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Research Article

Lipid Vesicles for the Skin Delivery of Diclofenac: Cerosomes vs. Other Lipid Suspensions

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Abstract

Purpose: Lipid suspensions as drug carriers, including conventional liposomes, ethosomes, transferosomes, proniosomes, niosomes, PEG-PPG-PEG niosomes and stratum corneum liposomes (cerosomes), were formulated and compared.

Methods: Lipid vesicles were formulated and assessed with regards to enhancement of skin permeation of diclofenac and stability profiles of the formulations. Formulation-induced changes of the biophysical structure of excised human skin were monitored using the Fourier transform infrared spectroscopy.

Results: The stability profiles of these suspensions over 12 weeks did not show any significant drug leakage from the vesicles of interest ($p > 0.05$). FTIR observations indicated that the vesicles increased stratum corneum (SC) lipid fluidization and altered protein conformation. Skin permeability experiments showed that the free unencapsulated drug in the cerosomal formulations caused significant increase in drug permeation across the skin ($p < 0.01$). Low skin permeability of drug from the other lipid suspensions could be due to the entrapment of diclofenac within these vesicles which decreased the solubility of the hydrophilic drug in the skin lipids and the partition coefficient of the drug from these vesicles into the SC.

Conclusion: Optimal drug entrapment in vesicles or alteration of the skin structure may not necessarily enhance the permeation of hydrophilic drugs across the human skin. These lipid vesicles may be further developed into carriers of both hydrophilic and hydrophobic drugs for topical and transdermal delivery, respectively.

Introduction

Diclofenac is a widely used non-steroid-type anti-inflammatory agent. Its administration is associated with adverse gastro-intestinal effects. It is extensively metabolized in the liver and has a short biological half-life. These challenges have been overcome via topical administration.[1-3]

Vesicular lipid systems are carriers for topical and transdermal drug delivery. However, it is generally agreed that *conventional liposomes* have little or no effect on the penetration of drugs through the skin and are chemically and physically unstable.[4,5] *Niosome* vesicles from nonionic surfactants were thought to be an improvement over conventional liposomes.[6,7] Alternatively, polyethyleneglycol (PEG) containing niosomes[8,9] and other formulations such as *proniosomes* containing cholesterol and non-ionic surfactants were developed.[10-12] Ethanol, a skin permeation enhancer, was added to the liposomes and termed *ethosomes*. These vesicles have the ability to permeate through the human skin and effect intracellular delivery.[13-15] *Transferosomes*, another form of lipid carriers, are regarded as deformable liposomes. These ultra-deformable carriers contain an edge activator and have high elasticity which enables them to squeeze through intercellular regions of stratum corneum (SC) under the influence of transdermal water gradient.[16-18] Recently, liposomes with similar composition to that of the SC (cerosomes) have also been formulated and used to enhance the skin delivery of drugs.[19,20]

The aim of this work was to identify the most effective formulation for delivering a hydrophilic drug in terms of drug encapsulation efficiency, stability and skin permeation properties. The vesicles of conventional liposomes, ethosomes, transferosomes, proniosomes, niosomes and polyethyleneglycol-block-polypropyleneglycol-block-polyethyleneglycol (PEG-PPG-PEG) niosomes were formulated and compared to SC liposomes (cerosomes) for their ability to increase skin permeation of diclofenac. Alterations of the biophysical structure of SC in the presence of these vesicles were determined using the Fourier transform infrared spectroscopy.

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Materials and Methods

Materials

Lipoid E 80 (Phosphatidylcholine from egg yolk lecithin) and Cerosome 9005 were gifts from Lipoid GmbH (Ludwigshafen, Germany). Diclofenac, cholesterol, sodium phosphate monobasic monohydrate and PEG-PPG-PEG were purchased from Sigma-Aldrich Singapore. Span 85 was obtained from Fluka (Singapore). Tween 80 was purchased from Bio-Rad Laboratories (Singapore). Acetonitrile HPLC grade was obtained from Tedia Company (United States of America). Absolute ethanol was purchased from VWR International Ltd. (England). All other regents were of analytical grade.

