Role of volatile oil pretreatment and skin cholesterol on permeation of ion-paired diclofenac sodium

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This study was designed to investigate the influence of volatile oil pretreated skin on in vitro permeation from films containing ionized and dodecylamine ion-paired diclofenac sodium (DS). The involvement of skin cholesterol was investigated to determine its possible role in enhancing the permeation of ion-paired DS. Cardamom oil produced the maximum (10 x) in vitro permeation enhancement for ion-paired DS. The carrageenan induced rat paw oedema reduction (up to 12 h) by cardamom oil was comparable to that of diclofenac injection (s.c.). Leaching of cholesterol from excised skin in addition to increased partition coefficient following volatile oil skin pretreatment appears to be responsible for in vitro permeation enhancement of DS. Whereas, a mild barrier perturbation effect due to altered cholesterol levels following pretreatment with volatile oils appears to increase the permeation of ion-paired DS across viable skin, thereby producing significant reduction of carrageenan induced paw oedema.

Transdermal drug delivery offers many biomedical advantages over conventional routes of drug administration. However, at the pH of skin normally experienced under physiological conditions, most of the drugs are in their ionized state. The partitioning of a drug through the skin under such a condition, is a function of its intrinsic charge.

The resistance to transdermal flux can be reduced by many methods. Out of the several methods, synthesis of lipophilic analogues and the use of permeation enhancers are being widely investigated. Ion-pairing is a method of increasing the lipophilicity of an ionized permeant. Our earlier investigations have shown that ion-pairing of diclofenac sodium (DS) with dodecylamine increases its partition coefficient between isopropyl myristate and phosphate buffer (pH 6.0). Although, the in vitro flux from films containing ion-paired diclofenac was five times greater as compared to that from films containing ionized DS, it was much less than that reported from pseudolatex dispersions. Hence, it was felt necessary to increase the permeation of ion-paired DS.

Terpenes in their pure form are increasingly being investigated for their percutaneous permeation enhancement effect. The permeation enhancement following topical application of pure terpenes is reported to be due to an increase in the stratum corneum lipid fluidity and perturbation of the integrity of the epidermis. However, the influence of terpenes on the microconstituents of skin that are involved in maintaining its barrier integrity has not been reported.

This investigation aims at studying the influence of different volatile oils on the permeation of ionized and ion-paired DS from transdermal films. In order to investigate the mechanism of permeation enhancement by volatile oils, the role of cholesterol, a marker of epidermal integrity was studied.

**Materials and Methods**

Diclofenac sodium (Ciba-Geigy, India), Eudragit L-100 (Rohm Pharma, Germany), HPMC (Shinetsu, Japan), carrageenan (Spectrochem, India) and dodecylamine (Lancaster, UK) were used as received. Clove oil was purchased from SD Fine Chemicals, India. Other oils (expressed) were procured from domestic manufacturers. Cholesterol was measured by using cholesterol test kit (Span Diagnostics Ltd., Surat, India). All other chemicals were of AR grade.

**Preparation of films**—Transdermal films with total polymer content of 12% w/w were prepared by mixing solutions of eudragit L-100 and HPMC (9:1). Eudragit solution was prepared in alcohol (1 part). Films containing ionized DS were prepared by dissolving DS in HPMC solution prepared in a mixture of phosphate buffer (pH 6.0) and alcohol (6:1). For preparing films containing ion-paired DS, the drug was dissolved in phosphate buffer (pH 6.0) and mixed with dodecylamine. This mixture was then incorporated...
into HPMC solution prepared as above. Polyethylene glycol 400 (15% w/w of total polymer) was added as plasticizer. The films were cast on mercury and dried at 45 °C for 4 hr. Amount of mercury was standardized or maintaining film thickness. Using the formula, Cp × Vd × Ke and Cp of 1.9 µg/ml, Vd of 0.17 L/Kg and Ke of 1.482/hr, the required release rate was calculated to be 478 µg/hr, corresponding to 11.48 mg/day. Hence, 22.944 mg of drug was loaded into films (3.63 cm²) to suffice for 48 hr study period.

