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Exploration of skin permeation mechanism of frusemide with proniosomes

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The present study explored the transdermal permeation enhancing mechanism of non-ionic surfactant vesicles (proniosomes) of frusemide across rat skin. Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), activation energy and histological examination were carried out to study the mode of action of the optimized proniosome formulations PGS [Span 40:soyalecithin:cholesterol (4.5:4.5:1)] and PGD [Span 40:dicetylphosphate:cholesterol (4.5:4.5:1)]. The IR spectra showed a prominent decrease in peak areas and heights of CH_2 stretchings but did not show shift of these peaks and shift in amide bands. DSC studies also confirmed the IR findings. It was concluded that the proniosomes disrupted the lipid bilayer by extracting the lipids thereby creating pathways for drug penetration. The significant decrease in activation energy for frusemide permeation across rat skin indicated the SC lipid bilayers were significantly disrupted ($p < 0.05$). Histological investigations were carried out. Disruption and extraction of lipid bilayers as distinct voids and empty spaces were visible in the epidermal region. Overall, our findings suggested that proniosomal formulations offer a promising means for non-invasive delivery of frusemide, especially due to their ability to modulate drug transfer and serve as non-toxic permeation enhancers.

1. Introduction

Delivery of the drug via the skin has many attractions including increased patient acceptability (non-invasiveness), enhanced and controlled therapeutic response, avoidance of gastrointestinal disturbances (Payne 1998) and first pass metabolism of the drug (Crook 1997), sustained delivery of drugs to provide steady plasma profiles, particularly for drugs with short half-lives and hence reduced systemic side effects (Thomas and Finnin, 2004). However, human skin is a very effective barrier and severely limits the transdermal delivery of drugs (Prausnitz et al. 2004). The stratum corneum (SC) which is the uppermost layer of the skin is primarily responsible for the resistance to drug permeation.
Many strategies have been suggested to overcome skin impermeability for transdermal drug delivery. These include mechanical disruption (Prausnitz et al. 2004), electrical disruption (Kaalia et al. 2004), chemical enhancers (Williams and Barry 2004), and so on. However, they suffer from various drawbacks such as irritancy, toxicity, inconvenient and impractical for daily usage, etc. Chemical penetration enhancement methods have received the most attention, where addition of various chemical agents, such as fatty acids, fatty esters, alcohols, terpenes, pyrrolidones, sulfoxides, etc. has been tested to increase the skin permeability (Williams and Barry 2004). Recent efforts have, therefore, been directed towards evaluation of biocompatible colloidal drug carriers such as liposomes (Fang et al. 2006), niosomes (Azeem et al. 2008a,b) and transferosomes (Choi and Maibach 2005).
Niosomes have been widely studied as a novel percutaneous penetration enhancement technology (Azeem et al. 2008a,b; Mura et al. 2007; Manconi et al. 2006; Carafa et al. 2002; Shahiwala and Misra 2002; Fang et al. 2001a). The provesicular approach (proniosomes) has been introduced recently to improve the stability of these deformable vesicles. The advantage of liquid crystalline proniosomes (or proniosomal gel) is that the system can be directly formulated into a transdermal patch and does not require the dispersion of vesicles into polymer matrix unlike the niosomes (Azeem et al. 2008b; Vora et al. 1998). Proniosomes have been evaluated for transdermal delivery of piroxicam (Alsarra 2008), flurbiprofen (Mokhtar et al. 2008), ketoprofen (Alsarra et al. 2005), aceclofenac (Solanki et al. 2008), levonorgestrel (Vora et al. 1998), estradiol (Fang et al. 2001b), and ethinylestradiol (Kumhar et al. 2003). However, these studies have not explored the mechanism of penetration enhancement by proniosomes.
The poor bioavailability of orally administered frusemide (approx. 50%) is due to site specific absorption in the GI tract (Ooi and Colucci 2001). The physicochemical and pharmacokinetic characteristics of frusemide (e.g. molecular weight, lipid solubility, elimination half-life, melting point) are in agreement with the ideal properties of a molecule for effective penetration through the SC (Barry 2001). Although parenteral preparations are available an improved frusemide formulation (proniosomes) with a high degree of skin permeation could be useful in the treatment of chronic diseases like hypertension, pulmonary edema and congestive heart failure. Earlier attempts have been made to increase the skin permeability of frusemide which included the use of chemical enhancers such as azone and oleyl alcohol and it was claimed that these formulations could be suitable for a possible transdermal delivery of frusemide for pediatric use (Gregorious 2004).
Until fairly recently not much is known about how the proniosomes interact with the components of the horny layer and reduce its barrier function. To our knowledge, the skin permeation mechanism of frusemide when formulated as a