Preparation of diclofenac-loaded vesicles

The compositions of different vesicle formulations are listed in Table 1. Diclofenac -loaded conventional liposomes were prepared by cast film method as reported previously with slight modification.[21] Briefly, Lipoid E 80 was dissolved in ethanol in a clean, dry round bottom flask followed by removal of the organic solvents using rotary vacuum evaporator above the lipid transition temperature to form a thin film on the wall of the flask. After removal of solvent traces, thin lipid film was hydrated with phosphate buffer saline (PBS) pH 7.4 containing diclofenac by magnetic stirring (700 rpm, 10 min) at the corresponding temperature.

Table 1. Composition of lipid suspensions

Formulation	Composition (% w/v)							
	PBS	Ethanol	Lecithin	Cholesterol	PEG-PPG-PEG	Tween 20	Tween 80	Span 85
Conventional liposome	65	30	5	-	-	-	-	-
Ethosome	65	30	5	-	-	-	-	-
Proniosome	20.3	31.6	22.8	2.5	-	22.8	-	-
Niosome	63	30	5	1	-	1	-	-
transferosome	64	30	5	-	-	-	1	-
PEG-PPG-PEG niosome	63	30	-	-	5	1	-	1

Ethosome colloidal suspensions, PEG-PPG-PEG niosomes, transferosomes, niosomes were prepared as reported elsewhere.[8,15,18,22] Ingredients were solubilized in absolute ethanol. PBS containing diclofenac was added gradually while mixing at 700 rpm with a magnetic stirrer.

Proniosomes were prepared as described previously with slight modification.[10] Briefly, Tween 20, Lipoid E 80, and cholesterol (9:9:2) were mixed with absolute ethanol and warmed in a water-bath sonicator at 65°C for 5 min. Then PBS containing diclofenac was added and the mixture was further warmed in the water bath for about 2 min until a clear solution was obtained. The mixture was allowed to cool at room temperature to form the proniosomal suspension.

All formulations were finely homogenized for 1 min at amplitude 30, and at pulser of 2 by means of an Ultrasonic processor (ITS Science).

Cerosomes (containing 6.6% v/v SC lipids) were used as obtained without future modification. Diclofenac dissolved in very small quantity of water was added to this readily made cream and stirred to make a homogenous formulation, therefore vesicle size and encapsulation efficacy was not calculated for this formulation.

A phosphate buffer saline solution of diclofenac was used as control. All formulations contained a total of 5 mg/ml diclofenac.

Encapsulation efficacy

The unentrapped diclofenac was removed following centrifugation at 17000 rpm for 45 min at 20°C and estimated by HPLC method. The encapsulation efficiency (EE %) was calculated using the follow equation:

$$EE\% = \frac{[total\ drug] - [unentrapped\ drug]}{[total\ drug]} \times 100 \quad (1)$$

Storage stability of vesicles

Storage stability studies are important in the development of pharmaceutically acceptable product. The ability of vesicles to retain the drug was assessed by keeping the formulation suspensions at 4±2°C (fridge) and 25±2°C (room temperature, RT) for a period of 60 days. Drug leakage was observed by measuring encapsulation efficacy of vesicular formulations. The diameters of the particles were measured on days 30 and 60 at two different temperatures. The vesicular suspensions were kept in sealed vials. Mean vesicle sizes of drug-loaded liposomes were determined using Zetasizer 300HSA (Malvern Instruments, Malvern, UK). Analysis (n=3) was carried out at room temperature and an angle of detection of 90°C.

Skin preparation method

This study was approved by the Institutional Review Board (IRB) of Singapore General Hospital, Republic of Singapore (IRB Reference Number 196/2006). This IRB operates in accordance with the International Conference on Harmonization/Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirements. The well-established and commonly used heat separation method was employed for the separation of epidermis.

