanol (6.00 ± 0.05 ml) was added and was stirred for 10 minutes with a SILVERSON SL2 Emulsifier until a stable homogeneous phase was obtained. The emulsion was stirred for 10 minutes daily up to 3 days and examined visually for stability.

formulation 2

dl-α-Tocopherol encapsulated liposomes were prepared using the thin-film hydration method [20]. Finally, unencapsulated free *dl*-α-tocopherol was removed from liposomes by dialyzing against cold deionized water. Preparation was carried out in triplicate.

formulation 3

Macro emulsions were prepared by mixing distilled water, Tween 80, Olive oil and liposomes containing *dl*-α-tocopherol at room temperature. First, Tween 80 (3.505 ± 0.001 g) was stirred with a magnetic stirrer for 5 minutes. Then, water (7.251 ± 0.001 g) and olive oil (1.242 ± 0.001 g) were added. Next, the liposomes containing *dl*-α-tocopherol (6.40 ± 0.05 cm³) were added and stirred for 10 minutes with a SILVERSON SL2 Emulsifier until a stable homogeneous phase was obtained. The emulsion was stirred for 10 minutes daily up to 3 days and examined visually for stability.

Determination of encapsulation efficiency

Encapsulation efficiency of *dl*-α-tocopherol encapsulated liposomes was determined using a spectrophotometric method. Briefly, liposomes were freeze dried and dry liposomes were disrupted using ethanol. The amount of encapsulated *dl*-α-tocopherol was determined by measuring absorbance at 291.4 nm using a spectrophotometer.

$$\text{Encapsulation efficiency} = \frac{\text{amount of encapsulated drug}}{\text{amount of total drug}} \times 100 \qquad \text{eq. 1}$$

The following equation was used to calculate the encapsulation efficiency.

Determination of the loading capacity

The loading capacity of *dl*-α-tocopherol encapsulated liposomes was determined using a spectrophotometric method using equation 2.

The following equation was used to calculate the loading capacity.

$$\text{Loading capacity} = \frac{\text{amount of entrapped drug}}{\text{amount of encapsulated liposomes}} \times 100 \qquad \text{eq. 2}$$

Determination of the particle size of dl-α-tocopherol encapsulated liposomes

The particle size of *dl*-α-tocopherol encapsulated liposomes was determined using a particle size analyzer (Malvern Zeta sizer Nano ZS).

In vitro skin permeability study of dl-α-tocopherol incorporated macro emulsion

First, the receiver compartment was completely filled with fresh PBS solution. A water supply at 25℃ was given to the water jacket. A sectioned pig ear was placed between donor and receiver compartments and was clamped tightly. The macro emulsion (1000 µl) was applied as a uniform layer on the epidermal side of the skin in the donor compartment. The magnetic stirrer was turned on and the receiver buffer was allowed to stir continuously throughout the experiment at a constant rate. Two 1000 µl aliquots were removed from the receiver solution at 30 min. intervals and were stored until analyzed. The receiver was refilled with fresh PBS solution. Sampling was continued at 30 min. intervals and was terminated after 5 hrs. The procedure was carried out in triplicate. The same procedure was followed for all trials and for the other two formulations.

Membrane permeability study of dl-α-tocopherol incorporated macro emulsion

The same procedure as in section 2.7 was followed except that instead of the sectioned pig ear a cellophane membrane was used as the permeation barrier.

Quantification of dl-α-tocopherol in samples

Methanol (2000 µl) was added to each 2000 µl buffer aliquot collected in centrifuge tubes, and the mixture was vortexed for 120 seconds with a VIBROFIX VF1 vibrator. The absorbance of each methanol extract was measured at 291.4 nm using a SHIMADZU UV-1601 UV-Visible spectrophotometer with respect to a reference containing methanol and PBS buffer.