proniosome employing different mechanistic techniques has not been reported although proniosomes are known to enhance the skin permeation of drugs. Thus, the main objective of the present study was to investigate the mechanism of skin permeation of frusemide on incorporation into proniosomes using various methods such as Fourier Transform Infra Red (FT-IR) spectral analysis, differential scanning calorimetry (DSC) spectral analysis, activation energy measurement and histological examination. Changes in stratum corneum lipid arrangement could be detected by these techniques. The FT-IR provides information about the molecular and conformational changes in SC lipids and proteins, whereas DSC provides information about their thermotropic behavior (Naik and Guy 1997). Therefore, both the methods are capable of providing independent and complementary data about the interaction of proniosomes with SC. Histological studies were performed to further assess the changes to SC structure and to ascertain their effect on skin for application suitability.

2. Investigations, results and discussion

The method of preparation of proniosomes is based on the principle of coacervation phase separation, when three phases like surfactant, alcohol and aqueous phase are mixed in a certain ratio; they form the concentrated proniosomal gel (or liquid crystalline proniosomes). Cholesterol was used as a stabilizing agent in order to prevent the leaching of drug content from proniosome gel. Its concentration was kept constant at 10% because increasing the cholesterol content decreases the transdermal drug delivery (Vanhal et al. 1996). The composition of the surfactants used for formulating proniosomes is depicted in the Table.

To understand the permeation mechanism of frusemide with proniosomes, we explored the morphological changes in the skin microenvironment in the presence of proniosomes using FTIR spectroscopy. Many of the IR spectra bands of SC can be attributed to lipid or protein molecular vibrations. The molecular vibrations of lipids and proteins are related to various peaks in the IR spectrum of the SC. The bands at 2920 and 2850 cm^{-1} were due to the asymmetric CH_2 and symmetric CH_2 vibrations of long chain hydrocarbons of lipids (Lewis and McEelhaney 1996). The height and area of these two bands are proportional to the amount of the lipids present. Therefore, any extraction of SC results in a decrease of peak height and area. The shift of CH_2 stretching peaks to a higher wavenumber (trans to gauche conformation) and increase in their peak widths indicate fluidization of the SC (Golden et al. 1986; Suhonen et al. 1999). The bands at 1650 cm^{-1} and 1550 cm^{-1} are due to the amide I and amide II band from C=O stretching and N-H bending vibration. The frequencies of these two bands, especially amide I band, are sensitive and shift to higher or lower frequencies according to the change in protein conformation (Tori and Tasumi 1996).

IR spectra were recorded in the frequency range 4000–1000 cm^{-1}. Figure 1 shows the typical FT-IR spectra of control (untreated) and proniosomal gel treated SC over the wavenumber range 3100-2800 cm^{-1}. It has been reported that the intercellular lipids are the major contributors to all the bands arising from C-H groups (Krill et al. 1992; Knuston et al. 1986). It was observed that in the IR spectra of the SC treated with proniosome formulations there was a prominent decrease in the height and area of the CH_2 stretchings indicating that the lipids were extracted from the SC. This disruption may lead to the formation of microcavities in the bilayer and, in turn, increases the volume available for drug diffusion. Extraction of the lipids from SC might have led to enhanced percutaneous absorption of the drug. Because peak shift to a higher wavenumber was not observed, the proniosome formulation did not fluidize the lipids. Our findings suggested that greater extraction of the SC lipids from proniosomal gel PGS led to greater permeability of frusemide when compared with PGD. These observations supported the *in vitro* skin permeation data of higher drug flux with PGS as compared to PGD (Azeem et al. 2008b).

