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Chemical enhancer solubility in human stratum corneum lipids and enhancer mechanism of action on stratum corneum lipid domain

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ABSTRACT

Previously, chemical enhancer-induced permeation enhancement on human stratum corneum (SC) lipoidal pathway at enhancer thermodynamic activities approaching unity in the absence of cosolvents (defined as *Emax*) was determined and hypothesized to be related to the enhancer solubilities in the SC lipid domain. The objectives of the present study were to (a) quantify enhancer uptake into SC lipid domain at saturation, (b) elucidate enhancer mechanism(s) of action, and (c) study the SC lipid phase behavior at *Emax*. It was concluded that direct quantification of enhancer uptake into SC lipid domain using intact SC was complicated. Therefore a liposomal model of extracted human SC lipids was used. In the liposome study, enhancer uptake into extracted human SC lipid liposomes (EHSCLL) was shown to correlate with *Emax*. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry (DSC) were used to evaluate lipid phase alterations in enhancer-treated intact SC. IR spectra demonstrated an increase in the lipid domain fluidity and DSC thermograms indicated a decrease in the phase transition temperature with increasing *Emax*. These results suggest that the enhancer mechanism of action is through enhancer intercalation into SC intercellular lipids and subsequent lipid lamellae fluidization related to enhancer lipid concentration.

1. Introduction

Skin, being a potential route of drug administration, has been studied extensively to ensure drug delivery at effective therapeutic levels (Ammar et al., 2006a,b). To overcome the skin barrier, numerous chemical penetration enhancers have been identified and their impact on the transport rate limiting barrier/domain in the stratum corneum (SC) has been studied (DiezSales et al., 1996; Lee et al., 2006; Mitragotri, 2000). It is generally agreed that the SC intercellular lipid domain is the site of action for many chemical penetration enhancers (Mills et al., 2003). Previous studies have suggested that a chemical enhancer targeting the SC lipid domain to enhance skin permeation requires the enhancer to orient itself within that microenvironment to perturb and alter the SC lipid lamellae structure (Warner et al., 2003, 2001). Once partitioned in the microenvironment, the mechanism of action of the enhancer is thought to be independent of its chemical structure (i.e., polar head group or alkyl chain length) and dependent only on the effective concentration of the enhancer within that domain (Chantasart et al., 2007).

In a previous study, the permeation enhancement across human epidermal membrane (HEM) induced by a chemical enhancer when it saturates the SC lipid domain in the absence of cosolvents was denoted as *Emax* (Ibrahim and Li, 2009a). *Emax* of the enhancers were determined by allowing HEM to equilibrate with the pure enhancers or enhancer saturated aqueous solutions. It was found that enhancer *Emax* decreased with increasing enhancer lipophilicities. Furthermore, a correlation was shown between *Emax* and the calculated n-octanol enhancer solubilities of the enhancers studied. It was therefore hypothesized that the maximum enhancer uptake into the SC lipid domain at saturation, a parameter related to enhancer solubility within the domain, would correlate with enhancer efficiency. In a different study using *Emax* to further study the mechanism of the enhancers, enhancer saturated SC was allowed to equilibrate with a model permeant, β-estradiol, and assessed for permeant uptake. It was deduced that enhancer-induced permeation enhancement across SC was attributed to the enhanced permeant partitioning into the lipid domain, and an enhancement in permeant diffusivity across the SC was not necessary to account for the permeation enhancement (Ibrahim and Li, 2009b). This has led to the hypothesis that a shift in the lipid domain polarity (rather than a change in the lipid domain microviscosity) would likely be the mechanism of action of the chemical enhancers studied.

Chemical enhancers have been postulated to cause a dynamic structural disorder in the SC intercellular lipids resulting in trans-

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dermal permeation enhancement (Bezema et al., 1996). Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry (DSC) have both been employed in the study of SC intercellular lipids and the effects of chemical enhancers on lipid membrane phase and organization (Golden et al., 1986; Kim et al., 2007). ATR-FTIR can provide insights into the vibrational frequencies of the components in intact SC, while DSC can be employed to study the phase behavior of SC lipids (Moore and Rerek, 1997; Narishetty and Panchagnula, 2005; Silva et al., 2006). Yet, the use of DSC and ATR-FTIR to study an array of different classes of chemical enhancers under the same enhancer treatment within a single study is not common, and a correlation between SC lipid phase behavior modifications and permeation enhancement induced by the enhancers has not been observed.

The objectives of the present study were to: (a) quantify chemical enhancer uptake into the SC intercellular lipids at saturation when the thermodynamic activities of the enhancers in the SC lipid domain approached unity, (b) determine enhancer uptake into liposomes of extracted human SC lipids as a model of the SC lipid domain, (c) examine a possible relationship between liposome enhancer uptake at saturation and *Emax*, (d) further depict the mechanism of action of the enhancers at the molecular level using DSC and ATR-FTIR with intact SC, and (e) study a possible correlation between the extent of enhancer-induced SC lipid phase alteration and enhancer *Emax*.

2. Experimental method

2.1. Materials

Posterior torso split thickness cadaver skin was obtained from the New York Firefighters Skin Bank (New York, NY). Isopropyl myristate (IPM) and n-hexanol (HL) were obtained from Alfa Aeser (Ward Hill, MA) at purities >98% and oleyl alcohol (OA) at purity >85%. 2-Phenoxyethanol (PHE), n-octanol (OC), 1-undecanol (UD), butylated hydroxyanisole (BHA), salicyaldehyde (SA) and sodium azide were purchased at purities ≥98% from Acrōs Organics (Morris Plains, NJ). 1-Octyl-2-pyrrolidinone (OP), N-dodecylpyrrolidinone (DoP), 2-ethyl hexylsalicylate (OS), and benzyl alcohol (BA) were purchased from Sigma–Aldrich, Co. (Saint Louis, MO) at purities >98%. Laurocapram (AZ) was purchased from NETQEM (Durham, NC) at 91% purity. Oleic acid (OL) was purchased from Fisher Chemicals (Pittsburgh, PA) at purity >95%. Padimate O (PADO) was purchased from Spectrum Chemicals (Gardena, CA) at purity >90%. Trypsin from bovine pancreas was purchased from MP biomedical (Santa Ana, CA). Phosphate buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) at pH 7.4, was prepared by dissolving phosphate buffer tablets in distilled deionized water and preserved using 0.02% sodium azide.

2.2. Isolation of human stratum corneum

Human epidermal membrane (HEM) was prepared from split thickness cadaver skin by heat separation (Chantasart et al., 2007; Raykar et al., 1988). Briefly, split thickness cadaver skin was immersed in PBS maintained at 60 °C for 1 min. After which, the dermis was carefully peeled off from HEM (viable epidermis and SC) which was then stored at −20 °C until later use. To isolate the SC from HEM, HEM was allowed to float in a Petri dish containing 0.2% trypsin in PBS (pH 7.4) with SC facing up. The Petri dish was covered and kept at 37 °C for 16 h. After epidermal digestion, the SC was lifted using a filter paper and was then placed over distilled deionized water. To ensure complete removal of the viable epidermis, the viable epidermis side was wiped gently using a cotton swab. The SC was then rinsed using distilled deionized water at least three times where excess water was removed using Kimwipes. The SC samples were weighed and placed in scintillation vials in a desiccator for 12 h at room temperature.

