Transepidermal Transport Enhancement of Insulin by Lipid Extraction and Iontophoresis

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Purpose. To study the effect of Ethyl acetate (EtAc), 1:1 ratio of EtAc and Ethanol (EtOH) and 2:1 ratio of chloroform (C) and methanol (M) on the extent of lipid extraction from the stratum corneum (SC) and *in vitro* passive and iontophoretic transport of insulin through porcine epidermis.

Methods. The porcine epidermis was pretreated for 40 min with the following solvents: 1) EtAc or EtAc:EtOH (1:1) and 2) C:M (2:1), which is a standard solvent combination for lipid extraction. Franz diffusion cells and Scepter™ iontophoretic power source were used for the transport studies. Cathodal iontophoresis was performed at 0.2 mA/cm^2 current density. Fourier transform infrared spectroscopy (FTIR) studies were performed to assess the extent of lipid extraction. Thin layer chromatography (TLC) and gas chromatography (GC) were used to quantitate the different classes of lipid and identify the composition of the fatty acids, respectively, extracted by solvent(s) treatments.

Results. Insulin flux was found to be significantly ($P < 0.05$) greater through solvent pretreated epidermis compared to untreated controls during both passive and iontophoretic transport. Pretreatment with EtAc:EtOH (1:1) exhibited an insulin flux of 15.29 x 10^{-8} nmoles/ cm^2/h compared to 52.71 × 10^{-8} nmoles/ cm^2/h during passive and iontophoretic transport, respectively. The passive and iontophoretic flux of insulin through EtAc:EtOH (1:1) pretreated epidermis was significantly greater ($P < 0.05$) than EtAc treated epidermis. The SC treated with solvents showed a decrease in peak areas of C-H stretching absorbances in comparison to untreated SC. A greater percent decrease in peak areas was obtained by EtAc:EtOH(1:1), in comparison to EtAc alone. Epidermal resistance measurements revealed its strong correlation with the amount of lipids present in the epidermis. The lipids extracted consisted of six series of ceramides, fatty acids, triglycerides, cholesterol, cholesterol esters, cholesterol sulfate and phospholipids.

Conclusions. The SC lipid extraction using suitable solvents followed by iontophoresis can synergistically enhance the transepidermal transport of insulin.

KEY WORDS: insulin; iontophoresis; fourier transform infrared spectroscopy; thin layer chromatography; gas chromatography; lipid extraction; permeability.

INTRODUCTION

The role of stratum corneum (SC) as the primary barrier to transdermal transport is well established (1). SC consists of a regular array of protein rich cells embedded in a multi lamellar lipid domain. Drug transport by the intercellular route involves the diffusion through the lipid pathways between the cells of the SC. There is significant evidence suggesting that the predominant pathway for transdermal delivery of ionic compounds during passive diffusion and iontophoresis is through the intercellular route (2,3). The intercellular route has been identified as the primary pathway for transport of charged substances using confocal microscopy (4) and vibrating probe electrode (5). Mercuric ions have been used to gather evidence to support transport of ionic solutes through the intercellular route (2,6). Hydrophilic permeants are also believed to follow aqueous pore pathways in the SC (7). Proteins and peptides, because of their hydrophilic nature and large molecular size, have limited permeability in the skin.

An increasing number of studies are supporting the hypothesis that removal of intercellular lipids helps decrease the SC barrier resistance and increase the transport of solutes (2,3,8). Iontophoresis is a noninvasive technique that uses a mild electric current to provide a driving force to facilitate transdermal delivery of peptides (8–9). The combining effect of iontophoresis and lipid extraction from the SC may permit the use of lower quantities of current within the delivery system and potentially circumvent adverse skin reactions, irreversible structural changes in the skin and dermatotoxicity (i.e., skin irritation).

Decreases in the absorbances of C-H stretching peaks under the FTIR spectrum of SC have been linked to the extraction of SC lipids (10). Extraction of the SC lipids with C:M (2:1) results in almost complete suppression of the C-H stretching absorbances (11). Therefore, C:M (2:1) can be used as a positive control for lipid extraction. The composition of the lipids in the epidermis has long been of interest because of the suspected differential contribution of different classes of lipids to the barrier function. The ceramides, together with cholesterol, fatty acids, cholesterol sulfate and cholesterol esters, fill the intercellular spaces of the SC, and the multiple lamellae produced from this mixture provide the diffusional resistance characteristic of the permeability barrier (12). Several attempts have been made to relate epidermal membrane lipids and permeability (13,14).