Calculation of cumulative drug permeation(Q), flux(J) and permeability (Kp)

Cumulative amount of drug permeating the skin(*Q*) at intervals is given by the following equation.

$$Q = \{ C_n V + \sum_{i=1}^{n-1} C_i\, S \}\, \text{eq3}$$

Where, *Q*= Cumulative amount of drug released (µg), Cn= Concentration of drug determined at nth sampling interval (µg ml^{-1}), V= Volume of Franz cell (ml), C_i= Concentration of drug in ith sample(µg ml^{-1}) and S = Volume of sampling aliquot (ml).

The average steady state flux (J) is given by the following equation.

$$J = \frac{dQ}{dt}\frac{1}{A}\, \text{eq4}$$

Where, A= Surface area of the skin and *dQ/dt* is the slope of the plot Q vs. t

The skin permeability (Kp) is given by the equation

$$Kp = \frac{J}{\Delta C}, \text{eq5}$$

Where, ΔC= Drug concentration difference between the donor and the receptor at a given time.

The average permeability can be calculated using the equation

$$(Kp)\text{ave} = \frac{\sum (J/\Delta C)}{N}, \text{eq6}$$

Where, N= Number of intervals.

RESULTS

Characterization of liposomes

dl-α-Tocopherol encapsulated liposomes prepared by the thin-film hydration method were characterized for their encapsulation efficiency, loading capacity, and size (Table 1).

Table 1: Average encapsulation efficiency, loading capacity, and size of liposomes

Liposomal formulation	EE (%)	LC (%)	Size (nm)
PC:CH:Vit E - 10:1:2	77.98 ± 1.23	13.49 ± 0.18	77.33

The average encapsulation efficiency and the average loading capacity were 78% and 13.5 %, respectively. Furthermore, the size distribution of *dl*-α-tocopherol encapsulated liposomes showed that the diameter of more than 99% of particles approximated to 77 nm. Moreover, the size distribution of liposomes by number seemed to be a normal distribution except for the unperceivable peak at 287.6 nm which accounted for only 0.9 % of the particles.

Permeation of dl-α-tocopherol through pig skin

The three formulations described above(i.e. *formulation 1, formulation 2, and formulation 3*)were tested for the permeability of *dl*-α-tocopherol through pig ear skin. The cumulative masses permeated are shown in Figure 1.

Results clearly show that   skin permeation of *dl*-α-tocopherol varied in the three formulations. The lowest degree of skin permeation was exhibited by *formulation 1* which was the macro emulsion containing *dl*-α-tocopherol.
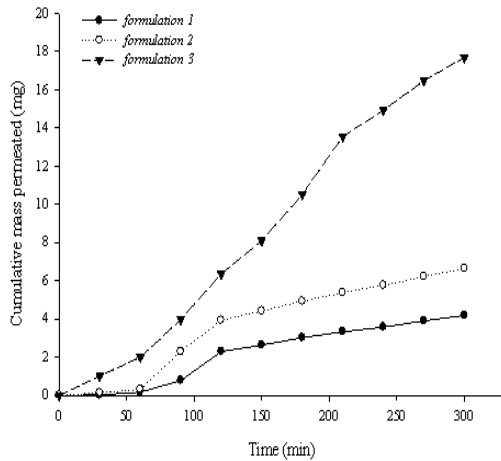
Fig. 1: Cumulative mass permeated through the pig skin: *formulation 1* (macro emulsion), *formulation 2* (liposomes) and *formulation 3* (*dl*-α-tocopherol incorporated liposome in emulsion)

The liposomal solution containing encapsulated *dl*-α-tocopherol (*formulation 2*) showed higher skin permeation than *formulation 1*, thus revealing the efficacy of liposomes as skin permeating vehicles carrying bioactive agents. As expected, *formulation 3* which was liposomal *dl*-α-tocopherol in macro emulsion exhibited the highest skin permeating ability.