2.3. n-Hexane treated and delipidized human stratum corneum preparation

n-Hexane treatment was used to remove the surface lipids of the SC in the present study. n-Hexane treated SC was prepared by rinsing dry SC with n-hexane for 3 × 10 s and patted dry using Kimwipes after each rinse (Chantasart et al., 2004). Delipidized SC was prepared by placing weighed dry n-hexane treated SC in scintillation vials filled with 10 ml chloroform/methanol (2:1) and kept on a low speed shaker for 48 h at room temperature (Chantasart et al., 2004). The SC was then removed and rinsed three times with fresh chloroform/methanol (2:1). n-Hexane treated SC and delipidized SC were then placed in scintillation vials and allowed to dry for 12 h in a desiccator at room temperature, and then carefully weighed. The chloroform/methanol SC lipid extracts were combined and evaporated using a rotavap DUCHI V-800 (Switzerland) under vacuum at 35 °C, which were then sealed under nitrogen and stored at −20 °C until later use.

2.4. Enhancer uptake and partitioning with n-hexane treated and delipidized SC

To completely hydrate n-hexane treated (or delipidized) SC, SC (n-hexane treated or delipidized) of known dry weight was placed in scintillation vials filled with 10 ml of PBS for at least 4 h. After hydration, n-hexane treated (or delipidized) SC was then carefully weighed.

For the relatively high lipophilic enhancers (enhancers with $\log K_{oct} > 3.3$), to determine enhancer uptake into n-hexane and delipidized SC at *Emax*, fully hydrated SC (n-hexane treated or delipidized) was lifted using a filter paper and, the filter paper was then placed on a cotton support wetted with PBS in a Petri dish. Enhancer (UD, OA, OS, IPM, DoP, PADO or AZ) solution was prepared at 8% (v/v) in ethanol, and 350 μl of which were used to treat SC (n-hexane treated or delipidized). This particular treatment has been demonstrated to result in HEM permeation enhancement with enhancement factors similar to *Emax* (Ibrahim and Li, 2009b). SC (n-hexane treated or delipidized) was then equilibrated for 20 min on the PBS wetted cotton support. After which, the SC (n-hexane treated or delipidized) was rinsed three times with distilled deionized water, patted using Kimwipes between each rinse, and placed in scintillation vials containing 20 ml of PBS. The SC was then placed in a shaker at 37 °C for 12 h. For the relatively low lipophilic enhancers (enhancers with $\log K_{oct} \leq 3.3$), PBS saturated with enhancers (HL, OC, SA, PHE, BHA, BA, or OP at 0.056, 0.006, 0.08, 0.18, 0.008, 0.38, and 0.0048 M, respectively) was prepared as described previously (Ibrahim and Li, 2009a). The n-hexane treated (or delipidized) SC was suspended in 20 ml of enhancer saturated PBS in a scintillation vial. The scintillation vial was then placed in a shaking water bath at 37 °C for 12 h.

After 12 h of equilibration, the enhancer equilibrated n-hexane treated SC (or delipidized SC) was removed from the vial and rinsed with distilled deionized water. The enhancer was then extracted from the SC samples with 5 ml of ethanol for 48 h on a medium speed shaker. Ethanol aliquots were centrifuged for 30 min (Fisher Centrific Model 228) then filtered using a 0.45 μm Millipore filter (MF™ membrane, Bioscience, life Science Products). The first part of the filtrate was discarded. The extraction supernatant was analyzed using HPLC or GC to determine enhancer uptake into SC (n-hexane treated or delipidized).

2.5. Calculation of enhancer uptake

The enhancer equilibrium uptake amount was calculated by:

$$A_{\text{corrected}} = \frac{A_{\text{extracted}}}{W_{\text{dry}}} - (W_{\text{wet}} - W_{\text{dry}})\frac{S_{\text{aq}}}{W_{\text{dry}}} \quad (1)$$

where $A_{\text{corrected}}$ is the amount of enhancer in the n-hexane treated SC (or delipidized SC) and expressed here as micromoles of enhancer uptake per milligrams of dry n-hexane treated SC (or delipidized SC), $A_{\text{extracted}}$ is the amount of enhancer extracted from the n-hexane treated SC (or delipidized SC), W_{dry} is the dry n-hexane treated SC weight (or dry delipidized SC weight), W_{wet} is the wet weight of n-hexane treated SC (or delipidized SC) and S_{aq} is the aqueous solubility of the enhancer. The second term of the equation was used to correct for enhancer uptake in the aqueous compartment within the SC.

2.6. Extracted human stratum corneum lipid liposomes (EHSCLL) preparation and characterization

2.6.1. Analysis of extracted human SC lipid and EHSCLL preparation

EHSCLL was prepared using the lipids extracted from human SC (Section 2.3). The methods of SC lipid extraction are well established in the literature and the SC lipid compositions have been identified (Hatziantoniou et al., 2007; Schreiner et al., 2000). To verify the SC lipid extraction procedure in the present study, the extracted SC lipids were analyzed. A known amount of extracted SC lipids was dissolved in chloroform/methanol (2:1) and the sample was analyzed under gradient elution (acetonitrile and 0.1% formic acid in deionized water at a gradient from 5 to 95% acetonitrile over a period of 20 min) in an analytical LC/ESMS system. Briefly the system consisted of an Alliance Waters 2695 separation module, connected to both a dual absorbance detector (Waters 2487), Waters micromass ZQ and a two position actuator control module (VICI Valco Instruments Co. Inc., Houston, TX).

For the preparation of EHSCLL, a known amount of the extracted dried lipids (after being accurately weighed on custom made aluminum weighing pan) was dissolved in a known volume of chloroform/methanol (2:1) in a round bottom flask (approximately 0.2 mg lipids per milliliter chloroform/methanol). The solvent was then evaporated using a rotavap DUCHI V-800 (Switzerland) under vacuum at 35 °C to obtain a thin lipid film. The thin film was then hydrated with a known volume of PBS and allowed to hydrate for 4 h, with cycles of warming to 70 °C and vortexing the dispersion (approximately 2 mg lipids per milliliter PBS). The mixture was then sonicated in a water bath ultrasonicator (Laboratory Supplies Co., Hicksville, NY) or by using a bath sonicator for 3 × 10 min cycles until large multilamellar liposomes were prepared (in the micron range). The mixture was then heated to 70 °C (Alonso et al., 2000) for 2 min and sonicated using a probe sonicator (VCX-130 Vibra Cell, Sonics & Materials, Inc., Newtown, CT) for 4 min at 100% amplitude with an on-off cycle of 10 and 1 s, respectively. The probe sonication step was repeated if the size of the liposomes was not within 100–400 nm range indicating the formation of large unilamellar vesicles (Luckey, 2008).