Insulin is used in the control of diabetes mellitus and is delivered by subcutaneous injection. Insulin is ionized across a wide pH range of physiological interest, exhibits poor bioavailability following oral administration and has a short biologic half-life. Thus, insulin is a potential drug to be evaluated for delivery through the transdermal route. Kari (15) observed that to lower the glucose levels in diabetic rabbits by transdermal iontophoretic delivery of insulin, the SC needs to be removed. Instead of removing the SC, another approach may be to reduce the amount and type of SC lipids thereby reducing the barrier properties of skin to passive and iontophoretic transport of macromolecules.

We tested the following hypotheses: 1) Iontophoresis through solvent pretreated epidermis would synergistically enhance the *in vitro* transport of insulin. 2) Pretreatment of the epidermis with solvents would extract the SC lipids and create free volume leading to formation of additional hydrophilic diffusional pathways in the membrane. Such additional hydrophilic pathways would decrease the epidermal electrical resistance and increase the passive and iontophoretic permeability of insulin. In this *in vitro* study, we investigated the effects of solvent pretreatment of the epidermis with EtAc, and 1:1 ratio of EtAc:EtOH on passive and iontophoretic

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transport of insulin through porcine epidermis, extent of lipid extraction from the SC by FTIR spectroscopy and the composition of the extracted lipids using TLC and GC.

MATERIALS AND METHODS

Materials

[^{125}I] Human recombinant insulin (specific activity – 2000 Ci/mmol) was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Ethyl acetate, chloroform, and standards (i.e., phosphatydylethanolamine, phosphatydylinositol, phosphatydylcholine, sphingomylin, cholesterol sulfate, cholesterol, triolein, cholesterol, cholesterol oleate and oleic acid) used in the TLC studies were purchased from Sigma Chemical Company (St. Louis, MO, USA). Hexane and methanol were purchased from EM Science (Gibbstown, NJ, USA) and Fisher Scientific (Pittsburgh, PA, USA), respectively. Toluene and ethanol were procured from Aldrich Chemical Company (Milwaukee, WI, USA). Acetic acid and ethyl ether were obtained from Mallinckrodt Chemical Inc. (Paris, KY, USA). Porcine SC ceramides were provided by Dr. P.W. Wertz (The University of Iowa, Iowa City, IA, USA). All other chemicals and reagents used were of analytical grade. Deionized water (Resistivity $\geq$ 18 MΩ-cm) was used to prepare all solutions and buffers.

Preparation of Epidermis and SC

Porcine ears were obtained from a local slaughterhouse. After cleaning under cold running water, the outer region of the ear was cut. The intact skin was carefully dissected free from the underlying cartilage using a scalpel. The method of Kligman and Christophers (16), with slight modification, was adopted to remove the epidermis. The epidermis was prepared by soaking the whole skin in water at 60°C for 45 s The skin was removed from water, blotted dry and pinned with the dermal side down. The intact epidermis was then teased off from the dermis with forceps, washed with water and used in the *in vitro* transport, epidermal resistance, TLC and GC studies.

The epidermis was incubated for 4 h in a 0.5% trypsin solution in phosphate-buffered saline (pH 7.4) at 37°C . The tissue was then smoothed out on a flat surface and the mushy epidermis removed by rubbing with a moistened cotton tipped applicator. The transparent SC obtained was briefly floated on water and lifted out on aluminum foil, blotted dry, and used in FTIR spectroscopic studies.

In Vitro Transport

The epidermis was pretreated by immersing it into the pretreatment solvents for 40 min and then washing it with de-ionized water. EtAc, EtAc:EtOH (1:1), and C:M (2:1) were used as solvents for pretreatment. Epidermis without pretreatment was used as a control.

Franz diffusion cells modified for iontophoresis with Ag/AgCl electrodes and Scepter™ iontophoretic power source (Keltronics Corporation, Oklahoma, USA) were used in the *in vitro* transport studies. The solvent pretreated or untreated (control) epidermis was sandwiched between the receptor and donor cells with the SC facing the top donor compartment. The maximum capacity of the donor and receiver compartments were 1 ml and 5 ml, respectively. The effective diffusional area of the epidermal membrane was 0.785 cm^2. The donor compartment contained 1 ml of insulin solution [0.2 μCi of insulin in 0.9% w/v sodium chloride (normal saline), pH 6.7], and the receiver compartment was filled with 5 ml of normal saline. The donor concentration of insulin used 0.1 μmoles/ml (0.6124 ng/ml). The cells were maintained at 37 $\pm$ 0.5°C by a PMC Dataplate® stirring digital dry block heater (Crown Bioscientific Inc., Somerville, NJ).

