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ORIGINAL ARTICLE

Effect of *O*-acylmenthol and salt formation on the skin permeation of diclofenac acid

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Abstract

Purpose: To enhance the transdermal delivery of diclofenac acid (DA) by using *O*-acylmenthol as a penetration enhancer and complexing with amines, or by a combination of the two methods. *Methods*: The skin permeability of diclofenac was tested in vitro across rat skin with each of the evaluated permeants in a saturated isopropyl myristate (IPM) donor solution. *Results*: A 4.5-fold increase in the flux of diclofenac was observed by ion-pair formation with diethylamine; however, the cations with hydroxyl groups had negative effects on the transdermal delivery of diclofenac. 2-isopropyl-5-methylcyclohexyl 2-hydroxypanoate and 2-isopropyl-5-methylcyclohexyl heptanoate produced significant increase in the permeation of diclofenac potassium (D-K); however, both of them were ineffective for the other diclofenac salts, including diclofenac diethylamine (D-DETA), diclofenac ethanolamine (D-EA), diclofenac diethanolamine (D-DEA), diclofenac triethanolamine, and diclofenac *N*-(hydroxylethyl) piperidine. 2-isopropyl-5-methylcyclohexyl tetradecanoate was effective on the penetration of D-K, D-DETA, D-EA, and D-DEA. Also, it is exciting to note that the combined use of diethylamine with 2-isopropyl-5-methylcyclohexyl tetradecanoate produced a 9.74-fold increase in accumulation amount of diclofenac compared with DA in IPM. *Conclusions*: The use of ion pair in combination with *O*-acylmenthol is necessary to further increase the diclofenac flux to provide better compliance for the patients undergoing clinical therapy.

Key words: *Diclofenac salts; hydrogen bond; ion pair; O-acylmenthol; transdermal*

Introduction

The transdermal route has many advantages for the administration of drugs for local and systemic therapy. However, the outermost layer of skin, the stratum corneum (SC), presents a strong barrier to most exogenous substances including drugs. Diclofenac acid (DA), which is a therapeutically important nonsteroidal anti-inflammatory agent, is extensively metabolized in the liver. Because of its short biological half-life, the drug needs to be administered quite frequently[1]. Thus, transdermal delivery of DA may provide better patient compliance and higher bioavailability compared with oral administration. However, DA, has a high log *P* (4.75)[2] and a relatively high melting point (170.69°C), and is not easily permeable, mainly due to its poor partitioning from the lipophilic SC into the hydrophilic dermis. One popular approach to deliver an effective dose of drug through skin is to reversibly reduce the barrier function of the skin with the aid of penetration enhancers or accelerants[3]. More recently, new types of *O*-acylmenthol derivatives (Figure 1) have been synthesized as candidates for percutaneous absorption enhancers and their promoting activities have been confirmed using model drugs with a range of lipophilicity[4,5]. Moreover, as esterases are present in the human and animal epidermis[6], *O*-acylmenthol derivatives are expected to be enzymatically hydrolyzed into nontoxic metabolites by esterases in the living epidermis. Similar investigations have been carried out by some other researchers[7,8] who found that some esters could be hydrolyzed into nontoxic metabolites in vitro using porcine esterase.

In the past two decades, in order to further improve the properties of DA making it more suitable for