2.6.2. EHSCLL characterization

EHSCLL were characterized based on size and zeta potential using Malvern Zetasizer® Nano (Malvern Instruments Ltd., United Kingdom). 1.5 ml of liposome dispersion was pipetted into VWR disposable cuvettes and the average diameter and polydispersity index (PDI) were determined. The zeta potential of the liposomes was determined by pipetting 0.75 ml of the dispersion into the folded capillary zeta potential cell and then analyzed. In order to determine the most suitable storage conditions and stability of liposomes, liposome dispersion was stored at 4 °C. Since some of the experiments proposed in the present study required the equilibration of liposomes in PBS and enhancer solutions at 37 °C, stability at this temperature was determined by pipetting 10 ml of the liposome dispersion into scintillation vials and kept under well stirred conditions in a circulating water bath. Under the two different storage conditions (4 and 37 °C), the size of EHSCLL was measured every 24 h for 8 days.

2.6.3. Quantification of EHSCLL

Since enhancer uptake into liposomes in the present study was determined based on the initial dry weight of the lipids used in the liposome preparation (the enhancer uptake results are presented in micromoles of enhancer per milligrams of liposomes in dry lipid weight), cholesterol was used as a marker to check for possible loss of liposomes in the various steps associated with the procedure of enhancer equilibration and separation of liposomes. Briefly, the amounts of cholesterol in 2 mg/ml extracted human SC lipids in chloroform/methanol (2:1) were determined by GC analysis and compared to those in the liposomes after being eluted through a G-25 Sephadex column (GE Healthcare, Piscataway, NJ) to estimate the percentage recovery of EHSCLL after elution through the column. The recovery of cholesterol in the liposomes after Sephadex separation was found to be ≈97%.

2.7. Enhancer uptake in EHSCLL

For the relatively lipophilic enhancers (enhancers with $\log K_{\text{oct}} > 3.3$), 1 ml of EHSCLL dispersion (~2 mg/ml) was placed in a scintillation vial containing 3 ml of PBS under well-stirred conditions in a circulating water bath at 37 °C. Enhancer (UD, OA, OS, IPM, PADO, or DoP) solution was prepared at 3.6% (v/v) in ethanol. The enhancer/ethanol solution was pipetted (1 μl) into the scintillation vial and stirred for 1 h. This titration step was repeated until the liposome dispersion system was saturated by the enhancer, evident by a thin enhancer film on the solution surface (Yang et al., 2007). The liposome dispersion was then stirred for a total of 12 h at 37 °C. For the relatively low lipophilic enhancers (enhancers with $\log K_{\text{oct}} \leq 3.3$), 100 μl of EHSCLL dispersion was pipetted into 9.9 ml PBS saturated with enhancer (HL, OC, SA, PHE, BHA, BA, or OP at 0.056, 0.006, 0.08, 0.18, 0.008, 0.38, and 0.0048 M, respectively). This dispersion was stirred at 37 °C for 12 h (Yoneto et al., 1997). After enhancer equilibration, 1 ml was taken from the EHSCLL dispersion and then passed through a Sephadex column G-25 (GE Healthcare, Piscataway, NJ) and the void volume fraction (1.5 ml) was collected (Bao et al., 2006). The eluted enhancer equilibrated liposome was then diluted with 500 μl of HPLC grade acetonitrile and placed on a shaker overnight, and the concentration of the enhancer was analyzed using the proper GC or HPLC chromatographic analyses. The ability of the Sephadex column to separate the enhancers and EHSCLL was tested in a control experiment, in which the enhancer/ethanol solution was pipetted into PBS and stirred for 12 h at 37 °C and then eluted through the Sephadex column as described above but without the liposome. No enhancer was detected in the void volume where the liposome was supposed to be eluted.

2.8. SC lipid organization alteration study with intact SC using DSC and ATR-FTIR

Intact SC, of three different donors, was isolated as indicated in Section 2.2, placed on aluminum foil sheets, and stored in tightly sealed scintillation vials at −20 °C till later use. Eight enhancers (OC, OP, DoP, OS, IPM, PADO, OL, and AZ), of varying *Emax* values and chemical classes, were selected for the DSC and ATR-FTIR studies.

SC was first allowed to fully hydrate in 20 ml of PBS in a Petri dish for 2 h. For equilibration with OP and OC, SC was placed in scintillation vials containing 20 ml PBS saturated with the enhancer (OC at 0.006 M and OP at 0.0048 M), and allowed to equilibrate in a shaking water bath for 12 h at 37 °C. For equilibration with DoP, OS, OL, PADO, IPM, and AZ, a direct enhancer treatment was carried out (Ibrahim and Li, 2009a). Briefly, SC was allowed to equilibrate with 20 ml of the enhancer saturated with PBS for 20 min then rinsed with PBS three times, and patted with Kimwipes between each rinse. SC was then allowed to equilibrate with 4 ml PBS in a scintillation vial for 12 h in a shaking water bath at 37 °C. After equilibration, the SC was lifted by an aluminum foil sheet for the DSC and ATR-FTIR studies.

2.8.1. DSC measurements

Approximately 20 ± 0.5 mg of enhancer-treated intact SC was sealed in platinum hermetic pans, and equilibrated for 1 h at room temperature before the measurement with DSC (STA 409 PC Luxx®—Thermobalance, NETZSCH Instruments Inc., Burlington, MA). The DSC resolution was 1 μW. The scanning rate was 2 °C/min over a temperature range of 20–110 °C. The thermograms were obtained, analyzed, and the endothermic lipid phase transition temperatures were determined. SC equilibrated with PBS was used as the control. SC samples prepared as described in Section 2.2 were delipidized (using 10 ml of chloroform/methanol (2:1) in a scintillation vial and kept on a medium speed shaker for 48 h) and was used as a negative control.

2.8.2. ATR-FTIR measurements

Before measurements, the ATR crystal was cleaned with a Kimwipe wetted with acetone. Enhancer-treated intact SC on an aluminum foil sheet was then placed on the ATR-FTIR (Nicolet 6700 FT-IR Spectrometer, Thermo Fisher Scientific Inc., Waltham, MA) with the SC facing the ATR crystal. All SC samples were analyzed at room temperature. Prior to the analysis of each sample, a blank background was collected. Each spectrum represented an average of 16 scans. The data spacing was 0.241–1.928 cm^{-1}. Three samples were taken from each skin donor, and the experiments were repeated using three different donors ($n = 9$ total). SC equilibrated with PBS was the PBS control. OMNIC professional software (Thermo Electron Corporation, Waltham, MA, USA) was used to analyze the IR spectra and determine the frequency band positions. SC delipidized using chloroform/methanol (2:1) was used as a negative control.