Insulin has an isoelectric point of 5.30, therefore it was negatively charged in normal saline (pH 6.7) (17). Cathodal iontophoresis was performed at a constant current density of 0.2 mA/cm^2. The contents of the receiver compartment were stirred with a magnetic stir bar at 100 rpm. At appropriate times, 0.5 ml samples were withdrawn from the receiver compartment. An equivalent amount of normal saline (0.5 ml) was added to the receiver compartment to maintain a constant volume. Experiments were performed in triplicate for each condition and results were expressed as the mean $\pm$ SD The samples from *in vitro* transport studies were analyzed for insulin using a gamma counter (Beckman Coulter ™, Gamma 5500B, Fullerton, CA, USA) for quantification of ^{125}I-labeled insulin.

Epidermal Resistance

The electric resistance of the epidermis pretreated with the solvents and the control (no treatment) was investigated using modified Franz diffusion cells and Scepter™ iontophoretic power source. The donor and receiver compartment contained 1 ml and 5 ml of normal saline, respectively. The solvent pretreated or control epidermis was sandwiched between the compartments, with the SC facing the donor compartment, and allowed to equilibrate in normal saline for 1 h. Direct current (DC), 0.2 mA/cm^2 (I), was applied by placing the anode in the donor and cathode in the receiver solution. The potential difference (V) across the epidermal membrane was measured using Scepter™. The resistance (R) was calculated according to Ohm's law ($V = I \times R$).

Fourier Transform Infrared (FTIR) Spectroscopy

After solvent pretreatment of the SC for 40 min, the samples were vacuum-dried (650 mmHg) at 21 $\pm$ 1°C for 3 days to remove the solvent from the SC. The samples were then subjected to FTIR spectroscopic study. For each SC sample, the peak areas of C-H stretching absorbances were measured before and after the solvent pretreatment using FTIR (Nicolet 210, Nicolet Instrument Corporation, Madison, WI). This experimental strategy allowed each sample to serve as its own control. Spectra were obtained in the frequency range 3000-2800 cm^{-1}. All spectra analyzed were Happ-Genzel apodized, had a zero-filling factor of none and represent an average of 64 scans with a resolution of 4 cm^{-1}. Attention was focused on the decrease in absorbance (measured in terms of peak area) of asymmetric and symmetric C-H stretching bands (near 2850 and 2920 cm^{-1}, respectively) associated with the alkyl chains of the lipids. OMNIC® FTIR software (Nicolet Instrument Corporation, Madison, WI) was used to calculate the peak areas of C-H stretching absorbances. FTIR experiments with each condition were performed in triplicate.

Thin Layer Chromatography

Glass preadsorbent plates (20 $\times$ 20 cm) coated with 250 μm thick silica gel G (Absorbosil-Plus-1, Alltech Associates,

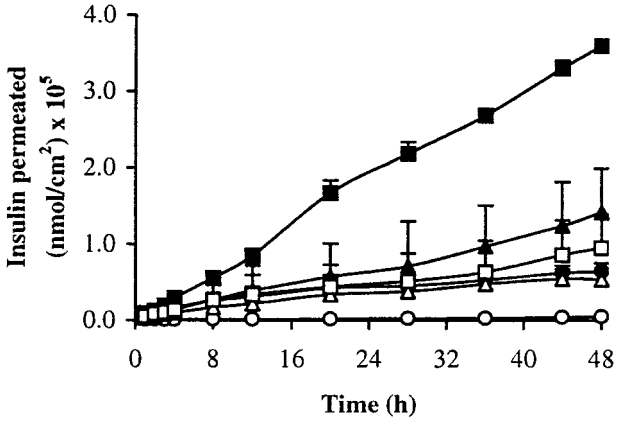

Fig. 1. Effect of solvent treatment on the *in vitro* passive (P) and iontophoretic (I) transport of insulin through porcine epidermis. Each data point is the mean ± SD of three determinations. Key: (○) P control; (Δ) P ethyl acetate; (□) P ethyl acetate:ethanol (1:1); (●) I control; (▲) I ethyl acetate; (■) I ethyl acetate:ethanol (1:1).

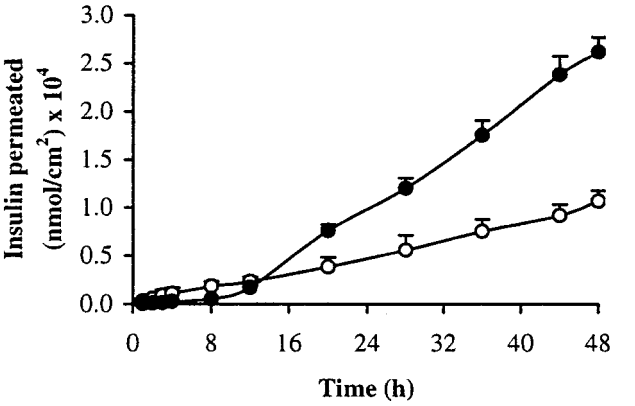

Fig. 2. Effect of chloroform:methanol (2:1) treatment on the *in vitro* passive and iontophoretic transport of insulin through porcine epidermis. Each data point is the mean ± SD of three determinations. Key: (○) passive; (●) iontophoresis.