Mid-line abdominal human skin samples were obtained from an adult donor with informed consent. Epidermal membranes were prepared by a heat separation technique.[23] Hypodermal and connective tissues were removed. The skin was then immersed in a water bath at 60°C for 2 min after which the epidermis was gently teased from the underlying dermis. Epidermis was stored at -80°C till use. Prior to skin permeation studies, the epidermal membranes were hydrated with SC side uppermost on 0.9% w/v for 2 h in covered petri-dishes.

In vitro skin permeation

Permeation profiles of drug-loaded ethosomes, niosomes, proniosomes, PEG-PPG-PEG niosomes, conventional liposomes, cerosomes and control samples through the skin were determined]using a flow-through diffusion cell described previously.[24] Skin was mounted with the SC side facing towards the donor compartment. The exposed surface area of the skin was 0.785 cm^2. A 1-ml formulation containing 5 mg/ml diclofenac was applied on the skin in the donor compartment. The receptor medium was phosphate buffer saline pH 7.4. The receptor compartment was maintained at 37°C. Samples were withdrawn at specific time intervals for a period of 48 h.

Fourier transform infrared spectroscopy (FT-IR)

After treating skin samples with each formulation for 48 h, samples were washed 3 times with PBS and vacuum-dried at room temperature. Samples were then subjected to FT-IR spectroscopy (Perkin Elmer Spectrum 100) at wavelengths of 500-4000 cm^{-1} at room temperature. Formulation-induced changes in the skin biophysical structure were monitored by alterations in band position, bandwidth, and band shape of the spectra compared to untreated epidermal samples.

HPLC assay of diclofenac

The quantitative determination of diclofenac was performed by HPLC using acetonitrile/pH 3 buffer solution (35:65 v/v) delivered at a flow rate of 1 ml/ min. A sample of 20 µl was eluted from the Agilent hypersil column, C18, 5 µm, 4.6×250 mm. Drug peaks, detected at wavelength 290 nm, were separated at 5.2 min.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and statistical analysis were carried out employing the ANOVA (Graph Pad Prism, Version 2), followed by the Tukey's post-test to determine the differences between treatment groups. A value of $P<0.05$ was considered statistically significant.

Results

Vesicle size

Figure 1 presents mean diameter of the vesicles over an 8-week storage period. The smallest vesicles were observed in transferosomes with a mean vesicle size of 118.9±9.8 nm. Proniosomes had the largest vesicles of 710.8±135.3 nm, as a consequence of the high concentrations of cholesterol and lecithin incorporated. Dynamic light scattering analysis showed that some of the samples were polydispersed (PI>0.35), but reproducibility of vesicle sizes appeared to be good.