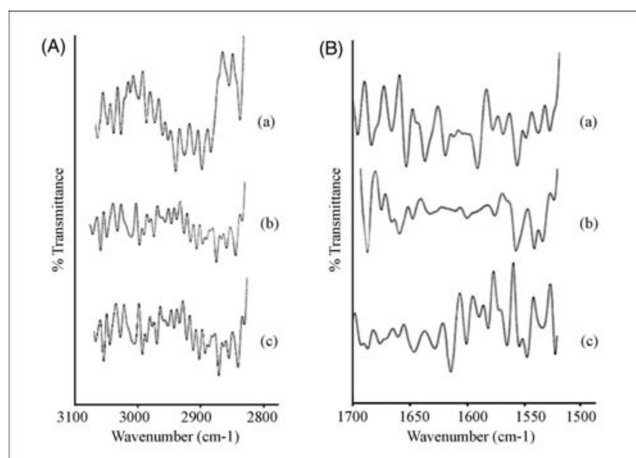

Fig. 1: FT-IR spectra of rat SC showing (A) asymmetric and symmetric C-H stretching absorbances with (a) control (b) PGS and (c) PGD (B) amide stretching vibrations with (a) control (b) PGS and (c) PGD after 24 h treatment

Table: Composition of surfactants in proniosomes

Ingredients (% w/w)	PGS	PGD
Span 40	45	45
Soya lecithin	45	–
Dicetyl phosphate	–	45
Cholesterol	10	10

The rate limiting barrier to transdermal drug delivery is the lipophilic part of the SC in which lipids (ceramides) are tightly packed as bilayers due to the high degree of hydrogen bonding. The amide I group of ceramide is hydrogen bound to amide I group of another ceramide forming a tight network of hydrogen bonding at the head of ceramide. This hydrogen bonding lends strength and stability to lipid bilayers and thus imparts barrier property to SC (Panchagnula et al. 2001; Jain et al. 2001). When skin was treated with a proniosome formulation ceramides got loosened because of competitive hydrogen bonding leading to breaking of the hydrogen bond network at the head of ceramides due to entry of niosomes into the lipid bilayers of SC. The tight hydrogen bonding between ceramides caused peak split at 1650 cm^{-1} (amide I) as shown in the control skin spectrum (Fig. 1Ba). Treatment with proniosomes resulted in single peak at 1650 cm^{-1} (Fig. 1Bb and c) which suggested breaking of hydrogen bonds.

The mechanism of skin permeation enhancement was further elucidated by DSC studies. DSC provides another method to probe changes in SC structure, based on its thermal properties (Brandys et al. 1989). It was used to characterize the thermal transitions in SC samples that were either untreated or treated with proniosome formulations. The DSC thermograms are shown in Fig. 2. Three expected transitions (T) were obtained in the control samples: T_2 around 69 °C (melting of bilayer lipids), T_3 at 80 °C (lipid melting) and T_4 around 105 °C (α-keratin conformational changes) (Barry and Williams 1995). There was no evidence for the peak T_1 (35 °C) although this

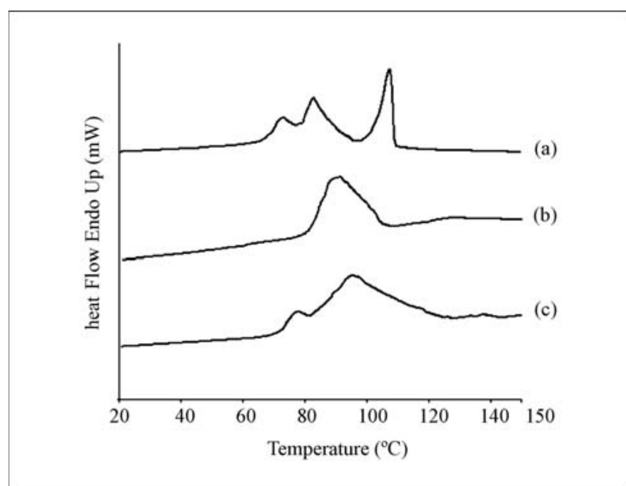

Fig. 2: DSC thermogram of SC (a) control (b) treated with PGS (c) treated with PGD for 24 h

is believed to arise from only a minor structural rearrangement within the SC lipids. Thermograms of the skin specimen treated with proniosome formulations were compared with the control sample. It was observed that both T_2 and T_3 endotherms shifted to lower melting points or disappeared in thermogram of the treated samples (Fig. 2). The effect was more pronounced with PGS as compared to formulation PGD. This indicated that the components of proniosomes enhanced skin permeation of drugs through extraction of SC lipids. Also, the proniosome formulation decreased the protein endotherm to lower melting points suggesting keratin denaturation and possible intracellular permeation mechanism in addition to the extraction of lipid bilayers.