Inc., Deerfield, IL) were used for the TLC studies. Porcine epidermal lipid extracts obtained using different solvents were dried with a nitrogen stream and were redissolved in C:M (2:1). Chromatograms were developed separately in three different mobile phases as follows: (a) C:M:water (40:10:1), (b) C:M:acetic acid (190:9:1), and (c) hexane followed by toluene followed by two developments to 11 cm with hexane:diethyl ether:acetic acid (70:30:1).

After development, the plates were dried, sprayed with 50% sulfuric acid and heated first to 60°C for 30 min and then 75°C for 3 h in an oven, to char all lipids present. Cholesterol [1, 2, 4, 8, 12, 16, 24, and 32 μg in 4 μl C:M (2:1)] was used as density reference. The chromatograms were scanned with a photodensitometer and the spots were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Values were expressed as μg lipid per g dry weight of epidermis.

Gas Chromatography

The dried lipid extracts were dissolved in ethyl ether and 0.1 M Sodium methoxide solution in methanol:ether (8:2) was added to convert fatty acids present in the samples into fatty acid methyl esters. Acetic acid was added to neutralize the solution. The ether layer was removed and made moisture free by adding anhydrous Na_2SO_4.

Samples were analyzed using a Varian 3600 Gas Chromatograph with a Flame Ionization detector and 30 m × 0.53 mm Hewlett Packard-FFAP (1.0 μm) column. For each sample, the column temperature was programmed to maintain 205°C for 1 min and then increased to 235°C at 3°C/min. A carrier gas of air (300 ml/min), H_2 (30 ml/min), and N_2 (30 ml/min) mixture was used.

Data Treatment

The receiver compartment concentration of insulin was corrected for sample removal (18). The cumulative amount of insulin permeated per unit skin surface area was plotted against time and the slope of the linear portion of the plot was estimated as the steady state flux (J_{ss}). The permeability coefficient, K_p was calculated as (19):

$$K_p = \frac{J_{ss}}{C_v} \quad (1)$$

where C_v is donor concentration of Insulin. Statistical comparisons were made using the students t test. The level of significance was taken as $P < 0.05$.

RESULTS AND DISCUSSION

In Vitro Transport Studies

Figures 1 and 2 demonstrate the *in vitro* passive and iontophoretic transport profiles of insulin through EtAc,

Table I. Epidermal Resistance, Flux of Insulin for Passive and Iontophoretic Transport, and Enhancement Ratio

Treatment	Resistance (KΩ.cm^2)	Flux (nmoles/cm^2/h) × 10^8		Enhancement ratio (ER)		
		P	I	ER_1	ER_2	ER_3
Control	10.27 ± 1.84	1.17 ± 0.42	8.50 ± 3.00[e]	—	—	7.26
EtAc	3.48 ± 0.24	7.90 ± 3.39[a]	30.58 ± 3.85[c,e]	6.75	3.60	26.14
EtAc:EtOH (1:1)	1.47 ± 0.20	15.29 ± 7.07[a]	52.71 ± 4.81[c,d,e]	13.06	6.20	45.05
C:M (2:1)	0.80 ± 0.05	238.87 ± 33.45[a,b]	666.03 ± 26.94[c,d,e]	204.16	78.36	569.26

Note. n = 3 for resistance and flux, except resistance (control) where n = 7; Control = No treatment. P = Passive. I = Iontophoresis; ER_1 = Passive flux of insulin with treatment/Passive flux of insulin with control; ER_2 = Iontophoretic flux of insulin with treatment/Iontophoretic flux of insulin with control; ER_3 = Iontophoretic flux of insulin with treatment/Passive flux of insulin with control; a = Significantly ($P < 0.05$) greater than control (passive); b = Significantly ($P < 0.05$) greater than ethyl acetate treatment (passive); c = Significantly ($P < 0.05$) greater than control (iontophoresis); d = Significantly ($P < 0.05$) greater than ethyl acetate treatment (iontophoresis); e = Significantly ($P < 0.05$) greater than the corresponding passive flux.

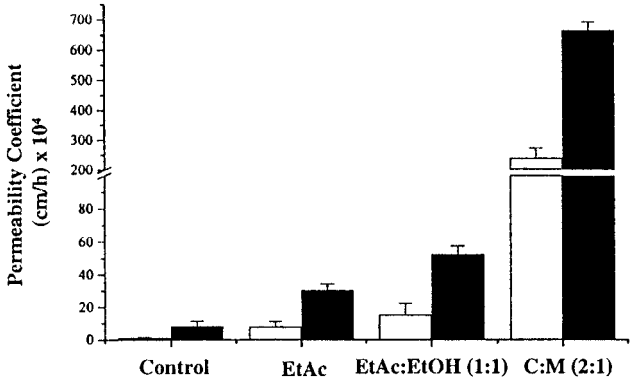

Fig. 3. Permeability coefficient of insulin through porcine epidermis after solvent treatment. Key: (□) passive; (■) iontophoresis.