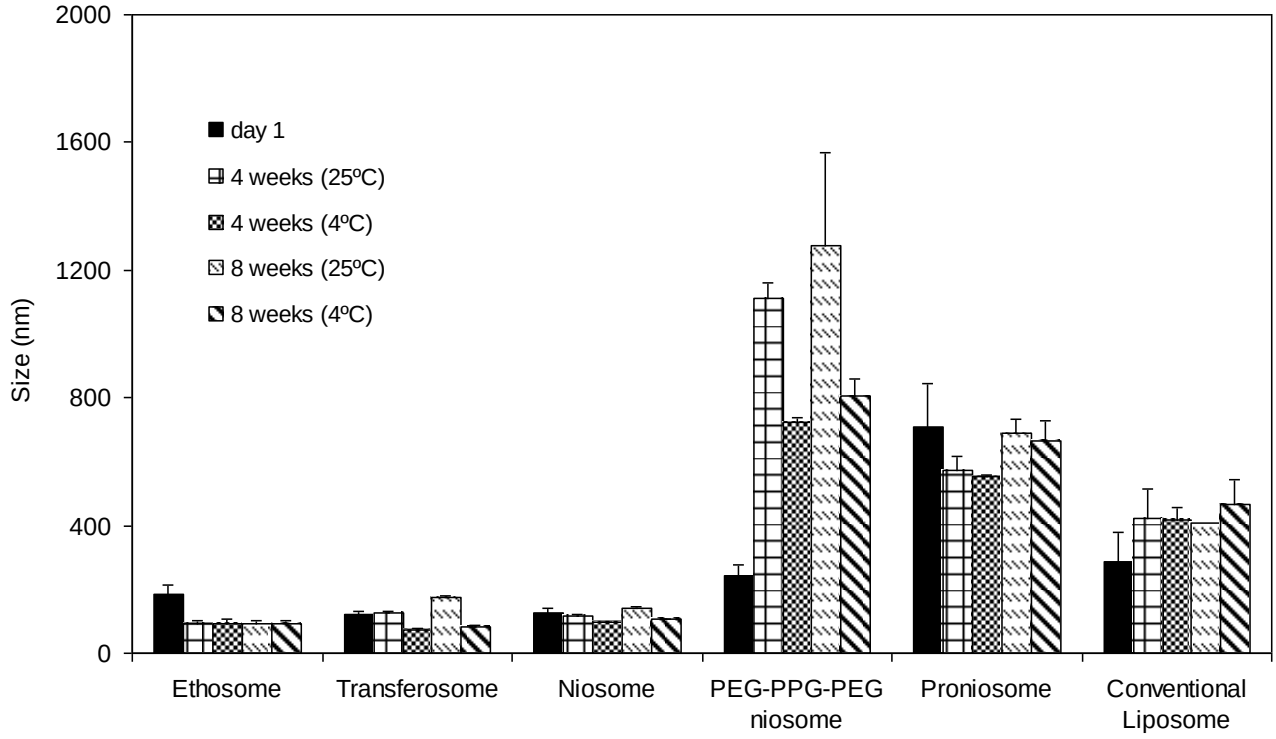

Figure 1. Stability of lipid formulations: Mean diameter of vesicle formulations (nm) with time.

Size of PEG-PPG-PEG niosomes increased upon 8-week storage compared to 1-h after preparation. The highest flocculation rate was observed for formulations stored at room temperature. Aggregation was not found to be temperature-dependent for the rest of the formulations as it was similarly observed during storage at room temperature and in the fridge. Lack of net electrical charge of conventional liposomes could have caused the aggregation of the vesicles. This effect might have resulted from storage of the liposomal formulation in PBS or other aqueous phase containing polyvalent ion.[25-27] However, ethosomes, transferosomes, niosomes and proniosomes show decrease in size after 8 week storage as compared to 1-h after preparation. The explanation could be the modification of net charges of these systems caused by ethanol.[13,28,29]

Encapsulation efficacy

Figure 2 presents the encapsulation efficacy of the vesicles. There was no significant difference in the amount of drug encapsulated in all formulations ($p>0.05$). Proniosomes and conventional liposomes had the highest encapsulation rate of 63.43 ± 9.31% and 67.15 ± 5.84%, respectively. The preparation methods required these vesicles to be hydrated at above 60°C while the rest of the formulations were hydrated at room temperature. The hydration temperature was thought to influence the extent of drug encapsulation, therefore less drug was entrapped at temperatures below the lipid transition point.[7] Lipid vesicles are generally thought to effectively entrap both hydrophobic and hydrophilic drugs.[30] However, some studies have demonstrated that the entrapment of hydrophilic molecules could be less efficient than that of hydrophobic molecules.[13] The latter supports our findings as relatively small amounts of diclofenac were encapsulated compared to those of hydrophobic drugs in the literature.