The magnitude of activation energy (E_a) for diffusion of a drug molecule across skin depends on its route of diffusion and physicochemical properties. It is affected by change in skin composition or phase behavior of lipids. Therefore, proniosomes by their action on SC lipid bilayers may change the E_a. Permeation studies were carried out at 27, 37 and 47 °C and permeability coefficients (K_p) were determined at all temperatures. Subsequently, log K_p values were plotted against 1/T as depicted in Fig. 3. The Arrhenius plot was found to be linear in the temperature range studied, indicating no significant structural or phase transition changes had occurred within the skin membrane. The activation energy for ion transport has been reported as 10.7 kcal/mol across rat phosphatidylcholine bilayer (Monti et al. 1995) and 4.1 kcal/mol across human epidermis (Pagano and Thompson 1968). The E_a value for the permeation of frusemide across rat skin was calculated from the slope of

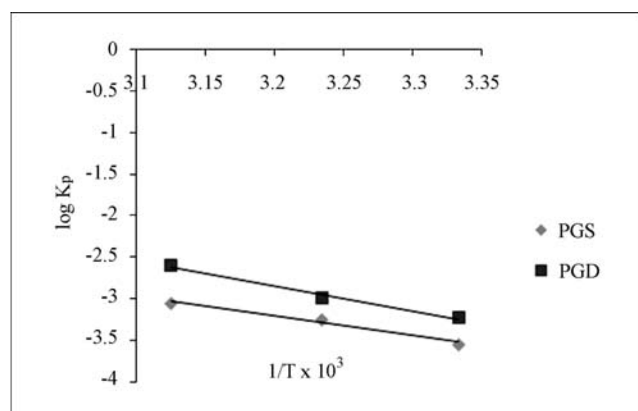

Fig. 3: Arrhenius plot of PGS and PGD permeation across rat skin

the Arrhenius plot. It was determined to be 2.351 kcal/mol and 3.034 kcal/mol from proniosome formulations PGS and PGD, respectively. The skin decrease in E_a for frusenide permeation across rat skin indicated that the SC lipid bilayers were significantly disrupted ($p < 0.05$). Therefore, it was inferred that proniosomes created pathways in the lipid bilayers of SC resulting in enhanced transdermal permeation of frusemide (Clarys et al. 1998).

The skin specimen was histologically examined to evaluate the cutaneous irritation potential of the optimized formulations (PGS and PGD). The photomicrographs of untreated rat skin (control) showed normal skin with well defined epidermal and dermal layers (Fig. 4a). Uniformly layered SC and loosely textured collagen in the dermis could be observed. Dermis was devoid of any inflammatory cells. When the skin was treated with proniosomes definite changes were observed in the skin morphology. The treated sections (Fig. 4b and 4c) showed a clear disruption of SC organization confirming the penetration enhancing capacity of these vesicular carriers. Dermis did not show any edema or inflammatory cell infiltration.

There were no apparent signs of skin irritation (erythema and edema) observed on visual examination of the skin specimens treated with proniosome formulations indicating absence of any skin irritation as a consequence of proniosome treatment. Proniosomes were comprised of non-ionic surfactants which fall under GRAS (Generally regarded as safe) category and are biocompatible and hence appeared to be safe for transdermal delivery.

In conclusion, the study sought to determine the mechanism by which proniosomes enhance transdermal transport. It was observed that the limited permeability of SC can be improved by application of proniosomes. In the lipid domains, proniosomal gel appeared to disturb the multilamellar lipid bilayers by extracting the lipids. Overall, the action of proniosomal gel on the keratin and lipids thus results in looser or more permeable structures which are presumably responsible for the observed increase in flux. Thus, barrier properties of SC, which are related to the composition, and complex structural arrangement of its lipids are decreased after treatment with these formulations subsequently leading to an increase in SC permeability.

3. Experimental

3.1. Materials

Frusemide was a gift sample from Aventis Pharma Limited, India. Span 40 (S.D. Fine Chemicals, India), soyalecithin (99% as phosphatidylcholine), cholesterol (99% purity), dicetyl phosphate (DCP) (98% purity), were purchased from Sigma Chemicals, USA. All other chemicals used in the study were of analytical grade.

3.2. Preparation of proniosomal gel

Proniosomal gel was prepared by the method reported earlier with a slight modification (Fang et al. 2001b). Precisely, the drug with surfactant mixture dispersed in 0.1 ml isopropyl alcohol (total surfactant concentration was kept at 100 mg) in a clean and dry wide mouth small glass tube. The compositions of surfactants are listed in the Table. After mixing all the ingredients, the open end of the glass tube was covered with a lid to prevent loss of solvent from it and warmed on a water bath at 65± 3 °C for about 5 min until the surfactants were dissolved completely. The aqueous phase, pH 7.4 phosphate buffer saline (PBS) was added and warmed on a water bath till a clear solution was observed which on cooling converts into a proniosomal gel.