EtAc:EtOH (1:1) and C:M (2:1) pretreated epidermis. Table I compares flux and enhancement ratios of flux for passive and iontophoretic transport of insulin through porcine epidermis.

All the solvent combination significantly ($P < 0.05$) increased the flux of insulin in comparison to the control (no solvent pretreatment). The flux enhancement ratios (ER_1 values) of insulin by EtAc and EtAc:EtOH (1:1) in comparison to the passive flux of the control was 6.75 and 13.06, respectively. A significant ($P < 0.05$) increase in iontophoretic flux and permeability coefficient of insulin by all pretreatment solvents was observed in comparison to both passive and iontophoretic flux compared to the control epidermis (Fig. 3). Furthermore, iontophoretic flux of insulin through the EtAc: EtOH (1:1) pretreated epidermis was significantly ($P < 0.05$) greater in comparison to the iontophoretic flux of insulin through EtAc pretreated epidermis.

C:M (2:1) is a standard solvent used for lipid extraction. Extraction of the epidermis in C:M (2:1) leads to almost complete (> 80–90%) removal of lipids (20). Forty min pretreatment of epidermis with C:M (2:1) resulted in an ER of 204.16 and 78.36 for passive and iontophoretic flux compared to controls, respectively (Table III).

EtAc has been reported as an effective penetration enhancer for a number of drugs in *in vitro* studies (21). The DSC and FTIR experiments on hairless mouse SC indicated greater lipid-extraction with longer exposures to ethyl acetate (22). EtAc has been examined in an occlusive patch test in humans over 24 h, during which it was found to be non-irritating and non-sensitizing (23). Higher concentrations of EtOH (>50%) are known to have lipid extraction properties (24). A synergism of iontophoresis and EtOH as enhancer was reported on the transport of peptides through human epidermis (25). Our *in vitro* transport studies indicate that use of EtAc with EtOH as a co-enhancer can be a good choice for iontophoretic transdermal delivery of insulin.

FTIR Spectroscopic and Epidermal Resistance Studies

FTIR studies were performed to understand the contribution of lipid extraction from the SC to the enhancement of insulin transport through porcine epidermis. Decreases in peak areas of the asymmetric (~2850 cm^{-1}) and symmetric (~2920 cm^{-1}) C-H stretching absorbances were observed indicating the extent of lipid extraction (Fig. 4). Table II presents the data derived from the FTIR spectra. SC treated with EtAc showed a decrease in peak areas for both asymmetric and symmetric C-H stretching in comparison to untreated SC. EtAc:EtOH (1:1) solvent showed a greater decrease in FTIR peak areas than EtAc alone. Percent decrease in FTIR peak area obtained by EtAc:EtOH (1:1) treated epidermis was 46.21 and 52.69 for asymmetric and symmetric C-H stretching absorbances, respectively (Table II). Pretreatment with C:M (2:1) showed a decrease of 75.94% and 89.94% in peak areas of asymmetric and symmetric C-H stretching absorbances, respectively.

The epidermal resistance of the control membrane was 10.27 $K\Omega.cm^2$ (Table I). Pretreatment of epidermis with C:M (2:1) showed the minimum resistance (0.80 $K\Omega.cm^2$). Exponential decrease in epidermal resistance was observed with decrease in asymmetric (correlation coefficient: 0.97) and symmetric (correlation coefficient: 0.96) C-H stretching peak areas suggesting extraction of lipids by solvent pretreatments (Fig. 5). This agrees with the work of Li *et al.* (26) where EtOH pretreatment had decreased the resistance of human epidermal membrane to a large extent. This study provides for the first time evidence that the electrical resistance of the epidermis is related to its lipid content.

TLC and GC Studies

TLC and GC studies were undertaken to investigate whether there was a difference in lipid composition of the extracted lipids, apart from the total amount, which reflected in the difference in the permeability of the epidermis to insulin.