Permeation studies (in vitro)—In vitro permeation of DS was investigated through dorsal skin of wistar rats excised 24 hr after removing the hair with mechanical clippers. Permeation was carried out in vertical Keshary-Chien diffusion cell containing phosphate buffer (pH 7.4) at 37° ± 1 °C in the receptor compartment. The stirring speed was 300 rpm. Aqueous formaldehyde (0.2% v/v) and polyethylene glycol 400 (5% v/v) were added for preservation and maintenance of sink condition, respectively. Pretreatment of skin was done by applying 1 ml of volatile oil to the stratum corneum surface immediately before application of the film formulation. DS permeated into the receptor compartment was analyzed at different time intervals at 276 nm using Beckman spectrophotometer (DU 640B).

Anti-inflammatory response in rat paw oedema—The anti-inflammatory studies were performed using a plethysmometer to measure carrageenan-induced paw oedema following the method of Winter et al. Ten wistar albino rats were used in each group. Hairs from the dorsal side (3 cm²) were removed with clippers. The transdermal film formulation was applied after 12 hr and occluded with an adhesive tape. After 30 min of application of film, 0.1 ml (1% w/v) solution of carrageenan was administered by intraplantar injection in one hind paw. The other hind paw served as control. The volume of both hind paws was measured at regular intervals up to 24 hr.

The treatments given to different groups were: group I, no drug (control); group II, injection (15 mg/kg, sc); group III, marketed gel formulation (containing 26.615 mg of diclofenac diethyl ammonium = 22.944 mg of DS); group IV, film containing DS at pH 2.0 (unionized); group V, film containing dodecylamine-DS ion-pair; group VI, film containing ionized DS and skin pretreated with cardamom oil; group VII, film containing ion-paired DS and skin pretreated with cardamom oil. Skin was pretreated by applying 1 ml of volatile oil on the shaved portion immediately before application of film formulation.

Effect of skin pretreatment with volatile oils on cholesterol leaching (in vitro)—Whole skin was clamped on the diffusion cell. The receptor fluid was stirred for 4 hr after which it was replaced with fresh phosphate buffer (pH 7.4). Volatile oil (1 ml) was applied on the stratum corneum side and stirring continued for 48 hr. At the end of this period the entire fluid was evaporated under vacuum and analyzed for cholesterol content by using cholesterol kit.

Effect of skin treatment with volatile oils on cholesterol in viable skin—Two patches (2 cm²), one on either side of the spinal cord of wistar rats were prepared by shaving and left open for 12 hr. One patch was treated with volatile oil (1 ml) and the area occluded with adhesive tape. The other patch served as control. The animal was sacrificed after 48 hr and both the portions of skin excised. After drying the skin to constant weight at 50°C, homogenizing in chloroform-methanol (1:1) followed by filtration and concentration (under vacuum), the cholesterol content was determined. All experiments were carried out in triplicate.

Results

The permeation of ion-paired DS across volatile oil treated skin (Fig. 1) is significantly greater (t-test, P < 0.05) than that of ionized DS (Fig. 2). Among the various oils tested, cardamom oil produced the maximum permeation enhancement for ion-paired DS. The

![Image](https://via.placeholder.com/150)

**Fig. 1**—Influence of volatile oil pretreatment on permeation enhancement ratio of ion-paired DS with respect to ionized DS and ion-paired DS across non-pretreated skin.
permeation profile of ion-paired DS across cardamom oil pretreated skin is depicted in Fig. 3. Increased flux of ion-paired DS following skin pretreatment with volatile oils is accompanied with an increase in partition coefficient (Fig. 4) and increase in the amount of cholesterol leached from excised skin (Fig. 5). In addition, cholesterol content in viable skin is found to slightly increase after topical application of any volatile oil (Fig. 5). This indicates a mild epidermal perturbation effect. Pharmacodynamic studies indicate that a combination of ion-pairing and cardamom oil skin pretreatment produces a sustained effect that is better than that obtained by single application of marketed gel formulation containing diclofenac diethylammonium (Fig. 6).