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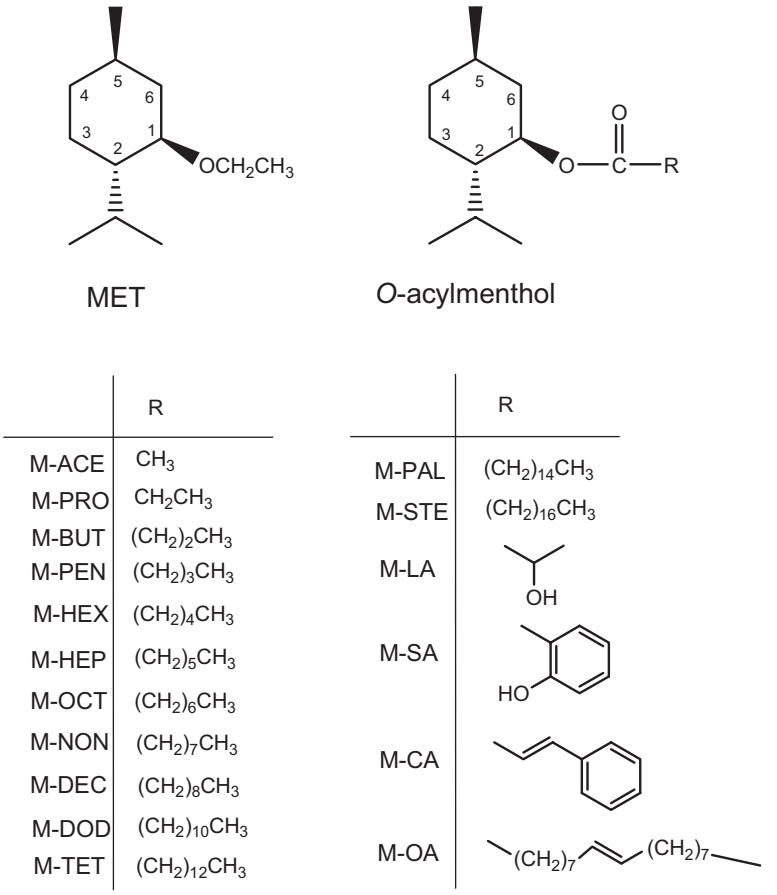

Figure 1. Chemical structures of MET and *O*-acylmenthol derivatives used as percutaneous absorption enhancers.

percutaneous absorption, such as a lower melting point and a higher water solubility, many diclofenac salts had been reported and identified[9–11]. However, ionized molecules are generally not well absorbed by biological membranes[12]. One possible method of transferring ionizable molecules across biologic membranes is via ion pairing or complexation with large bulky counterions[13]. A mutual affinity between the cation and the anion present in the salt allows the formation of a complex or weakens the ionic dissociation, producing species such as ion pairs. These species, where ions are in close proximity and their charges are masked or shielded by the low dielectric constant of the hydrocarbon moieties of the functional groups, exhibit a lower hydrophilicity than the two ions considered separately and offer unusual behavior for an ionic species, such as a high solubility in apolar solvents or increased partition in a lipid phase[14]. Many researchers have used ion pairs to increase the skin permeability of relatively lipophilic or hydrophilic molecules in ex vivo permeation experiment[12,15–17], but they only investigated the effect of alkylamines on the absorption behavior of a certain anion, such as diclofenac and salicylic anions. There is little information about the permeability of diclofenac salts when combined with the use of penetration enhancers in isopropyl myristate (IPM) solution. The only study that involved a comparison of the effect of different counterions on the percutaneous absorption of DA through human skin has been finished elsewhere[18]. In that work, the vehicles evaluated separately in donor phase were water, propylene glycol, transcutol, and oleic acid; however, the synergistic effect of the combined use of enhancers has not been investigated.

In this study, we initially examined the effects of *O*-acylmenthol on the in vitro percutaneous absorption of DA and diclofenac potassium (D-K) through excised rat skin and chose *O*-acylmenthol, which had significant promoting effects on the permeation of DA or D-K, for experimental studies. Then, we investigated the effects of five organic diclofenac salts upon the penetration of DA in the saturated solutions of IPM. One is a commercial product of diclofenac diethylamine (D-DETA), and the other four salts prepared by us were diclofenac ethanolamine (D-EA), diclofenac diethanolamine (D-DEA), diclofenac triethanolamine (D-TEA),