The first mobile phase separated the polar lipids (phospholipids and cholesterol sulfate) in the lower half of the chromatogram, but the ceramides and non-polar lipids were incompletely resolved on the upper half of the chromatogram (Fig. 6A). The second solvent system resolved the ceramides (ceramides 1–6) and the third solvent development system

Table II. Changes in Symmetric and Asymmetric C-H Stretching Absorbance Peak Areas after Treatment

	Peak area (mean ± SD, n = 3)					
	Asymmetric			Symmetric		
Treatment	Control	Treatment	% decrease[a]	Control	Treatment	% decrease[a]
EtAc	12.77 ± 0.50	9.61 ± 0.56	24.63	2.91 ± 0.16	2.06 ± 0.23	28.79
EtAc:EtOH (1:1)	14.15 ± 0.67	7.63 ± 0.94	46.21	3.04 ± 0.15	1.43 ± 0.05	52.69
C:M (2:1)	6.09 ± 0.21	1.12 ± 0.94	75.94	2.36 ± 0.62	0.23 ± 0.10	89.94

[a] % decrease = 100 – [(absorbance peak area due to treatment/absorbance peak area due to control) × 100].

Table III. Quantitation of Epidermal Lipids Extracted by Different Solvent Treatments

Lipid	Amount of lipid (μg/g of dry epidermis)		
	C:M (2:1)	EtAc	EtAc:EtOH (1:1)
Cholesterol	3197.6 ± 335.3	2664.6 ± 315.9	3331.9 ± 319.4
Cholesterol esters	976.6 ± 81.8	1132.0 ± 166.9	1199.5 ± 143.0
Triglycerides	655.3 ± 209.1	1010.1 ± 108.05	954.7 ± 29.8
Fatty acids	412.3 ± 43.9	338.0 ± 28.4	410.7 ± 19.1
Ceramide 1	140.7 ± 64.5	149.7 ± 76.9	103.3 ± 62.6
Ceramide 2	272.4 ± 95.0	136.3 ± 80.4	194.1 ± 82.0
Ceramide 3	977.7 ± 246.4	476.5 ± 267.6	782.1 ± 221.7
Ceramide 4	438.0 ± 146.6	162.4 ± 70.6	312.6 ± 132.4
Ceramide 5	400.0 ± 230.3	233.1 ± 166.5	374.0 ± 224.6
Ceramide 6	324.5 ± 249.4	148.4 ± 81.7	290.1 ± 143.9
Cholesterol sulphate	672.3 ± 438.7	8.8 ± 15.3	437.6 ± 497.8
Phosphatidyl ethanolamine	483.5 ± 30.9	0.0 ± 0.0	230.4 ± 152.2
Phosphatidyl choline	134.7 ± 79.2	6.6 ± 11.4	60.1 ± 45.3
phosphatidyl inositol	886.7 ± 205.2	10.5 ± 11.0	371.9 ± 251.2
Sphingomyelin	611.8 ± 92.9	9.8 ± 12.4	188.2 ± 90.5
Total	10584.0	6486.6	9241.0

separated the non-polar lipids (cholesterol, triglycerides, fatty acids and cholesterol esters) as seen in Fig. 6B,C.

The three major groups of lipids contributing to the extracted lipids were ceramides, phospholipids and cholesterol. The amount of cholesterol extracted was comparable for pretreatment of epidermis with EtAc, EtAc:EtOH (1:1), and C:M (2:1). However, the amount of phospholipids varied widely. The total amount of phospholipids extracted from epidermis by EtAc, EtAc:EtOH (1:1), and C:M (2:1) were 26.9, 850.6 and 2116.7 μg/g of epidermis, respectively, indicating that almost all the phospholipid contribution in EtAc:EtOH (1:1) extracted lipids is by ethanol.

Increase in total ceramide content was observed from 1891.9 to 2689.3 to 3228.2 μg/g of epidermis for EtAc, EtAc:EtOH (1:1) and C:M (2:1), respectively. Ceramides have been associated with the barrier function because they are thought to be chemically well suited for the formation of impermeable membranes (27). Of special importance to the barrier function is ceramide 1, which is believed to function in the fusion of the disks extracted from the lamellar granules and in stabilization of the resulting multilamellar sheets (27). *In vitro* experiments have demonstrated that the ceramides are capable of promoting the stacking, flattening, and fusion of liposomes (28).

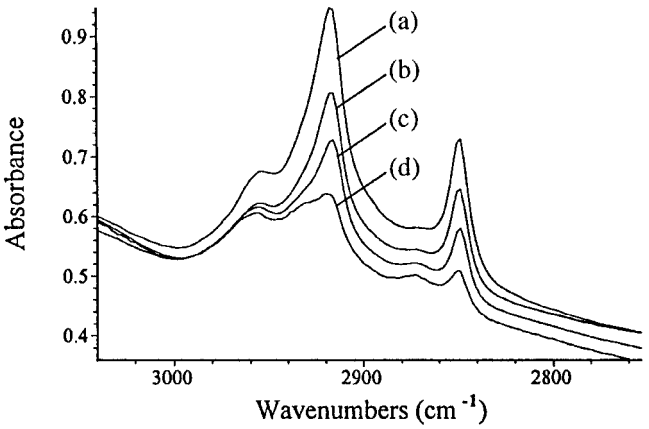

Fig. 4. FTIR spectra (3000–2800 cm^{-1}) of porcine stratum corneum treated with different solvents for 40 min and the control. Key: (a) control; (b) ethyl acetate; (c) ethyl acetate:ethanol (1:1); (d) chloroform:methanol (2:1).