2.9. HPLC and GC analysis

The same HPLC and GC systems and analytical procedure described previously were used to analyze the enhancers (Ibrahim and Li, 2009a), except the following. The GC methods for a number of enhancers were modified from the referenced procedure to a splitless injection. The injector temperature, FID detector temperature, and column oven temperature were: 350, 350, 150 °C for 1 min then to 250 °C at a rate of 30 °C/min and held at 250 °C for 0.5 min for IPM; 350, 350, 100 °C for 1 min then to 250 °C at a rate of 30 °C/min and held at 250 °C for 1 min for UD; 250, 250, 50 to 120 °C at a rate of 15 °C/min and held at 120 °C for 0.25 min for HL; 250, 250, 70 °C for 1 min then to 120 °C at a rate of 20 °C/min and held at 120 °C for 0.5 min for OC; 450, 450, 150 °C for 0.5 min then to 300 °C at a rate of 50 °C/min for AZ, respectively. A split injection (split ratio = 20) was used for cholesterol assay where injector temperature, FID detector and column oven temperature were 400, 400, 200 °C for 1 min then to 360 °C at a rate of 20 °C/min, respectively. The limits of quantitation (LOQ) of the HPLC and GC methods were in the ~0.01–0.1 μg/ml range.

2.10. Statistical analysis

The mean and standard deviation (SD) of the results were presented. Data were analyzed using two-tailed student's *t*-tests for the comparison of two groups and ANOVA for multiple groups when appropriate. Linear regression analyses were used to examine possible correlations and determine the correlation coefficients.

3. Results and discussion

3.1. Enhancer uptake and partitioning into SC

It was previously postulated that the efficiency of an enhancer to enhance transdermal permeation, expressed as *Emax*, was related to the solubility of the enhancer in the SC lipid domain. In the present study, enhancer solubility within the SC lipid domain was determined to examine a possible relationship between the amount of enhancer in the lipid domain and enhancer efficiency (*Emax*) by assuming the existence of three distinct compartments in the human SC: (a) the intercellular lipid domain (the lipoidal transport domain), (b) the protein domain including the cross-linked keratin and other protein cellular components of keratinocytes along with the covalently bound lipids (non-extractable lipids), and (c) the aqueous solvent domain that was assumed to have similar properties to bulk water (Raykar et al., 1988). Table 1 presents the amount of enhancer uptake into the n-hexane treated SC and into the delipidized SC. The amount of enhancer uptake into the SC intercellular lipid domain was calculated by subtracting the amount uptake into the delipidized SC from that into n-hexane treated SC [(Table 1 column 2) minus (Table 1 column 4)].

Fig. 1 presents a plot of the enhancer uptake in SC intercellular lipid domain and enhancer *Emax*. The *Emax* values in the figure were obtained from a previous study (Ibrahim and Li, 2009a). The figure shows large data scattering in the relationship of enhancer uptake into the lipid domain versus *Emax* for the majority of the chemical enhancers studied. In addition, the results of PHE, AZ, and IPM are not presented due to the difficulty of determining enhancer uptake into the SC intercellular lipid domain; this is attributed to the comparable enhancer uptake in n-hexane treated and delipidized SC. For enhancers UD, PADO and DoP, the amounts of enhancer uptake in SC (by weight) were surprisingly high and larger than the total amount of lipids in the SC. In other words, the ratio of the weight of enhancer uptake in the lipid domain to the total weight of SC intercellular lipids exceeded unity. The uncertainties in enhancer uptake

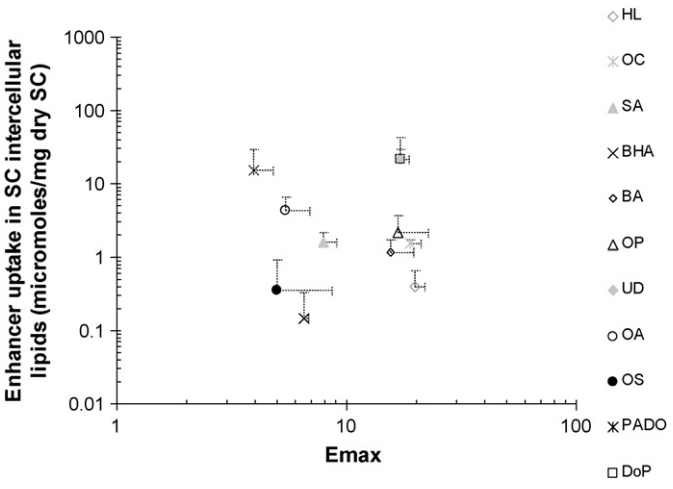

Fig. 1. Relationship between log of enhancer concentration in the intercellular lipid domain and log *Emax*. DoP and UD results superimpose. Mean ± SD ($n \geq 4$).

Table 1
Enhancer uptake in n-hexane treated and delipidized human stratum corneum.

Chemical enhancer	Amount of chemical enhancer uptake into n-hexane treated human SC[a]	Amount of chemical enhancer uptake into delipidized human SC[a]	
	$A_{corrected}$[b] (μmol/mg dry n-hexane treated human SC)	$A_{corrected}$[b] (μmol/mg dry delipidized human SC)	$A_{corrected}$[b,c] (μmol/mg dry delipidized human SC)
HL	0.8 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
OC	1.7 ± 0.2	0.29 ± 0.09	0.24 ± 0.07
SA	2.0 ± 0.5	0.5 ± 0.2	0.4 ± 0.2
PHE	7.4 ± 1.1	9.4 ± 3.5	7.8 ± 2.9
BHA	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.2
BA	1.9 ± 0.5	0.9 ± 0.2	0.8 ± 0.2
OP	2.7 ± 1.5	0.61 ± 0.06	0.51 ± 0.05
UD	22.4 ± 8.2	1.2 ± 0.2	9.6 ± 0.2
OA	4.3 ± 2.4	0.11 ± 0.03	0.09 ± 0.03
OS	1.0 ± 0.6	0.7 ± 0.2	0.6 ± 0.2
IPM	0.4 ± 0.1	0.81 ± 0.07	0.67 ± 0.06
PADO	17 ± 14	2.0 ± 0.4	1.6 ± 0.2
DoP	13.0 ± 1.0	1.0 ± 0.2	0.8 ± 0.2
AZ	0.09 ± 0.06	0.5 ± 0.2	0.4 ± 0.2

[a] Mean ± SD ($n \geq 4$).
[b] Corrected for the uptake into the aqueous compartment.
[c] Normalized by the weight of n-hexane treated SC. Hence the uptake data were multiplied by the weight percent of the delipidized component of SC (83.6%).

measurements of the SC lipid and non-lipid domains and the unreasonably high enhancer uptake of some enhancers into the SC lipids are believed to be related to enhancer-SC interactions. The extent of these interactions is likely to depend on the physicochemical properties of the enhancers. For the low lipophilic enhancers, e.g., PHE, enhancer uptake into the delipidized and n-hexane treated SC would not differ significantly (*t*-test, $p = 0.4$) possibly due to the relatively high uptake of these enhancers into the SC protein domain and the significant contribution of enhancer uptake in the aqueous solvent domain. For the highly lipophilic enhancers, e.g. PADO, considerable enhancer uptake into the keratinocyte covalently bound lipids and enhancer binding to delipidized SC could occur. Therefore, the present uptake study with intact n-hexane treated and delipidized SC did not provide any conclusive data for the enhancers.