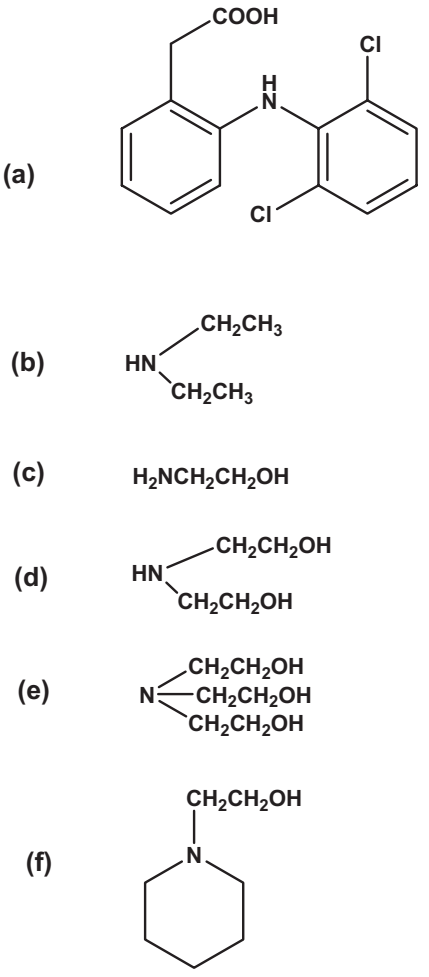

Figure 2. Chemical structures of DA and organic amines. (a) Diclofenac acid, (b) diethylamine, (c) ethanolamine, (d) diethanolamine, (e) triethanolamine, and (f) *N*-(hydroxylethyl) piperidine.

and diclofenac *N*-(hydroxylethyl) piperidine (D-HEPP), the structures of DA and amines are shown in Figure 2. Finally, we also examined the effects of the combined use of *O*-acylmenthol, which has been screened by the transdermal delivery of DA and D-K on the skin permeation of DA.

Materials and methods

Materials

DA, D-DETA, and D-K were purchased from Tieling Tiande Pharmaceutical Co. Ltd. (Tie ling, China); ethanolamine, diethanolamine, triethanolamine, acetone, and glacial acetic acid were all supplied by Tianjin Bodi Chemical Reagent Co. Ltd. (Tianjin, China); *N*-(hydroxylethyl) piperidine was obtained from Alfa Aesar (Johnson Matthey Company, West Chester, PA, USA); propylparaben, *l*-menthol, and IPM were supplied by China National Medicines Co. Ltd. (Shanghai, China); *O*-ethylmenthol (MET) and all the *O*-acylmenthol derivatives including 2-isopropyl-5-methylcyclohexyl acetate (M-ACE), 2-isopropyl-5-methylcyclohexyl propionate (M-PRO), 2-isopropyl-5-methylcyclohexyl butyrate (M-BUT), 2-isopropyl-5-methylcyclohexyl pentanoate (M-PEN), 2-isopropyl-5-methylcyclohexyl hexanoate (M-HEX), 2-isopropyl-5-methylcyclohexyl heptanoate (M-HEP), 2-isopropyl-5-methylcyclohexyl octanoate (M-OCT), 2-isopropyl-5-methylcyclohexyl nonanoate (M-NON), 2-isopropyl-5-methylcyclohexyl decanoate (M-DEC), 2-isopropyl-5-methylcyclohexyl dodecanoate (M-DOD), 2-isopropyl-5-methylcyclohexyl tetradecanoate (M-TET), 2-isopropyl-5-methylcyclohexyl palmitate (M-PAL), 2-isopropyl-5-methylcyclohexyl stearate (M-STE), 2-isopropyl-5-methylcyclohexyl cinnamate (M-CA), (E)-2-isopropyl-5-methylcyclohexyl octadec-9-enoate (M-OA), 2-isopropyl-5-methylcyclohexyl 2-hydroxybenzoate (M-SA), and 2-isopropyl-5-methylcyclohexyl 2-hydroxypanoate (M-LA) were synthesized in previous reports[4,5]. The structures of these compounds were confirmed by nuclear magnetic resonance (ARX-300, Bruker, Faellanden, Switzerland) and high-performance liquid chromatography mass spectrometry (HPLC-MS; ZQ-2000, Waters, Milford, MA, USA). Methanol of HPLC grade was obtained from the Yuwang Pharmaceutical Co., Ltd. (Shandong, China). All other chemicals were of the highest reagent grade available.