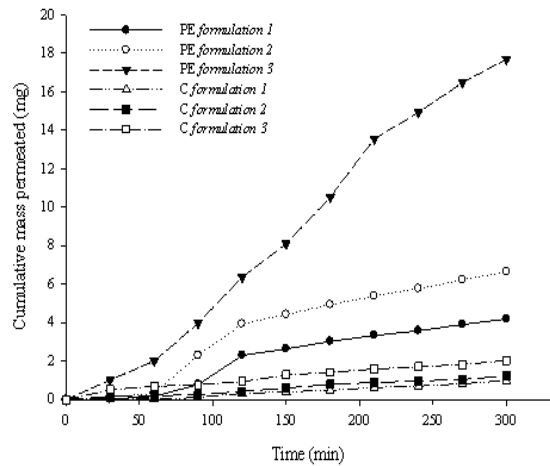**Permeation of dl-α-tocopherol through cellophane membrane**

The three formulations were tested for permeation through a cellophane membrane using the Franz cell apparatus. The cumulative masses permeated for the three formulations are shown in figure 2.

According to Figure 2, skin permeation of *dl*-α-tocopherol through cellophane membrane from formulations 1-3 showed a similar pattern

as the pig ear skin. *Formulation 3* exhibited the highest skin permeation; while *formulation 2* showed lower skin permeation than *formulation 3* but showed higher skin permeation than *formulation 1*.
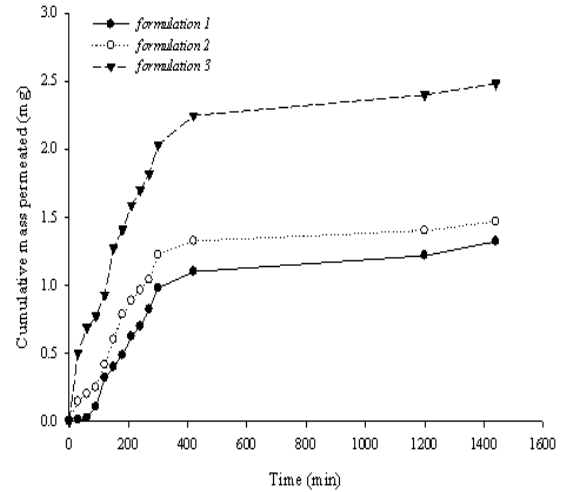
Fig. 2: Cumulative mass of *dl*-α-tocopherol permeated through cellophane membrane in three formulations

Fig. 3: Average cumulative masses of *dl*-α-tocopherol permeated through pig ear (PE) and cellophane membranes (C) during 5 hours

According to Figure 2, skin permeation of *dl*-α-tocopherol through cellophane membrane from formulations 1-3 showed a similar pattern as the pig ear skin. *Formulation 3* exhibited the highest skin permeation; while *formulation 2* showed lower skin permeation than *formulation 3* but showed higher skin permeation than *formulation 1*.

Table 2: Permeability results obtained for the two membrane types

Formulation	Membrane type	Study period (h)	Q total (µg)	Average percent permeation (%)	K_p average (10^{-6} cm min^{-1})
1	Pig ear	5	4.17	1.21	5.82
2	Pig ear	5	6.63	1.93	9.28
3	Pig ear	5	17.68	5.26	3730
1	Cellophane	24	1.32	0.38	1.31
2	Cellophane	24	1.48	0.43	1.59
3	Cellophane	24	2.48	0.74	2.64

Comparison of the permeability parameters for permeation through the two membrane types

As shown in Table 2, the membrane type affects the permeability of *dl-α*-tocopherol. In both cases, the highest permeability (Kp 3730 x 10^{-6} cm min^{-1} for pig ear and Kp 2.64 x 10^{-6} cm min^{-1} for cellophane membrane) was observed when the liposomes were incorporated in the emulsion (*formulation 3*). The lowest permeation (Kp 5.82 x 10^{-6} cm min^{-1} for pig ear and Kp 1.31 x 10^{-6}cm min^{-1} for cellophane membrane) was seen with the free *dl-α*-tocopherol in the macro emulsion (*formulation 1*). The cumulative mass of *dl-α*-tocopherol permeated through the pig ear from *formulation 3* in 5 hours showed more than a 4 fold increase over permeation of free *dl-α*-tocopherol (*formulation1*). However, permeation of *dl-α*-tocopherol through the cellophane membrane from *formulation 3* showed only a 2 fold increase over that from *formulation 1* (figure 3).