3.2. EHSCLL and enhancer uptake in EHSCLL

Due to the difficulty of assessing enhancer uptake into SC lipid domain by the direct determination of enhancer uptake using intact n-hexane treated and delipidized SC, EHSCLL were employed as a model system for the SC lipid domain. The extracted human SC lipids were analyzed using LC/MS, and the spectra obtained were compared to SC lipid spectra previously presented (Pons et al., 2002; Raith et al., 2000) to support the lipid extraction procedure. The size of EHSCLL ranged from 100 to 400 nm with polydispersity index (PDI) less than 0.25 (on PDI scale from 0 to 1) and zeta potential within −40 ± 19 mV. EHSCLL were considered no longer stable if their average size increased to above 400 nm or PDI exceeded 0.25. Based on the size of the liposomes in storage over time, EHSCLL were determined to be stable for up to 8 days when stored at 4 °C whereas at 37 °C the liposomes were stable only for up to 48 h (data not shown).

Table 2 summarizes the enhancer uptake data of EHSCLL. For the majority of the enhancers, the uptake of enhancers into EHSCLL (in μmol/mg) is smaller than that determined using intact SC. This can be attributed to enhancer uptake into the different SC compartments and enhancer-SC binding in the intact SC experiments. OL was excluded from this study due to the inability to distinguish between ionized and unionized forms of OL in EHSCLL and the existence of the ionized form of OL that may alter the structure of the EHSCLL (preliminary studies on the uptake of a number of fatty acids into EHSCLL also demonstrated excessively high uptake of fatty acids, data not shown). Fig. 2 shows a relationship between enhancer uptake into EHSCLL and *Emax*. The figure shows an increase in enhancer uptake into EHSCLL with an increase in the enhancement factor *Emax* with a log–log linear regression

Table 2
Enhancer uptake in EHSCLL.

Chemical enhancer	Amount of chemical enhancer uptake into EHSCLL[a] (μmol/mg EHSCLL)
HL	0.6 ± 0.3
OC	2.2 ± 0.4
SA	0.09 ± 0.05
PHE	29 ± 4
BHA	0.10 ± 0.03
BA	5 ± 1
OP	1.7 ± 0.2
UD	0.03 ± 0.02
OA	0.10 ± 0.04
OS	0.032 ± 0.002
IPM	0.018 ± 0.008
PADO	0.039 ± 0.007
DoP	0.4 ± 0.1
AZ	0.24 ± 0.03

[a] Mean ± SD ($n = 4$).

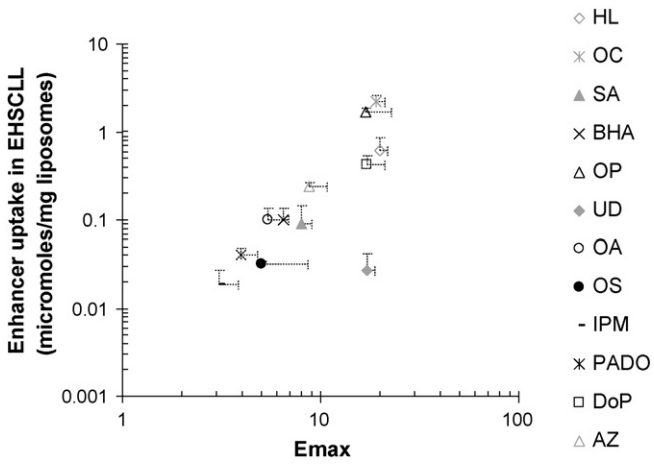

Fig. 2. Relationship between log of enhancer concentration in EHSCLL and log *Emax* with linear regression of $r^2 = 0.55$ ($r^2 = 0.89$ without UD). Mean ± SD ($n = 4$).

of $r^2 = 0.55$ ($r^2 = 0.89$ when UD is excluded). UD shows lower enhancer uptake into EHSCLL in comparison to other enhancers with similar *Emax*. PHE and BA have been excluded from the figure due to their high weight fraction in EHSCLL (exceeding 50% by weight of dry lipids in liposomes). These enhancers have relatively high aqueous solubilities when compared to the other enhancers studied. The high uptake values of PHE and BA may be attributed to their aqueous solubilities resulting in comparable enhancer amounts in the lipid phase and internal aqueous phase of EHSCLL and/or excessive amounts of the enhancers in the liposome bilayer altering the liposome structure and consequently compromising the enhancer uptake data in the EHSCLL model. The figure shows that the relationship between EHSCLL enhancer uptake and *Emax* was independent of the chemical class of the enhancers studied (except UD), and suggests that the permeation enhancing effect of the enhancers depends mainly on the effective concentration of the enhancers in the SC lipid domain: the larger the number of enhancer molecules intercalating the lipids, the larger the permeation enhancement induced.

Previous enhancer uptake study of enhancers with hairless mouse skin showed that approximately 0.2 μmol of the enhancers per milligram dry SC lipids (estimated by converting the per milligram dry SC data to per milligram dry SC lipids) were required to induce a ten-fold permeation enhancement ($E = 10$) on the SC lipoidal pathway (He et al., 2003, 2004). The enhancer uptake data in the present EHSCLL study for enhancers of *Emax* values between 7 and 10 (e.g., BHA, SA and AZ) showed enhancer uptake of ~0.09 to 0.24 μmol/mg of dry lipids, consistent with those determined previously using intact SC at approximately the same enhancement factor (He et al., 2003, 2004). This result further supports the hypothesis that the EHSCLL is a good model for studying SC lipid domain enhancer uptake and for chemical enhancer evaluations.

3.3. SC lipid organization alteration study with intact SC and DSC

DSC has been commonly used to study the phase transitions (thermal phase properties) of the SC intercellular lipids. SC phase transitions normally occur between 35 and 90 °C (Narishetty and Panchagnula, 2005). Typical *in vitro* analyses of isolated human SC have previously demonstrated three to four endothermic temperature transitions. The first typical endothermic peak occurs at 40 °C and has been attributed to either lipid lamella phase transition from a crystalline to a gel like phase (Golden et al., 1986) or melting of sebaceous lipids (Barry and Williams, 1995). At higher temperatures ranging from 75 to 85 °C, the lipids further change from the gel phase to a more liquid state (Golden et al., 1986). Two peaks around 62 °C (lower range lipid phase transition temperature) and 79 °C (upper range lipid phase transition temperature) have also been reported (Vaddi et al., 2002). These peaks generally correspond to the transformations from a lamellar to disordered state in the lipid structure and protein associated lipid transition from gel to liquid state, respectively. The endothermic peak associated with the denaturation of keratinocyte keratins occurs at 105 °C (Golden et al., 1986).

In the present study, the endothermic lipid phase transition temperatures in the DSC thermograms were investigated in the range of 20–110 °C. The phase transition temperatures of the enhancer-treated SC and the control (PBS treated) SC of the same skin donors are summarized in Table 3. Among the DSC endotherms of the PBS control SC samples, two donors showed a single endothermic peak at 84.1 and 78.1 °C, respectively, whereas the third donor showed two endothermic peaks at 53.2 and 79.7 °C. These results demonstrate the broad range of the phase transition profiles among different skin samples from different donors. In all the thermograms, there appeared no peaks associated with keratinocyte denaturation at 105 °C. The keratin denaturation peak has also been reported to occur at 130 °C (Baby et al., 2006) which may explain the absence of this peak in the thermograms in the present study. Furthermore, the number of endothermic peaks in enhancer-treated SC was not equivalent to the number of endothermic peaks in the PBS control SC of the same donor as demonstrated in Table 3. For example, all OP SC equilibrated samples showed two endothermic peaks, whereas all OL and OC treated SC showed a single endothermic peak.