**Discussion**

DS exhibits a pKa of 4.0\(^{11}\). At pH values greater than 4.0, it will exist in ionized form. Primary fatty amines being cationic at higher pH values, form ‘ion-pairs’ with DS to yield a lipophilic molecule. Our earlier investigations showed that among various amines dodecylamine is most effective in enhancing both partition coefficient (isopropyl myristate / phosphate buffer) and in vitro permeation of DS across rat skin. Film formulation containing ion-paired DS (at pH 6.0) exhibited five times greater in vitro flux than that from films containing ionized DS\(^2\).

In order to further increase the in vitro permeation
of DS, pretreatment of skin with different volatile oils was carried out. The enhancement ratio for various volatile oils followed the order, cardamom > clove > turpentine > eucalyptus (t-test, \( P < 0.05 \)). The maximum permeation of DS from films containing ion-pairs obtained using cardamom oil pretreated skin is approximately 10 times greater as compared to that from films containing ionized DS (Fig. 1). These results demonstrate the influence of a combination of ion-pairing and volatile oil skin pretreatment in increasing the permeation of DS.

It is interesting to note that the permeation of ionized DS is increased after skin pretreatment as compared to that across non pretreated skin with cardamom oil producing the maximum enhancement (Fig. 2). However, the permeation enhancement for ionized DS by the respective oils was significantly less than that for ion-paired DS (t-test, \( P < 0.05 \)). The order of permeation enhancement for ionized DS was, cardamom > clove > lemon > eucalyptus > turpentine (t-test, \( P < 0.05 \)). It is noteworthy, that the permeation of ionized DS across oil pretreated skin was significantly greater as compared to that of ion-paired DS across non pretreated skin. These results indicate that volatile oils are capable of enhancing the permeation of ionized form of DS and their presence is essential for enhancing the percutaneous permeation of highly lipophilic ion-paired DS molecules.

The change in order of enhancement ratio for ion-paired and ionized DS can be explained on the basis of the active constituents of the respective volatile oils. Limonene, the major constituent of lemon oil, has been reported to enhance the in vitro transport of lipophilic molecule, oestradiol and decrease the transport of hydrophilic molecule, 5-fluorouracil\(^{12}\). The present investigation also reveals a higher enhancement ratio for ion-paired as compared to that for ionized DS by lemon oil. Clove oil contains eugenol, a hydroxyl group containing terpene, that can be more effective in enhancing the permeation of hydrophilic drugs by formation of hydrogen bonds. Hence, clove and lemon oil rank second and third in enhancing the permeation of ionized DS and ion-paired DS, respectively. Similarly, eucalyptus was more effective than turpentine for ionized DS, while the reverse is true for ion-paired DS. This finding is in agreement with the earlier report that aramon-drene (the constituent of eucalyptus oil) is more effective than longifolene (the major constituent of turpentine oil) in enhancing the in vitro transport of 5-fluorouracil\(^{13}\). Also, eucalyptus oil is reported to be more effective than peppermint oil for 5-fluorouracil\(^{14}\). Separation and purification of volatile oils for obtaining the active constituents in pure form is a costly process. The results of this study suggest

![Graph 1](image1.png)

**Fig. 4**—Influence of volatile oil pretreatment on in vitro percutaneous permeation parameters of ion-paired DS (Note: actual values are, Flux \( \times 1000 \); Diffusivity \( \times 10^{-11} \); Partition coefficient \( \times 0.1 \)).

![Graph 2](image2.png)

**Fig. 5**—Influence of volatile oil skin pretreatment on flux of ion-paired DS and cholesterol (Chl) leached from excised skin and in viable skin (Note: actual values are, Flux \( \times 1000 \); Chl (leached) \( \times 10 \) wrt control; Chl \( \times 10 \) (viable skin) \% control).
that it would be possible to increase the permeation of a drug by simply utilizing a polar or non-polar solvent extract of the oil yielding part of the plant. The choice of extracting solvent will depend upon the lipophilic / hydrophilic character of the drug.