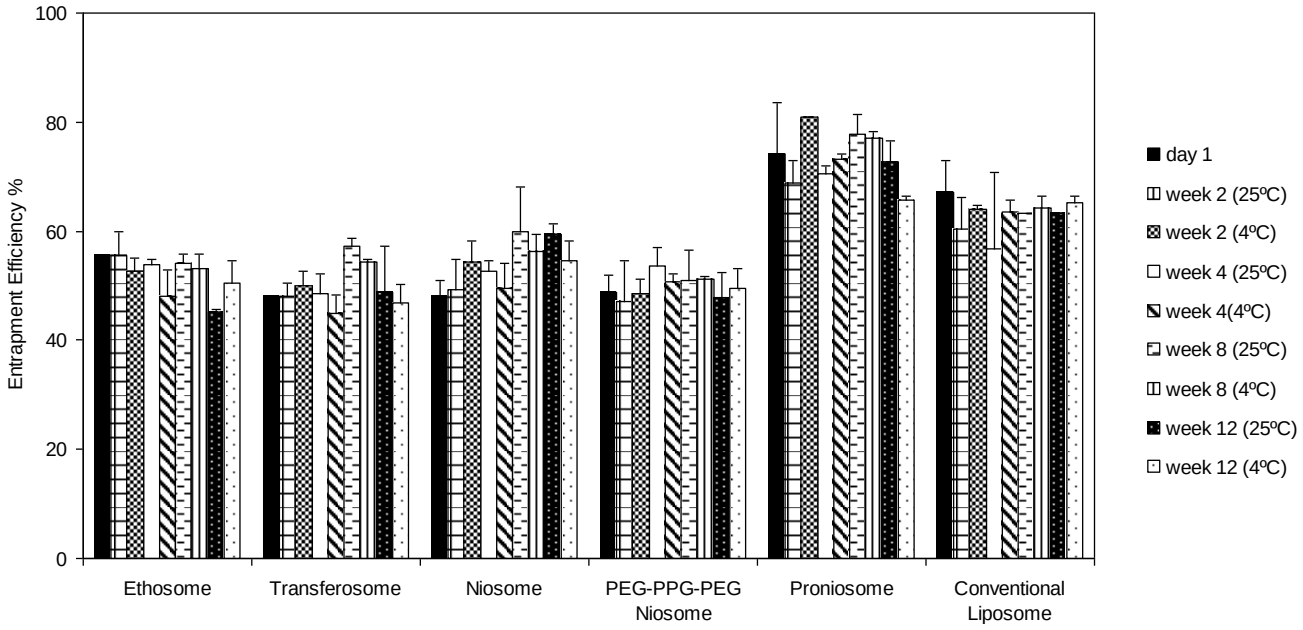

Figure 2. Stability of lipid suspensions: Encapsulation efficacy of the vesicles with storage

Results show that drug encapsulation efficacy studies for a 12-week storage at 25°C and 4°C did not show any difference from that of a freshly prepared sample. These formulations were relatively stable with minimal drug leakage ($p > 0.05$).

In vitro drug permeation

The effects of 6 vesicular formulations on the in vitro percutaneous permeation of diclofenac through human epidermis are shown in Figure 3. Percentage of drug permeation acoss the human epidermis is shown in Figure 4. Cerosomes significantly increased drug penetration compared to other formulations ($p < 0.01$), and this could be attributed to the penetration enhancement of the fatty acids in these formulations.[3,9,31-33]

Transferosomes gave a slight increase in drug permeation relative to aqueous solution ($p > 0.05$, not significant) and this could be due to the small vesicle size and the flexibility of the lipid membrane which enables the vesicles to penetrate through the skin to deliver more drug. Recent studies have demonstrated the enhancing effect of ultradeformable vesicles on enhancing the skin delivery of drugs.[1,16,34-39] Interestingly, drug penetration across human epidermis was not significantly different in other vesicle types and this was also reported in previous findings.[40] A few possible mechanisms could explain this effect. It is known that the degree of hydration of the SC influences skin permeability. Skin hydration loosens the packing of tight cell junctions which make skin more permeable.[41] Presence of ethanol in the formulations would reduce the