Salts preparation

Apart from D-K and D-DETA, four other salts were prepared in our laboratory. For this, 1 M DA was dissolved in acetone, an equivalent amount of the appropriate base was added to the solution, and the final product was allowed to slowly crystallize from solution. In the case of the preparation of the D-EA, chloroform was employed as a solvent and the reaction temperature was kept at 55°C. Differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) analyses have been used to compare the physicochemical characteristics of DA and its salts (presented in Table 1). Then, the desired diclofenac salts were heated in an oven at 100°C for one night to obtain a completely desolvated form, which was stored in a desiccator.

Drug analysis

The HPLC system for determining drug concentrations was equipped with an L-2420 variable wavelength ultraviolet absorbance detector and an L-2130 pump (Hitachi High-Technologies Corporation, Tokyo, Japan). A reversed phase stainless steel column (20 cm × 4.6 mm) was packed with Diamonsil C-18 (5 μm particle size;

Table 1. Physicochemical properties of DA and its salts.

	Permeants						
Parameters	DA	D-DETA	D-EA	D-DEA	D-TEA	D-HEPP	D-K
Melting point (°C)	170.69	126.6	158.9	130.95	137.68	131.14	—
ΔH(KJ/mol)[a]	−18.95	−20.59	−40.50	−45.07	−53.90	−43.46	48.49
MW (g/mol)	296.15	369.29	357.23	401.29	445.34	425.42	334.24
S_W at 32°C (μmol/mL)[b]	0.048	42.92	15.77	22.25	21.41	18.91	144.55
S_O at 32°C (μmol/mL)[c]	96.38	115.3	15.28	17.19	10.33	26.08	28.39

[a]ΔH represents enthalpy of fusion or decomposition. [b]S_W is solubility in water. [c]S_O is solubility in *n*-octanol.

Dikma Technologies, Beijing, China). The HPLC conditions were as follows: the mobile phase for DA consisted of methanol and 0.5% acetic acid in distilled water (3:1 v/v), the pH was adjusted to 6.0 with triethylamine, with detection at 280 nm, propylparaben was employed as the internal standard, and the retention times for DA and internal standard were 6.8 and 4.3 minutes, respectively. The flow rate under the above conditions was 1.0 mL/min.

Solubility determination

To determine the saturation solubility (C_s) of the DA, D-K, D-DETA, D-EA, D-DEA, D-TEA, and D-HEPP in water, in *n*-octanol, and in IPM with or without enhancers, excess drug was added to known volumes of vehicle, vortexed for 2 minutes followed by sonication for 10 minutes to dissolve the drug and then equilibrated at 32 ± 0.5°C for more than 48 hours[19]. Finally, the suspensions were filtered through a 0.45-μm membrane filter and aliquots of the supernatant saturated solution were diluted and analyzed by HPLC. The experiments were performed in triplicate.

Permeation experiments

Donor solutions of the DA and its salts, including D-K, D-DETA, D-EA, D-DEA, D-TEA, and D-HEPP, were obtained by equilibration of excess amounts of solute in IPM, with and without selected concentration enhancers, then vortexed for 2 minutes followed by sonication for 10 minutes to dissolve the drug, and an excess amount of solute was present throughout the experiments.

Male Wistar rats weighing 180–220 g (6–8 weeks old) used in all experiments were supplied by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All experiments in this study were carried out in accordance with the guidelines for animal use published by the Life Science Research Center of Shenyang Pharmaceutical University. The rats were anesthetized with urethane (20%, w/v, i.p.) and the abdomen was carefully shaved with a razor after removal of hair by electric clippers (model 900, TGC, Tokyo, Japan). Full thickness skin (i.e., epidermis with SC and dermis) on the abdomen was excised and the integrity of the skin was carefully confirmed by microscopic observation, and any skin that was not completely uniform was rejected. After removing the fat and subdermal tissue, the skin was kept frozen at –20°C and used within 1 week. Before starting the experiments, the skin was allowed to reach room temperature for at least 10 hours.