Figure 3 (inset) depicts the 'burst release' pattern obtained for permeation of ion-paired DS from films across cardamom oil pretreated skin. Calculation of lag time by extrapolation to X-axis is not possible in such graphs. Hence, an attempt was made to transform the data of Fig. 3 (inset) by using the approach used by Shah et al.\textsuperscript{15} The initial points that were not linear in cumulative amount (CA) Vs time plot were left out. All the other experimentally obtained points were treated through a regression analysis and a CA Vs log time plot was constructed (Fig. 3) that resulted in a higher \( R^2 \) value. For comparison purpose, the permeation profiles of DS reported by Vyas et al.\textsuperscript{2} and Huang et al.\textsuperscript{16} were also transformed using this method. The reported flux of 118 and 74.15 \( \mu g/cm^2/hr \) upon transformation become 3504.692 and 2976.885 \( \mu g/cm^2/log \) hr, respectively. The highest flux obtained in the present investigation from ion-paired films across cardamom oil pretreated skin is 3775.392 \( \mu g/cm^2/log \) hr. This is 1.07 and 1.26 times greater than that reported by Vyas et al.\textsuperscript{2} and Huang et al.\textsuperscript{16}, respectively.

A comparison of permeation parameters following skin pretreatment with various volatile oils shows that an increase in partition coefficient may play a role in increasing the flux (Fig. 4). Diffusivity does not seem to be important because no particular trend is evident. Interestingly, lag time is found to increase following skin pretreatment as compared to control because of slow redistribution leading to 'conditioning' of the membrane during early stages of diffusion process\textsuperscript{13}. In addition to partition coefficient, volatile oil treatment also increases the amount of cholesterol leached from excised skin (Fig. 5). An alteration of enthalpy and entropy of lipid transitions\textsuperscript{17} and perturbation of stratum corneum lipid fluidity\textsuperscript{18} following skin treatment with terpenes have been reported to increase skin permeability and enhance the permeation of drugs. Moreover, increased epidermal cholesterol synthesis is known to accompany altered barrier function and transepidermal water loss following perturbation\textsuperscript{16}. Therefore, changes in transepidermal water loss\textsuperscript{17,18} appear to arise from altered cholesterol levels in skin. Fig. 5 shows increase in the cholesterol content in viable skin after pretreatment with oils, indicating a mild epidermal perturbation effect. This suggests that pretreatment with volatile oils increases the permeation of ion-paired DS both by increasing its partition coefficient and by mild epidermal perturbation effect due to their influence on epidermal cholesterol. Hence, continuous use of volatile oils at the same site needs to be critically considered. In addition, their effect on other epidermal constituents cannot be ruled out and needs further investigation.

Fig. 6 shows that oedema at 12\textsuperscript{th} hour follows the order, I = IV > V = III > VI > VII = II (t-test, \( P < 0.05 \)). Hence, the permeation of ion-paired DS across cardamom oil pretreated skin (gr.VII) is not significantly different from that following subcutaneous injection (gr. II). Cardamom oil pretreatment also enhanced the permeation of ionized DS (gr. VI) and the oedema reduction is comparable to that produced by marketed gel formulation containing an equivalent dose of diclofenac diethyl ammonium (gr. III). Therefore at the end of 12 hr, single application of the marketed gel is significantly less effective than application of ion-paired DS with cardamom oil pretreatment. However, oedema at the end of 24 hr followed the order, III = I = IV = V = VI > VII > II (t-test, \( P < 0.05 \)). No significant difference in oedema between the control group and the group that received an application of marketed gel at the end of 24 hr is because the drug is rapidly absorbed and metabolized.
following application of gel formulation and hence, does not produce any reduction in oedema after 10 hr. Group VII exhibits lower oedema with respect to other groups. This indicates that a combination of ion-pairing and skin pretreatment with cardamom oil enhances systemic delivery of DS. However, the oedema of group VII is significantly higher than that obtained following subcutaneous injection. This can be attributed to the sustained release of DS from ion-paired films.

A flux of 118 µg/cm²/hr (transformed flux 3504.692 µg/cm²/log hr) has been reported to give a Cmax of 1.92 µg/ml and is effective in vivo. The present study reveals a higher flux in vitro (3775.92 µg/cm²/log hr) from films containing ion-paired DS across cardamom oil treated skin that is pharmacodynamically effective. Hence, a combination of ion-pairing and cardamom oil skin pretreatment may provide a new formulation strategy for percutaneous permeation enhancement of DS.

References