The phase transition temperature between 70 and 85 °C was suggested to be associated with enhancer-induced fluidization effect (Glass, 2007), and therefore was used to evaluate the impact of the enhancers on SC lipid phase behavior in the present study. To further validate that the transition temperatures occurring within the above stated range are associated with SC intercellular lipids, thermograms of negative control SC samples (delipidized SC samples, $n = 3$) were analyzed and showed complete absence of any endothermic peaks within the temperature range studied. Fig. 3 shows a trend of a decrease in this phase transition temperature with an increase in *Emax* ($r^2 = 0.81$). The decrease in the phase transition temperature of SC (ΔT_m) observed in the present study is consistent with those in previous studies. An example is the decrease in the phase transition temperature of AZ treated SC in the present study (ΔT_m of 6.2 °C) and that of a previous study when azone was deposited on SC from an ethanol solution ($\Delta T_m \approx 5$–8 °C) (Harrison et al., 1996).

The decrease in transition temperature suggests an increase in the gross fluidity of the SC lipids in the present study. This is consistent with the general view that the enhancer mechanism of action is attributed to the alteration of the lipid organization and an increase in lipid lamellae disorder in SC. For enhancers with low and moderate *Emax*, i.e., OS, PADO, IPM, and AZ, there was no significant change in the phase transition temperature when compared to the PBS control (ANOVA, $p > 0.05$), whereas the enhancers of higher *Emax* (i.e., OC, OP, DoP, and OL) showed a significant decrease in transition temperature (ANOVA, $p < 0.05$). Thus, it is interesting that there exists an initial stage with no noticeable change in transition temperature at low *Emax* followed by a gradual but statistically significant decrease in the transition temperature with increasing *Emax* (Fig. 3). The lack of a significant transition temperature decrease at the lower *Emax* range can be related to the lack of sensitivity to show a difference due to skin-to-skin variability. Or, this may be attributed to the existence of a target domain (microenvironment) within the SC intercellular lipids that enhancer-induced lipid fluidization was masked and was not detected by DSC even when permeation enhancement or *Emax* was as high as 9. Under this hypothesis, in order for a significant decrease in the phase transition temperature to occur, a significant alteration of the gross fluidity of SC lipid domain is necessary. This requires the "activation" of permeation enhancement in the other lipid domains that would occur with the high *Emax* inducing enhancers.

3.4. SC lipid organization alteration study with intact SC and ATR-FTIR

To further investigate the impact of an enhancer on the molecular organization of the lipids in the SC lipid domain, ATR-FTIR measurements were conducted. Table 3 provides a summary of the wavenumbers of interest for the skin donors studied under different enhancer conditions and of the PBS control. The C—H_2 symmetric and asymmetric stretching (~2850 and 2920 cm^{-1}) frequencies were first analyzed in the present study because the C—H_2 symmetric and asymmetric stretching were often used to study the alterations of SC lipid hydrocarbon chain packing and possible conformational changes induced by chemical enhancers (Golden et al., 1986; Yokomizo, 1996). Normally, hydrocarbons within the lipid

Table 3
Summary of frequency band signals of ATR-FTIR spectra and phase transition temperature(s).

Condition	Donor	C—H_2 asymmetric stretching (cm^{-1})[a]	C—H_2 symmetric stretching (cm^{-1})[a]	Amide I C=O stretching (cm^{-1})[a]	DSC phase transition temperature(s) (°C)
PBS (Blank)	A	2918.2 ± 0.2	2850.2 ± 0.1	1642.8 ± 0.5	84.1
	B	2918.6 ± 0.2	2850.3 ± 0.1	1642.1 ± 0.4	78.1
	C	2919.5 ± 0.5	2850.7 ± 0.1	1643.5 ± 0.7	79.7, 53.2[b]
OC	A	2921.2 ± 0.8	2851.4 ± 0.3	1642.4 ± 1.1	58.6
	B	2920.9 ± 0.6	2851.3 ± 0.3	1642.6 ± 0.2	72.7
	C	2921.5 ± 0.8	2851.4 ± 0.1	1641.6 ± 1.2	60
OP	A	2920.9 ± 1.4	2851.6 ± 0.7	1643.5 ± 0.8	66.6, 54.3[b]
	B	2921.7 ± 0.4	2851.7 ± 0.5	1644.2 ± 0.4	71.5, 36.2[b]
	C	2922.1 ± 0.2	2852.2 ± 0.2	1644.7 ± 0.3	67, 54.3[b]
DoP	A	2923.4 ± 0.0	2853.1 ± 0.1	1646.2 ± 0.2	54.8
	B	2922.8 ± 0.2	2852.7 ± 0.1	1645.2 ± 0.8	52.5
	C	2922.6 ± 0.1	2852.5 ± 0.2	1645.4 ± 0.2	55.2
OS	A	2919.1 ± 0.3	Weak	1643.4 ± 0.3	77.9
	B	2920.0 ± 0.6	Weak	1643.9 ± 0.6	84.7
	C	2919.9 ± 1.0	Weak	1644.5 ± 0.6	86.7, 55.4[b]
IPM	A	2920.0 ± 0.3	2851.1 ± 0.1	1641.4 ± 0.5	82.9
	B	2920.8 ± 0.3	2851.4 ± 0.1	1642.9 ± 0.6	85.9
	C	2920.9 ± 0.3	2851.2 ± 0.1	1643.3 ± 0.8	93.6, 69.5[b]
PADO	A	2918.9 ± 0.6	2851.0 ± 0.5[c]	1642.6 ± 0.8	82.6, 56.8[2]
	B	2919.3 ± 0.2	Weak	1642.5 ± 0.3	92.4
	C	2919.8 ± 0.5	Weak	1644.5 ± 0.2	78.9
OL	A	2922.7 ± 0.0	2852.8 ± 0.1	1645.5 ± 0.6	55.3
	B	2922.3 ± 0.5	2852.3 ± 0.5	1645.0 ± 0.3	72.9
	C	2922.3 ± 0.2	2852.7 ± 0.1	1650.1 ± 0.3	64.1
AZ	A	2922.8 ± 0.2	2852.3 ± 0.2	1641.8 ± 0.1	69.9
	B	2921.7 ± 0.2	2851.6 ± 0.2	1643.6 ± 0.3	77.2, 39[b]
	C	2922.6 ± 0.1	2852.0 ± 0.1	1641.8 ± 0.5	76.1

[a] Mean ± S.D, $n = 3$, 3 samples from each skin donor of A–C.
[b] Indicates the existence of two phase transition temperatures.
[c] One of the SC samples showed a weak signal.