According to Figure 3, the cumulative masses of *dl-α*-tocopherol permeated through pig ear skin from all three formulations were much greater than that permeated through the cellophane membrane. These results reveal that *dl-α*-tocopherol can penetrate pig ear skin to a greater extent than the cellophane membrane.

DISCUSSION

Characterization of liposomes

dl-α-Tocopherol encapsulated liposomes prepared in this study were conventional liposomes composed of egg yolk phosphatidylcholine and cholesterol. Since *dl-α*-tocopherol is a lipophilic molecule, it is expected to reside in the lipid bilayer rather than in the aqueous core. The method adopted in this study to prepare liposomes, thin-film-hydration method, is known for yielding liposomes with high encapsulation efficiencies and loading capacities when lipophilic drugs or bioactive agents are encapsulated. In fact, the relatively high average encapsulation efficiency (i.e. 77.98 ± 1.23 %) and loading capacity (i.e. 13.49 ± 0.18 %) observed in this study further strengthens the above claim. However, Cagdas and co-workers illustrated, in their report on the effect of method of preparation of liposomes on the encapsulation of drugs, that *dl-α*-tocopherol can be encapsulated with maximum encapsulation efficiency (i.e. 100 %) using either the sonication or extrusion method. Furthermore, they demonstrated that the method of preparation of liposomes had no effect on the encapsulation of *dl-α*-tocopherol**.** They also argued that the ratio of lipids may serve as the determining factor of the degree of encapsulation of *dl-α*-tocopherol [21].

Indeed, the types and ratios of phospholipids used by Cagdas and coworkers, and those used by us were different, which may account for the observed differences in encapsulation efficiency between the two studies. Even though the mechanism of action of liposomes in enhancing skin penetration of encapsulated drugs is still under debate, numerous authors have demonstrated the importance of the size of liposomes. Du Plessisand co-workers illustrated that liposomes of intermediate size performed better in increasing drug deposition into the skin than other liposomes [22]. However, El Maghraby and co-workers reported that the structure of liposomes was important for skin permeation of oestradiol [23]. Furthermore, Folvari and co-workers showed that the outer lipid bilayers of large multi lamellar vesicles may disintegrate during skin penetration allowing small unilamellar liposomes to penetrate up to the dermis [24]. This observation suggests that intact small unilamellar liposomes may penetrate the stratum corneum. According to our results the diameter of *dl-α*-tocopherol encapsulated liposomes approximated to 77 nm with a size distribution close to a normal Gaussian distribution. This small size may have been favourable if penetration of intact liposomes occurred in topical delivery of *dl-α*-tocopherol from liposomes.

Permeation of dl-α-tocopherol through pig skin

In this study, *ex vivo* skin penetration experiments were carried out using a Franz-diffusion cell where the temperature was maintained at 25.0 ± 0.5 C. The macro emulsion containing *dl-α*-tocopherol (*formulation 1*) used in this study contained anoil phase, aqueous phase and surfactant. Each of these constituents plays a significant role in topical formulations. Hydration of the skin may have occurred due to water in macro emulsions used in this study [25]. Moreover, the oil phase - olive oil - may have served as an occlusive barrier rendering a moisturizing effect [26]. In addition, olive oil droplets may solubilize *dl-α*-tocopherol in the emulsion because this bioactive agent is a small lipophilic molecule. Furthermore, surfactants are known to enhance penetration of bioactive compounds through the skin by interacting with the lipid matrix, which may result in alteration of the structure of the stratum corneum [26]. Thus, the presence of Tween 80 in macro emulsions may have contributed to further increasing the skin permeation of *dl-α*-tocopherol.