Skin permeation experiments were performed according to the method of Fang[20]. A diffusion cell consisting of two half cells with a water jacket connected to a water bath at 32 ± 0.5°C was used. Each half cell had a volume of 2.5 mL and an effective area of 0.95 cm^2. The dermis side of the skin was in contact with the receiver compartment and the SC with the donor compartment. The donor compartment was filled with the drug suspension (about twice the solubility) and the receiver compartment with pH 7.4 phosphate buffered saline (PBS). To ensure the sink conditions and maintain the thermodynamic activity of the drug during the transdermal experiment, an excess amount of solute was present in the donor compartments. Both donor and receiver compartments were stirred with a star-head bar driven by a constant speed synchronous motor at 600 rpm. At predetermined time intervals, 2.0 mL of receptor solution was withdrawn from each receiver compartment for analysis and replaced with the same volume of fresh solution to maintain sink conditions, the experiments lasted for 8 hours. The drug concentration was determined by reversed phase HPLC with reference to a calibration curve.

Data analysis

The cumulative amount of each drug permeating through the skin was plotted as a function of time. The skin flux was determined from Fick's law of diffusion:

$$J_s = \frac{dQ_r}{Adt}, \quad (1)$$

where J_s is the steady-state skin flux in μmol/cm^2/h, dQ_r is the change in quantity of the drug passing through the skin into the receptor compartment in μmol, A is the active diffusion area in cm^2, and dt is the change in time. The flux was calculated from the slope of the linear portion of the profiles[21].

The permeability coefficient (P) was calculated as[22]

$$P = \frac{J_s}{C_s}, \tag{2}$$

where C_s is the saturated solubility of drug in donor solution.

To evaluate the promoting activity of each enhancer, enhancement ratios (ERs) were calculated as Q for the enhancer-containing group divided by the Q for the control (no enhancer present). Controls were assigned a value of 1.00. Statistical analysis was carried out using analysis of variance. The level of significance was taken as $P < 0.05$.

Results

DSC analysis

DSC measurements were performed using a Shimadzu DSC60 thermal analysis system (Shimadzu Co., Kyoto, Japan). Figure 3 shows the DSC curves of DA and its salts over the experimental temperature range from 30 to 350°C (heating rate, 5°C/min). The melting point and the enthalpy of fusion or decomposition for DA and its salts are listed in Table 1. The DSC curve of DA shows