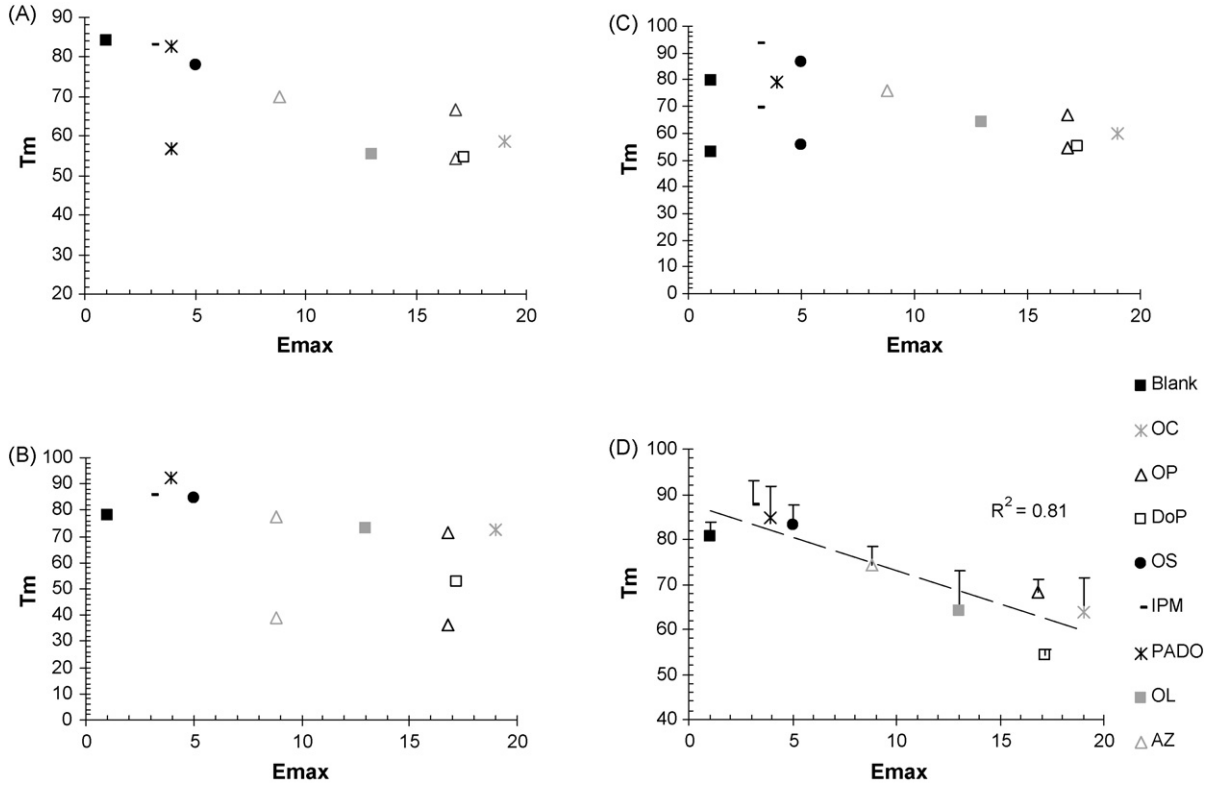

Fig. 3. Relationship between the phase transition temperature of SC lipids and enhancer *Emax*. Graphs A–C represent the lipid phase transition temperature of individual skin donors. Graph D represents the average lipid phase transition temperature (the higher phase transition temperature) of the different skin donors studied. Mean ± SD ($n = 3$).

domain below their transition temperature exist in a trans conformation. When the lipid domain becomes fluidized, a *trans gauche* shift to a higher frequency (blue shift) would occur (Mantsch and Mcelhaney, 1991; Prasch and Forester, 2002). In an IR spectrum, the intensity of a band (height and area) is related to the concentration of the energy absorbing component. When an enhancer extracts SC lipids, these lipid bands would show a decrease in band height and/or area or the bands would completely disappear (Laugel et al., 2005). For untreated SC samples (the PBS control) in the present study, the C—H_2 symmetric ($2850.4 \pm 0.3\,cm^{-1}$) and asymmetric ($2918.8 \pm 0.6\,cm^{-1}$) band frequencies support the existence of SC lipid hydrocarbon in a mostly *trans* conformation and are comparable to those observed in a previous study (Yokomizo, 1996). To further validate the association of these bands with the intercellular lipids, the negative control SC samples (delipidized SC, $n = 9$) in the present study showed complete disappearance of both symmetric and asymmetric C—H_2 bands (data not shown). Fig. 4 shows an increase in the C—H_2 asymmetric and symmetric frequency (indicative of the occurrence of *gauche* rotational conformers along the lipid domain alkyl chains) in the SC treated with the enhancers as *Emax* increases. This trend supports the hypothesis that the enhancers in the present study induce SC intercellular lipid fluidization. The C—H_2 symmetric stretching band for the SC samples treated with OS and PADO showed a weaker signal compared to the PBS control (t-test, $p < 0.05$). This may be attributed to enhancer interference with the IR spectrum and is probably not due to enhancer lipid extraction since OS and PADO did not reduce the asymmetric band signal in the spectrum of the same SC samples. The insignificant changes in the signal height and width of the asymmetric C—H_2 signals (t-test, $p > 0.05$) in all the enhancer-treated SC samples in comparison to the PBS control support that the studied enhancers do not result in significant SC lipid extraction.

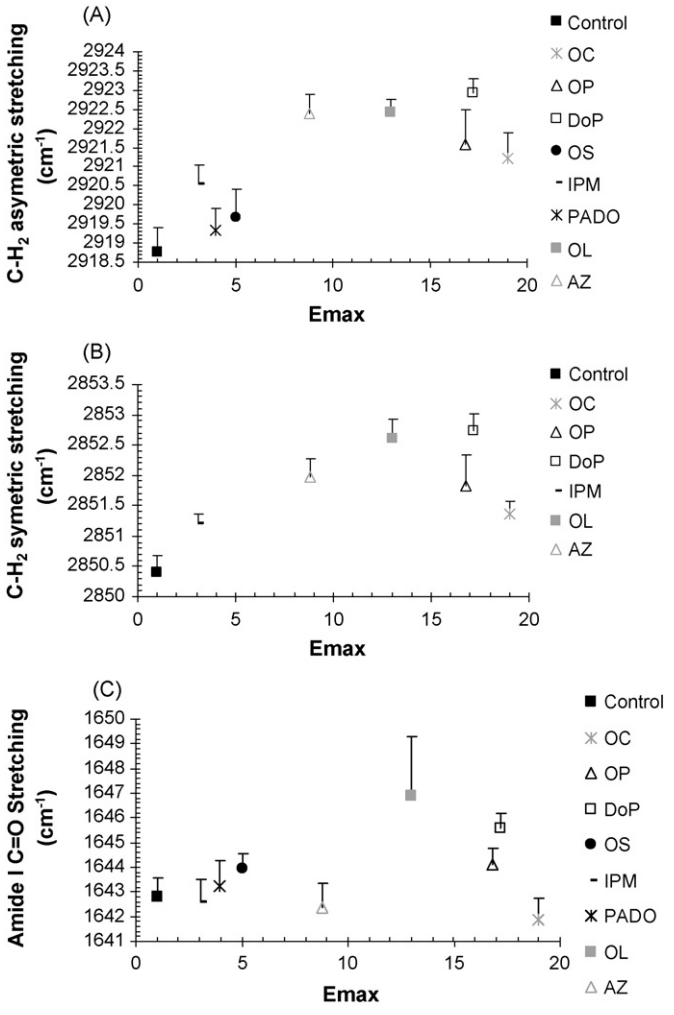

Fig. 4. Relationship between enhancer *Emax* and (A) asymmetric, (B) symmetric C—H_2 stretching frequency of the SC lipid components, and (C) amide I C=O stretching vibration. Mean ± SD, $n = 9$, 9 measurements on 9 SC samples (3 skin donors and 3 SC samples from each donor).