Despite the absence of Tween 80 in formulation 2, our study revealed that the skin permeation of *dl-α*-tocopherol from *formulation 2* was higher than that from *formulation 1*. This observation shows that the effect of liposomes supersedes that of macro emulsions, made of water, olive oil and Tween 80, during the skin penetration of *dl-α*-tocopherol. As Mezei and Gulasekharam have reported, liposomes, being spherical vehicles containing *dl-α*-tocopherol, may penetrate the stratum corneum as intact particles [27]. Foldvari and coworkers have suggested that the outer bilayers of phosphatidylcholine may interact with the stratum corneum allowing the inner spherical particles to penetrate this barrier, if the liposomes are multilamellar vesicles [24]. Moreover, phosphatidylcholine may serve as a skin penetration enhancer interacting with the stratum corneum [28]. In addition, phosphatidylcholine may hydrate the stratum corneum [26]. Thus, the increase in skin penetration of *dl-α*-tocopherol exhibited by *formulation 2* compared to that exhibited by *formulation 1* may be either due to one or more modes of action of phosphatidylcholine liposomes described above. The macro emulsion containing liposomal *dl-α*-tocopherol (*formulation 3*) showed the highest skin penetration of *dl-α*-tocopherol. This observation is consistent with the fact that *formulation 3* is a combination of *formulation 1* and *formulation 2*, and thus, it may exert the effects of both macro emulsions and liposomes on skin penetration of *dl-α*-tocopherol. Interestingly, *formulation 3* reveals a much greater skin penetration effect than the effect of macro emulsion and liposomes. This observation may be at least partially due to the presence of both Tween 80 and phosphatidylcholine. As Karande, Jain and Mitragotri have demonstrated, the presence of two skin penetration enhancers may show synergistic interactions towards increasing skin permeation of radiolabeled inulin [29].

Permeation of dl-α-tocopherol through cellophane membrane

The efficacy of the three formulations containing *dl-α*-tocopherolas delivery vehicles facilitating skin permeation was evaluated using a cellophane membrane as the barrier of a Franz-diffusion cell. As expected, the relative skin permeability of *dl-α*-tocopherol in the three formulations remained the same as in the case of pig ear skin.

However, several differences in the results are worth analyzing. Firstly, the cumulative amount of *dl-α*-tocopherol permeated through the cellophane membrane is much lower than that through pig ear skin. This decrease suggests that the cellophane membrane is a more stringent barrier than pig ear skin for *dl-α*-tocopherol. Secondly, the ratio of *dl-α*-tocopherol permeated within 5 hours from *formulation 3* to *dl-α*-tocopherol permeated within 5 hours from *formulation 1*approximated to4 in experiments carried out using pig ear skin while it approximated to only 2 in experiments conducted using the cellophane membrane.

Moreover, the performance of *formulation 3* as a delivery vehicle of *dl-α*-tocopherol through the cellophane membrane was not as efficient as that through pig ear skin. These differences illustrate the importance of interactions between the chemicals of different formulations and stratum corneum in enhancing skin permeation of *dl-α*-tocopherol. For example, the effects of surfactants, lipids and water on skin permeation depend, mainly, on the interactions of those species with the stratum corneum. Since such interactions are absent with cellophane membranes, the increase of its skin permeability is not as pronounced as that through pig ear skin.

CONCLUSION

Liposomal preparations are superior to macro emulsions in delivering *dl-α*-tocopherol, through the stratum corneum of the pig ear skin most probably due to the small size and skin penetration enhancing effects of liposomes. Thus, liposomal preparations may be superior to delivering other bioactive agents, including drugs, through the stratum corneum. In addition, increased delivery of bioactive agents may be achievable when liposomes are dispersed in macro emulsions rather than having liposomes in solution. Enhanced penetration of drugs through the skin from macro emulsions containing liposomes is most probably a result of the interactions of the constituents of the emulsion and liposomes with the stratum corneum. Furthermore, this increased skin penetration of *dl-α*-tocopherol may be a result of the presence of two penetration enhancers – egg phosphatidylcholine and Tween 80 - that may act synergistically in *formulation 3*.

ACKNOWLEDGEMENTS

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