thermodynamic activity of the drug and result in skin dehydration, hampering drug permeation from these vesicles.[42-44] Some researchers ascribed the structural similarity of vesicles and components of the skin, vehicles introduce another lipid barrier by creating a drug reservoir which eventually retards diffusion of the drug. This may result in prolonged drug release and retention by the skin.[45] Literature reports support that there is no correlation between entrapment efficiency and permeation of a drug across the skin.[18,46] Greater drug permeation from cerosomes could be due to free uncapsulated diclofenac, thus favoring drug uptake into skin. Drug solubility in SC lipids and affinity of the drug to the vesicles may influence the skin penetration of diclofenac. The low skin permeability of a hydrophilic drug such as diclofenac could be explained by the limited solubility of the drug in the skin lipids and the low partition coefficient of the drug from the vesicle formulation into the SC.[18,39,47-51]

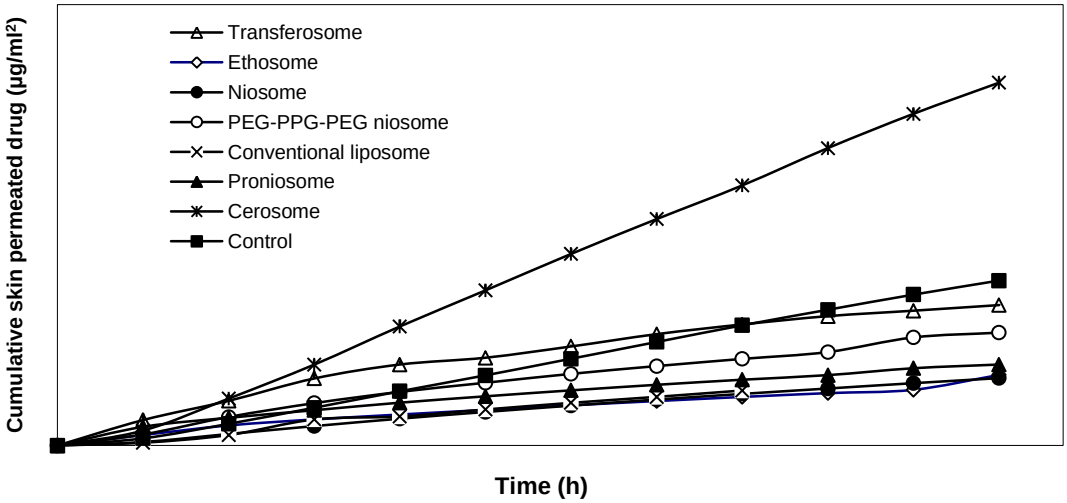

Figure 3. Cumulative concentrations of diclofenac across human epidermis (n = 3 or 4).