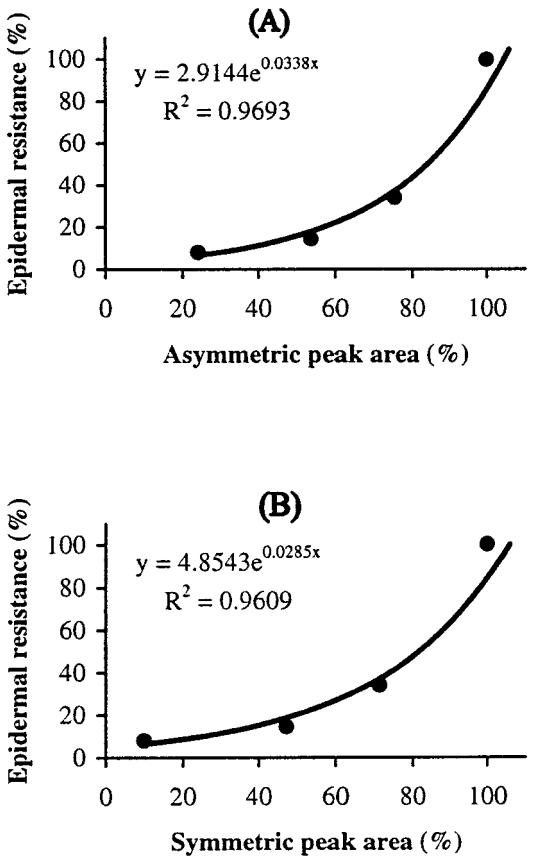

Fig. 5. Relationship between the epidermal resistance and SC lipid content. Resistance with no solvent treatment is taken as 100%. Asymmetric (A) and symmetric (B) peak areas of SC prior to solvent treatment are taken as 100%.

Table IV. Fatty Acid Composition Extracted from SC by Solvents[a]

Fatty acid	C:M (2:1)	EtAc	EtAc:EtOH (1:1)
Myristic acid (C14:0)	0.91 ± 0.03	1.17 ± 0.04	1.24 ± 0.02
Myristoleic acid (C14:1)	0.66 ± 0.11	0.61 ± 0.24	0.34 ± 0.06
Palmitic acid (C16:0)	12.01 ± 0.10	17.62 ± 1.11	19.07 ± 0.46
Palmitoleic acid (C16:1)	2.91 ± 0.06	2.56 ± 0.20	2.69 ± 0.07
Heptadecanoic acid (C17:0)	0.50 ± 0.01	0.56 ± 0.08	0.44 ± 0.02
Stearic acid (C18:0)	7.28 ± 0.12	7.85 ± 0.55	8.64 ± 0.11
Oleic acid (C18:1)	15.15 ± 0.31	22.70 ± 1.12	24.36 ± 1.42
Linoleic acid (C18:2)	12.89 ± 1.01	3.69 ± 0.16	8.02 ± 0.20
Nonadecanoic acid (C19:0)	0.28 ± 0.01	0.00 ± 0.00	0.09 ± 0.08
Linolenic acid (C18:3)	3.11 ± 0.62	3.31 ± 1.11	2.21 ± 0.60
Arachidic acid (C20:0)	0.77 ± 0.10	0.00 ± 0.00	0.39 ± 0.04
Eicosenoic acid (C20:1)	0.54 ± 0.03	0.52 ± 0.03	0.56 ± 0.08
Eicosedienoic acid (C20:2)	0.96 ± 0.38	0.46 ± 0.43	0.88 ± 0.18
Homo gamma linolenic acid (C20:3)	0.19 ± 0.01	0.00 ± 0.00	0.17 ± 0.19
Behenic acid (C22:0)	2.05 ± 0.13	0.74 ± 0.32	0.92 ± 0.04
Lignoceric acid (C24:0)	1.77 ± 0.11	1.01 ± 0.31	0.78 ± 0.06
Sum (Misc.)	38.03 ± 0.43	37.22 ± 3.02	29.21 ± 1.23

[a] Values expressed as percentage of total fatty acid extracted using different solvents.

It has been suggested that cholesteryl sulfate may be necessary to maintain the bilayer structure of the lipids by acting as a cementing material (29). It was noticed that large amounts of cholesteryl sulfate were extracted with EtAc: EtOH (1:1) and C:M (2:1), whereas almost none was extracted with EtAc.