Details of the preparation method, characterization and optimization of proniosome formulations are provided in our previous report (Azeem et al. 2008b).

3.3. Preparation of stratum corneum (SC)

The rat epidermis with SC side up was incubated in a petridish over filter paper soaked with 0.1% trypsin solution in PBS (pH 7.4) at 37 °C for 4 h (Vaddi et al. 2002; Krishnaiah et al. 2002). The SC was removed,

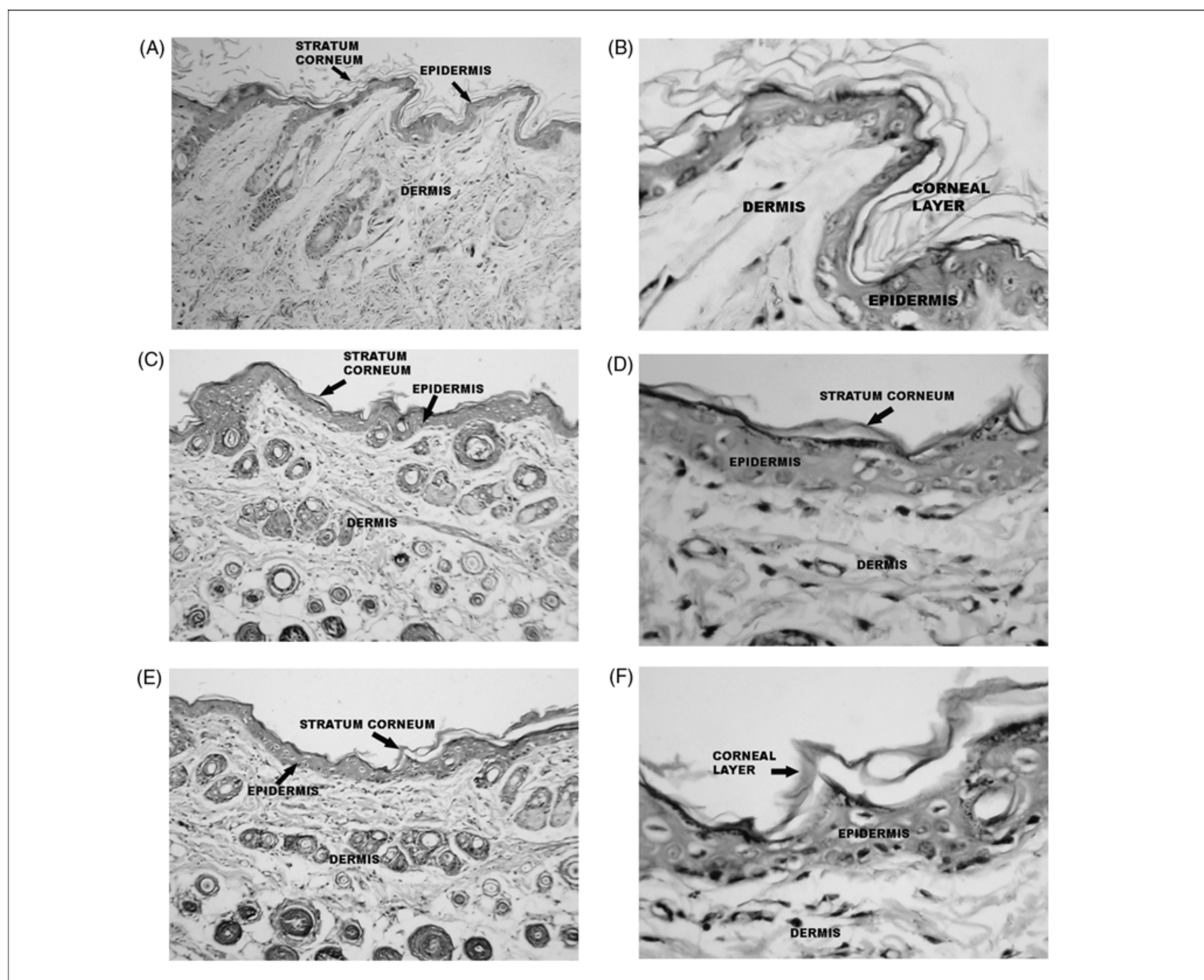

Fig. 4: Photomicrographs of rat skin sample (A) control group showing normal epidermis, dermis and subcutaneous tissues at low power view (HE × 100) (B) control group at high power view (HE × 400) (C) skin sample from PGS treated animal at low power view (HE × 100) (D) PGD treated group at high power view (HE × 400) (E) skin sample from PGD treated animal at low power view (HE × 100) (F) PGD treated group at high power view (HE × 400)

thoroughly washed, and dried in a vacuum desiccator and used for further studies.