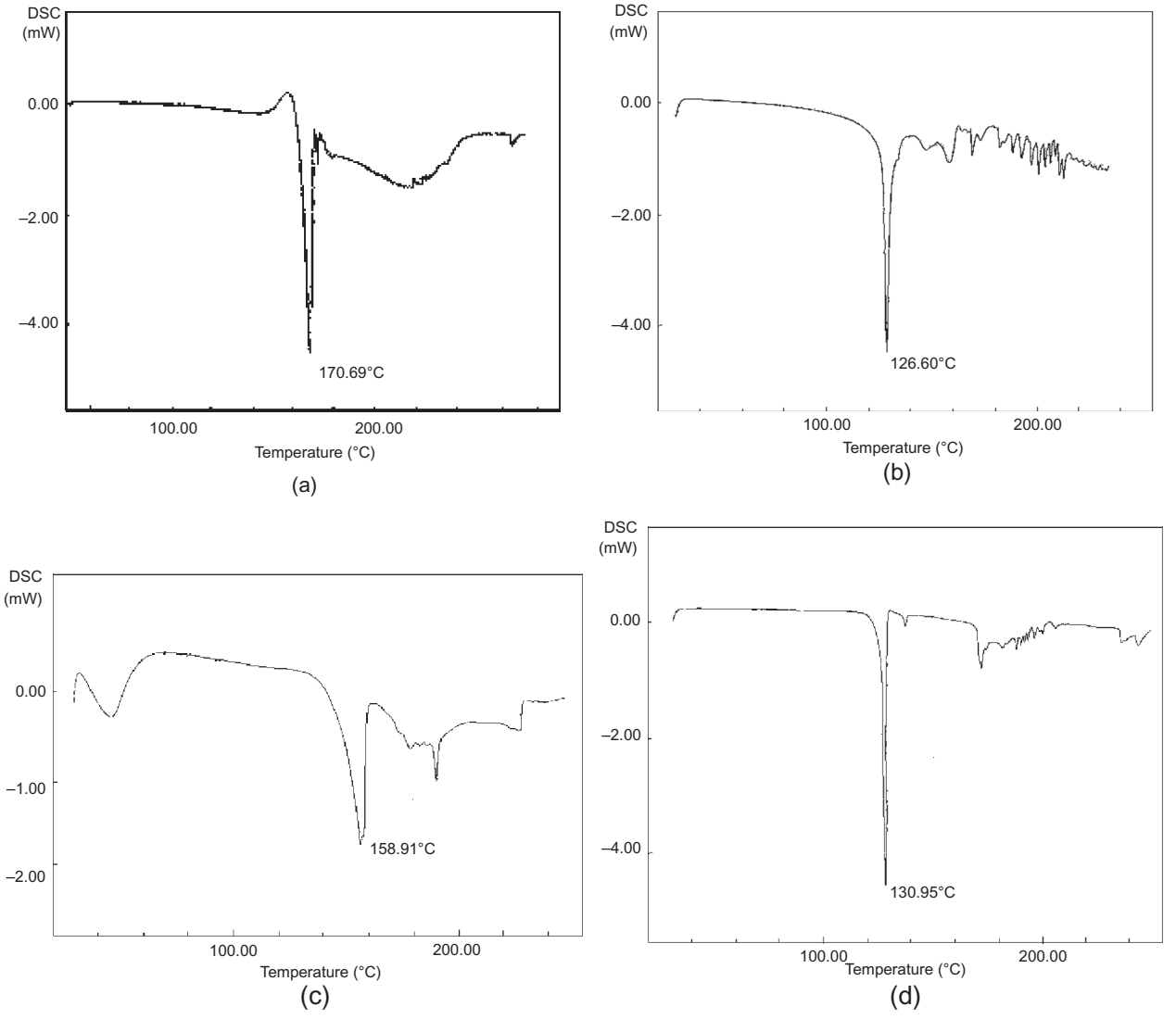

Figure 3. DSC curves of DA and its salts. (a) DA, (b) D-DETA, (c) D-EA, (d) D-DEA, (e) D-TEA, (f) D-HEPP, and (g) D-K.