Table IV gives the composition of the fatty acids extracted by different solvent treatments measured by GC. The C:M (2:1) extract shows that the major components are palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:0), linoleic acid (C18:1), linolenic acid (C18:3), behenic acid (C22:0) and lignoceric acid (C24:0). It has been suggested that the long chain, saturated fatty acids (C16:0, C18:0, C22:0, C24: 0) may be able to form a stable hydrophobic barrier (30).

The percentage of linoleic acid (18:2) based on the total fatty acids extracted was 3.69, 8.02, and 12.89 for EtAc, EtAc: EtOH (1:1), and C:M (2:1) pretreatment, respectively. The large loss observed for linoleic acid among all the fatty acids extracted from the SC becomes more significant in the light of its major contribution to epidermal barrier function (30).

Current literature supports the existence of intercellular and transappendageal routes for passive and iontophoretic transport of solutes, with the transcellular route considered being unlikely. Also, it should be noted that with follicular transport the pathway is an intercellular route between hair follicles and epidermal cells (6). The intercellular pathways are localized between the sheaths of the intercellular lipid matrix. Bodde *et al.* (2) after studying the transport pathway of mercury in the SC concluded that the prominent route of transport was through the intercellular pathway. EtOH and

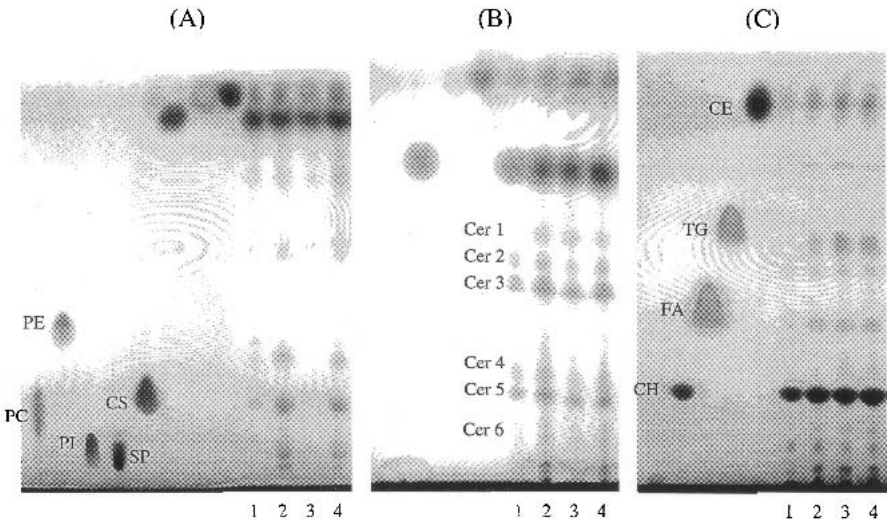

Fig. 6. Thin layer chromatographic separation of lipids for (A) polar lipids (B) ceramides (C) neutral lipids. Lane 1. Porcine SC ceramide standard; Lane 2. Chloroform:methanol (2:1) treatment; Lane 3. Ethyl acetate treatment; Lane 4. Ethyl acetate:ethanol (1:1) treatment. Abbreviations. PC- Phosphatydylcholine; PE- Phosphatydylethanolamine; PI- Phosphatydylinositol; CS- Cholesterol sulfate; SP- Sphingomyelin; Cer- Ceramide; CH- Cholesterol; FA- Fatty acids; TG- Triglycerides; CE- Cholesterol esters.

C:M (2:1) treatment removed the intercellular lipids, reduced the tortuosity of the pathways and increased permeability of the skin (26). Based on these studies, it is reasonable to suggest that the SC lipid extraction by the solvents studied would have created free volume leading to formation of additional "electrically conductive" diffusional pathways in the epidermis. Such additional pathways in the pretreated epidermis have enhanced both the passive and iontophoretic transport of insulin compared to the control epidermis.

CONCLUSIONS

The present studies identify the synergy between lipid extraction from SC using solvents [EtAc, EtAc:EtOH (1:1) and C:M (2:1)] and iontophoresis as a technique to enhance and control the transdermal delivery of insulin. Pretreatment of the epidermis with solvents and subsequent rehydration of the membrane significantly ($P < 0.05$) increased the flux of insulin in comparison with the control (no solvent treatment). Iontophoresis further increased the flux of insulin through solvent pretreated epidermis. FTIR investigation revealed that SC treatment with the solvents decreased the peak areas for both asymmetric and symmetric C-H stretching absorbances suggesting lipids were extracted from the membrane. Correlation between a decrease in epidermal electrical resistance and reduction in lipid content was observed. Greater permeability of the epidermis to insulin pretreated with EtAc: EtOH (1:1) in comparison to EtAc alone was found to be coincident with greater extraction of ceramides, cholestryl sulfate, phospholipids and linoleic acid.

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