3.4. *FT-IR spectral analysis of proniosomes treated and untreated rat skin*

The SC was cut into small circular discs with approximate diameter of 1.5 cm (Gao and Singh 1997). 0.9% w/v solution of sodium chloride was prepared containing 0.01% w/v sodium azide which acted as an antibacterial and antimycotic agent (Jain et al. 2001). Equal volume of 0.9% w/w of sodium chloride was placed in different conical flasks and SC was floated over it for 3 days. After 3 days of hydration, these discs were thoroughly blotted over filter paper and FT-IR spectra (Perkin Elmer, Germany) of each SC disc were recorded. After recording the FT-IR spectra, the same discs were treated with proniosome formulations for 24 h (equivalent to the permeation studies). Each SC disc after treatment was washed, blotted dry and then air dried for 2 h. Samples were kept under vacuum in dessicators to remove the traces of solvent completely. The FT-IR spectra of treated SC discs were recorded again. Each sample served as its own control. Attention was focused on characterizing the occurrence of peaks near 2850 and 2920 cm^{-1} which were due to the symmetric and asymmetric C-H stretching, respectively.

3.5. *DSC studies of proniosomes treated and untreated rat skin*

Approximately 20 mg SC was taken and hydrated over saturated potassium sulfate solution for 3 days. Then the SC was blotted to get hydration between 20–25%. The SC was treated with the prepared nanoemulsion systems for 24 h. After treatment, SC was washed with water and blotted dry. It was cut (to obtain weight about 5 mg) and sealed in aluminium hermetic pans. The changes in the structure of SC were assessed by DSC. The SC samples were scanned on a DSC6 Differential Scanning Calorimeter (Perkin-Elmer) at a scanning rate of 5 °C/min. over the temperature range of 30–150 °C (Panchagnula et al. 2001; Vaddi et al. 2002).

$$\text{Percentage of hydration} = \frac{\text{weight of hydrated SC} - \text{weight of dry SC}}{\text{weight of dry SC}} \quad (1)$$

3.6. *Determination of activation energy*

Ex vivo skin permeation studies of frusemide across rat skin was carried out at various temperatures (27, 37 and 47 °C of receptor medium). Receptor medium comprised 30% PEG400-water. In the donor compartment, proniosome gels PGS and PGD containing frusemide were taken. Permeability coefficients (K_p) were calculated at each temperature and activation energies of frusemide were then calculated from Arrhenius relationship (Shakeel et al. 2008; Narishetty and Panchagnula 2004).

$$P = P_0\, e^{-\mathrm{Ea/RT}} \text{ or} \quad (2)$$

$$\mathrm{Log}\, P = E_a/2.303\,\mathrm{RT} + \log\, P_o \quad (3)$$

where E_a is the activation energy, R is gas constant (1.987 kcal/mol), T is absolute temperature in K, P is the permeability coefficient, and P_o is the Arrhenius factor.

3.7. *Histological evaluation*

Histological studies were undertaken to evaluate any irritation evoked *in vivo* on rat skin after application of proniosome gels. The albino wistar

rats were prepared for the drug administration a day before by manually trimming the hair of the abdominal region of the rats to the length of 2 mm maximally with a pair of scissors followed by careful shaving with an electrical shaver. All investigations were performed after approval by Institutional Animal Ethical Committee, Jamia Hamdard (Approval No. 253). The animals were maintained in separate cages with food and water freely available throughout the experiment. After application of the optimized nanoemulsion gels on the abdominal skin for a period of 24 h, the rats were sacrificed and specimens of the exposed areas and of adjacent untreated skin area were taken for histological examination. The skin pieces were immediately fixed in 10% formalin. Subsequently, each tissue was rinsed with running water, dehydrated using a graded series of alcohols and embedded in paraffin wax and sections of 5 μm thickness were cut from each sample. The sections were then stained with haematoxylin-eosin for microscopic observation (Motic, Tokyo, Japan) (Wu et al. 2001). Skin not treated with the formulation served as a control. The skin specimens were evaluated for the mechanism of penetration enhancement.

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