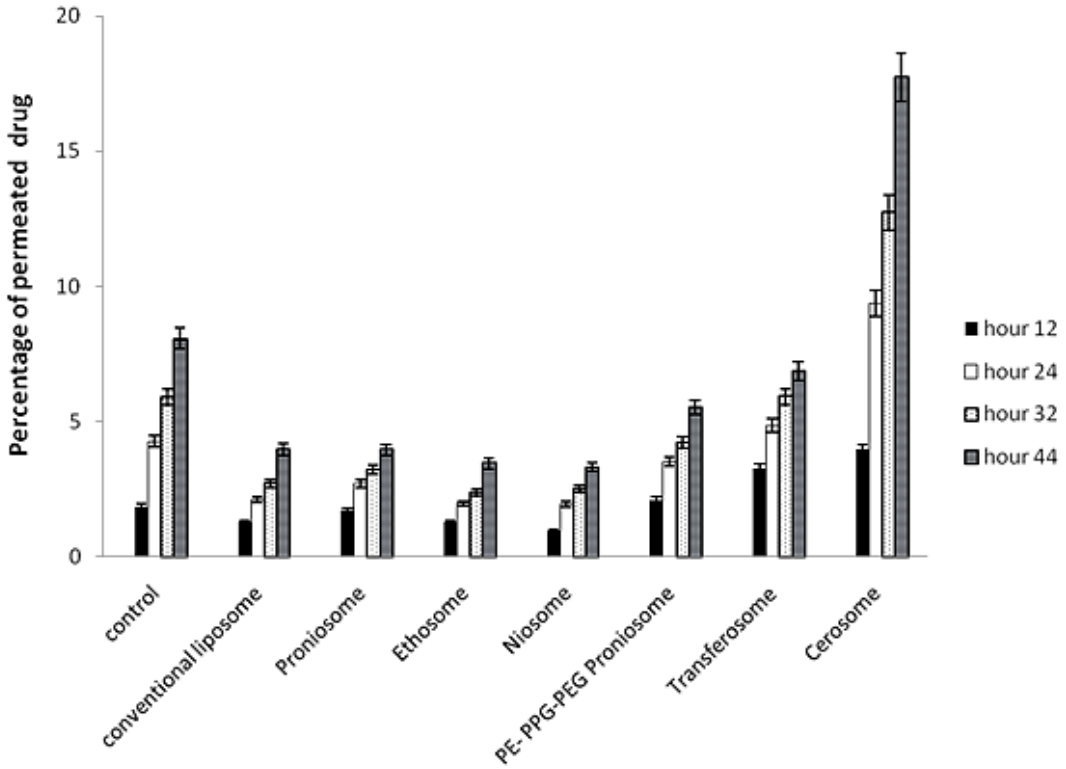

Figure 4. Percentage of drug permeation across human epidemis (n=3 or 4)

Fourier transform infrared spectroscopy (FT-IR)

Vesicle-induced changes in the skin structure were monitored by FTIR. Spectra of the skin samples were taken after the in vitro permeation study. The vesicle-induced alterations in the epidermis are presented in Figure 5.

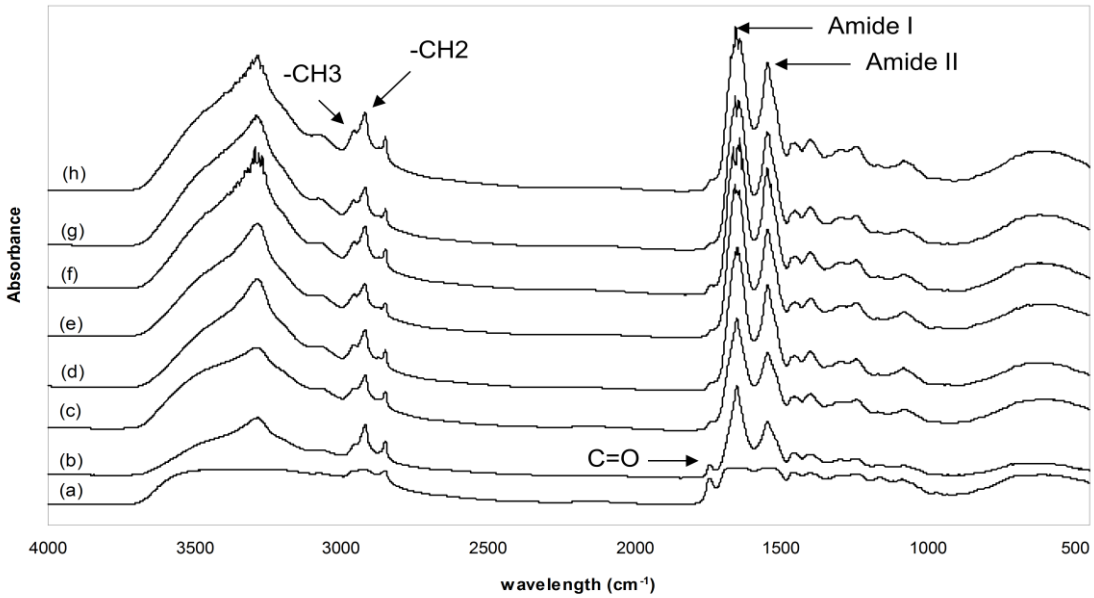

Figure 5. Representative FTIR spectra of (a) untreated human epidermis, and skin in the presence of (b) control, (c) ethosome (d) transferosome (e) proniosome (f) niosome (g) PEG-PPG-PEG niosome and (h) cerosome.