The small but statistically significant shifts of band signals associated with the SC lipid domain induced by the enhancers in the present study are similar to the magnitude of those in previous studies (e.g., Potts et al., 1991). The larger shifts in the asymmetric C–H_2 band signals in comparison to the C—H_2 symmetric band signals observed in the present study are also consistent with those in previous studies (e.g., Narishetty and Panchagnula, 2005). In a recent study, N-lauroyl sarcosine in an ethanol solution was shown to enhance fluorescein permeation across HEM by approximately 15 times, and FTIR was used to assess the impact of the formulation on the SC showing a shift of $2.6\,cm^{-1}$ in the C—H_2 ($\sim 2920\,cm^{-1}$) asymmetric band in comparison to the control (Kim et al., 2007). This value is consistent with the magnitude of the asymmetric band shifts observed with the enhancers inducing *Emax* ~ 15 in the present study. As a further validation of the present FTIR method, SC samples were treated using the same protocol as stated in a previous study for 2 h in 0.15 M oleic acid in ethanol for comparison (Clancy et al., 1994). The result showed a shift in the asymmetric C—H_2 band to $2923.5 \pm 0.2\,cm^{-1}$, which is comparable to the $2923.9 \pm 0.3\,cm^{-1}$ reported in the previous study. Although previous FTIR results supported SC lipid extraction as a possible enhancer mechanism (e.g., Zhao and Singh, 2000), lipid extraction was not evident in the present study. This difference may be attributed to the use of cosolvents such as ethanol or propylene glycol that may have extracted a considerable amount of SC lipids in the previous studies (e.g., Krishnaiah et al., 2002).

To investigate the impact of enhancers on SC protein conformation, the amide I C=O stretching frequency was studied. This amide I band typically occurs at 1600–1700 cm^{-1} and is sensitive to changes in protein conformation. In Fig. 4, the enhancers OL, DoP, OP and OS appear to alter the protein conformation in the non-lipid domain of SC when compared to the PBS control (t-test, $p < 0.05$), although this shift does not correlate to *Emax* of the enhancers. The shift in amide I wavelength may also be a secondary effect of lipid organization change and not associated solely with the protein conformation in SC. The lack of parallelism between protein conformation change and *Emax* supports that the enhancers mainly target the SC lipid domain and not the protein domain for permeation enhancement across SC.

3.5. Mechanism of chemical penetration enhancer

The present study investigated a number of chemical permeation enhancers that are commonly used in transdermal and topical formulations. Under the condition when the enhancers are in equilibrium with SC at saturation and in the absence of a cosolvent, the present results of permeation enhancement induced by the enhancers (previously defined as *Emax*) provide insights into the enhancer mechanism(s) of action in transdermal and topical formulations such as aerosols, topical sprays, and hydro-alcoholic and polymer based gels. The mechanism(s) by which these enhancers induce permeation enhancement in transdermal transport of a lipophilic compound could be attributed to the alteration of the polarity and/or microviscosity of the transport rate limiting pathway in the SC lipid domain. Previously, it was demonstrated that a plausible enhancer mechanism was the alteration of polarity since any changes in microviscosity would result in the alteration of permeant diffusivity in SC, which was not observed in

a previous enhancer study—the transport lag time of the permeant (or its apparent diffusivity in SC) remained essentially the same in the presence of the enhancers (Ibrahim and Li, 2009b). To further understand the enhancer mechanism(s), the impact of the enhancers on SC lipid fluidity was investigated using DSC and ATR-FTIR in the present study. Both the DSC and ATR-FTIR results showed a more fluidized SC lipid state in the presence of the enhancers. The fluidization and consequently a change in the microviscosity of the lipid domain now appear to be a main mechanism of chemical enhancer-induced permeation enhancement for the enhancers investigated. This finding together with the results in the previous study would therefore suggest that the enhancers induce both a polarity shift in the SC lipid domain and a decrease in lipid microviscosity through lipid fluidization in the SC. The absence of a change in the transport lag time of the permeant in the previous study may be attributed to enhancers altering the effective volume or pathlength of the SC lipid domain. Hence, the change in the permeant diffusivity (microviscosity) is masked, and no apparent change in the lag time (apparent diffusivity) occurs.

4. Conclusion

To ensure proper selection of chemical permeation enhancers and to reduce the time required for enhancer screening in transdermal formulations, the mechanism(s) of action of the enhancers must be determined. The chemical enhancers investigated in the present study are commonly used in transdermal and topical formulations. The mechanisms of many of these chemical enhancers, though comprehensively studied, have not yet been clearly understood. Recently, enhancer *Emax*, defined as permeation enhancement across HEM induced by an enhancer in equilibrium with the SC at saturation such that the thermodynamic activity of the enhancer approaches unity in the absence of a cosolvent, was determined and used to study the effectiveness of the enhancer. The study of enhancer effects without a cosolvent under this particular condition (*Emax*) could provide important insights into percutaneous absorption with topical dosage forms of aerosols, topical sprays, and hydro-alcoholic and polymer based gels.

In the present study, SC lipids were extracted and used to prepare EHSCLL that were then used as a model to evaluate enhancer solubility in SC lipid domain. It was shown that the concentration of enhancer uptake in EHSCLL increased with increasing *Emax*. This finding suggests that the effectiveness of an enhancer in transdermal permeation enhancement on the lipoidal pathway across SC is related to enhancer solubility in the SC lipids. This also suggests that the extent of skin permeation enhancement induced by an enhancer is via lipid fluidization and is directly related to the amount of the enhancer in the SC lipids; the enhancement is relatively independent of the nature of the enhancer such as its chemical structure (i.e., alkyl chain length and polar head group), consistent with the hypothesis in the previous *Emax* studies, in which no specific interaction between the studied enhancers and SC lipids for permeation enhancement across the SC lipoidal pathway was observed.

To further understand the mechanism of action of enhancer in the present study, DSC and ATR-FTIR analyses of enhancer-treated intact SC samples were performed. A semi-quantitative correlation between the phase transition temperature, the asymmetric and symmetric $C—H_2$ stretching frequencies, and *Emax* was observed with the enhancers. These results suggest that the main mechanism of action of the enhancers is the fluidization of the SC lipids. The endothermic transition temperature profiles and IR spectra show that the enhancers result in SC lipid disorganization and increase SC lipid fluidity. The results also suggest that SC lipid extraction is not a major mechanism of action of the enhancers in the present study.

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