Control samples containing diclofenac in aqueous solutions did not significantly alter the skin conformation. However, treatment of SC with vesicles (Figure 4c-h) resulted in an increase in the wavenumber of the $–CH_3$ asymmetric stretching from 2955 to 2958 cm^{-1}. This indicates fluidization of the SC lipids. Carbonyl (C=O) stretching at wave number 1740 cm^{-1} corresponds to the esterified ester lipids. This peak became negligible after skin treatment with vesicles which indicates fusion of the vesicles with the epidermal layer and alteration of SC protein conformation.[44,52-54]

Protein secondary conformation is characterized by Amide I bond which corresponds to different component bands, each representing a different state of peptide secondary structure, β-helix protein (1620-1635, 1670, and 1692-1697 cm^{-1}) and α-sheet protein structure (1649-1656 cm^{-1}). In the presence of vesicles, both low (1634, 1640, 1645 cm^{-1}) and high (1660, 1667, 1671 cm^{-1}) wavenumber shoulders were observed within the Amide I region. This pattern is diagnostic for formation of β-sheet structure and indicates change in the conformation of protein secondary structure in the presence of these vesicles.[52,55] The extent of the changes in Amide I band position varied for different formulations however the change was almost negligible for skin samples treated with ethosome. It is important to consider the influence of vehicle in order to delineate the effect of lipids or non-ionic surfactants on the skin structure and FTIR spectra. Amide I band observed for ethosomes was comparable to that produced by aqueous mixture alone, inferring that the branching effect of amide I area observed for the other formulations is not due to the presence of lecithin or ethanol. Non-ionic surfactants, cholesterol, ceramides or fatty acids seem to play an important role in the branching effect of amid I region. Also the intensity of this effect appears to be concentration dependent and influenced by the type of components used in each formulation. Transferosomes which contained 1% v/v Tween 20 demonstrated less effect as compared to PEG-PPG-PEG niosomes with higher concentration of the surfactant.

The skin permeation studies showed that percutaneous permeation of diclofenac from these vesicles may not be affected by the fluidity of the SC lipid layer as all formulations exerted similar effects on the SC but only cerosomes produced significantly higher drug permeation. Hydrophilic drugs such as diclofenac were thought to permeate the skin mainly via the polar transcellular pathway which is a protein rich domain whereas alteration in the lipid structure of the skin is thought to be responsible for the non-polar passage of hydrophobic drugs.[56] Results of the permeation study suggest that the solubility of the drug in SC lipids and the partitioning of the drug molecule from the vesicles to skin may be key factors that influence diclofenac permeation across the skin.

Conclusion

In this study, different vesicle systems containing hydrophilic diclofenac were prepared and their vesicular sizes, drug encapsulation efficacy, stability, in vitro epidermal permeation and effect on skin structure were investigated. Through this it was found that entrapment efficacy and size of the lipid suspension is related to

vesicle component and method of preparation. FTIR observations indicate that lipid suspensions increase SC lipid fluidization and altered protein conformation. Results of this work have shown that drug solubility and partition in SC lipids are important factors for enhanced skin permeability of diclofenac. Therefore the optimum drug entrapment in vesicles or alteration of the skin structure may not necessarily enhance skin permeation of hydrophilic drugs. These vesicles may be used for the topical delivery of hydrophilic drugs and transdermal delivery of hydrophobic molecules.

Ethical Issues

Not applicable.

Conflict of Interest

No potential conflicts of interest.

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