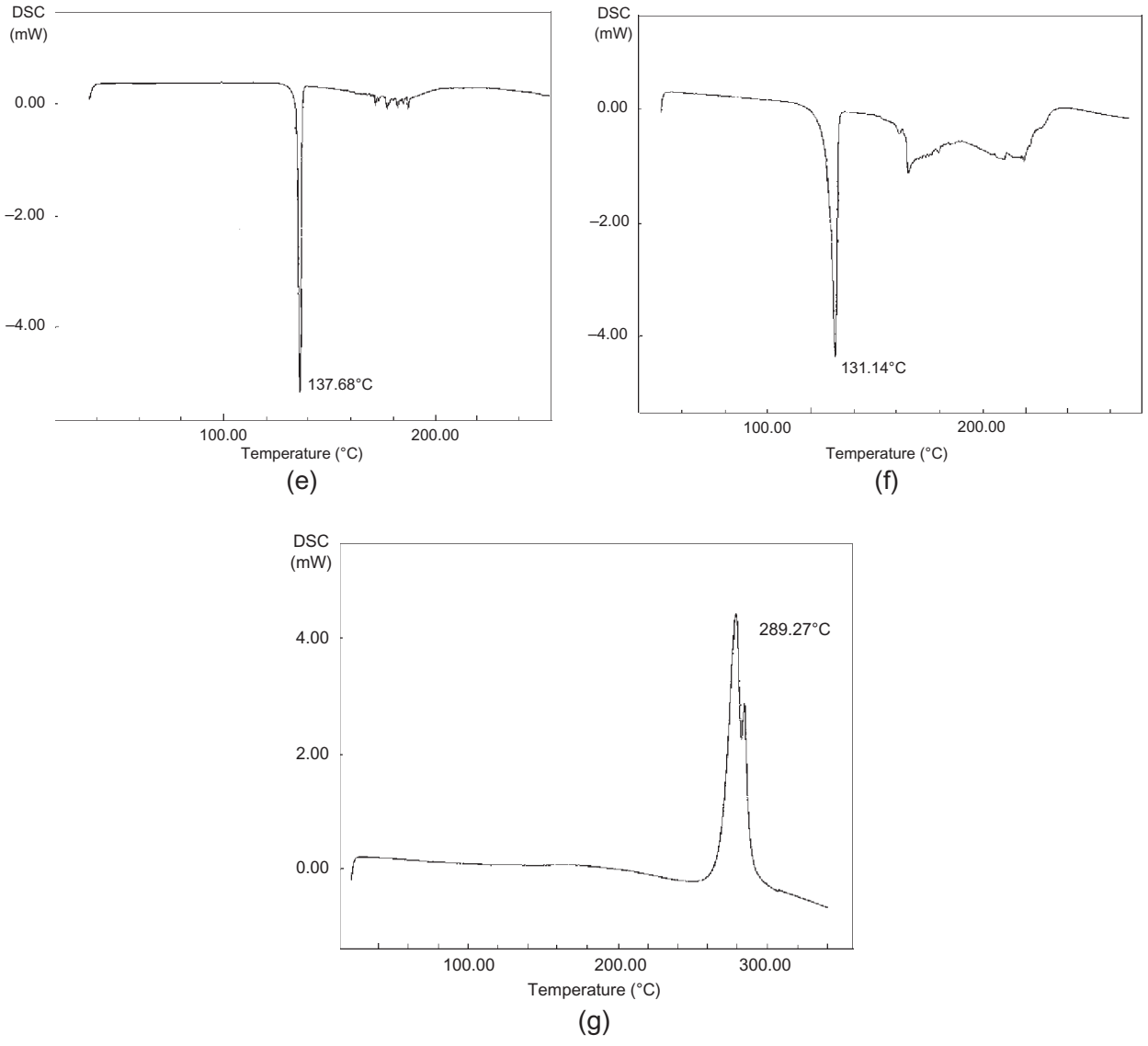

Figure 3. (Continued).

a characteristic sharp endothermic peak of the drug at 170.69°C corresponding to its melting point. This value is in good agreement with that in the literature[23]. The melting points of D-DETA, D-EA, D-DEA, D-TEA, and D-HEPP were markedly reduced compared with pure DA. In the case of D-EA, the endothermic peak at a temperature of about 50°C was attributed to solvent volatilization. The broad endothermic peaks, at a higher temperature in the range 150–230°C, were due to complex decomposition consistent with the published literature on the thermal analysis of some diclofenac salts[24]. In the case of D-K, exothermic peaks were obtained at 289.27°C, which could be attributed to the decomposition of salts and was consistent with literature reports[25]. The shift in melting point and change in the molar enthalpy are the result of salt formation of DA.

FTIR spectroscopy

To further investigate the formation of DA salts, more information was obtained from IR spectroscopy. The infrared spectra of DA and its salts were obtained with an FTIR spectrometer (Bruker IFS-55, Faellanden, Switzerland) and shown in Figure 4. Any sign of interaction is reflected by shifts in the N–H or C=O vibrations. The signal at 1684 cm^{-1} in the spectrum for DA was attributed to C=O stretching of the carboxylic acid group[26], whereas, in this study, DA exhibited a strong narrow signal around 1694.0 cm^{-1}, characteristic of the carbonyl stretching vibration. However, the carbonyl peaks of the DA salts were shifted to a lower wavenumber (D-DETA 1629.0, D-EA 1575.8, D-DEA 1638.7, D-TEA 1603.9, D-HEPP 1608.6, and D-K 1705.2 cm^{-1}), whereas

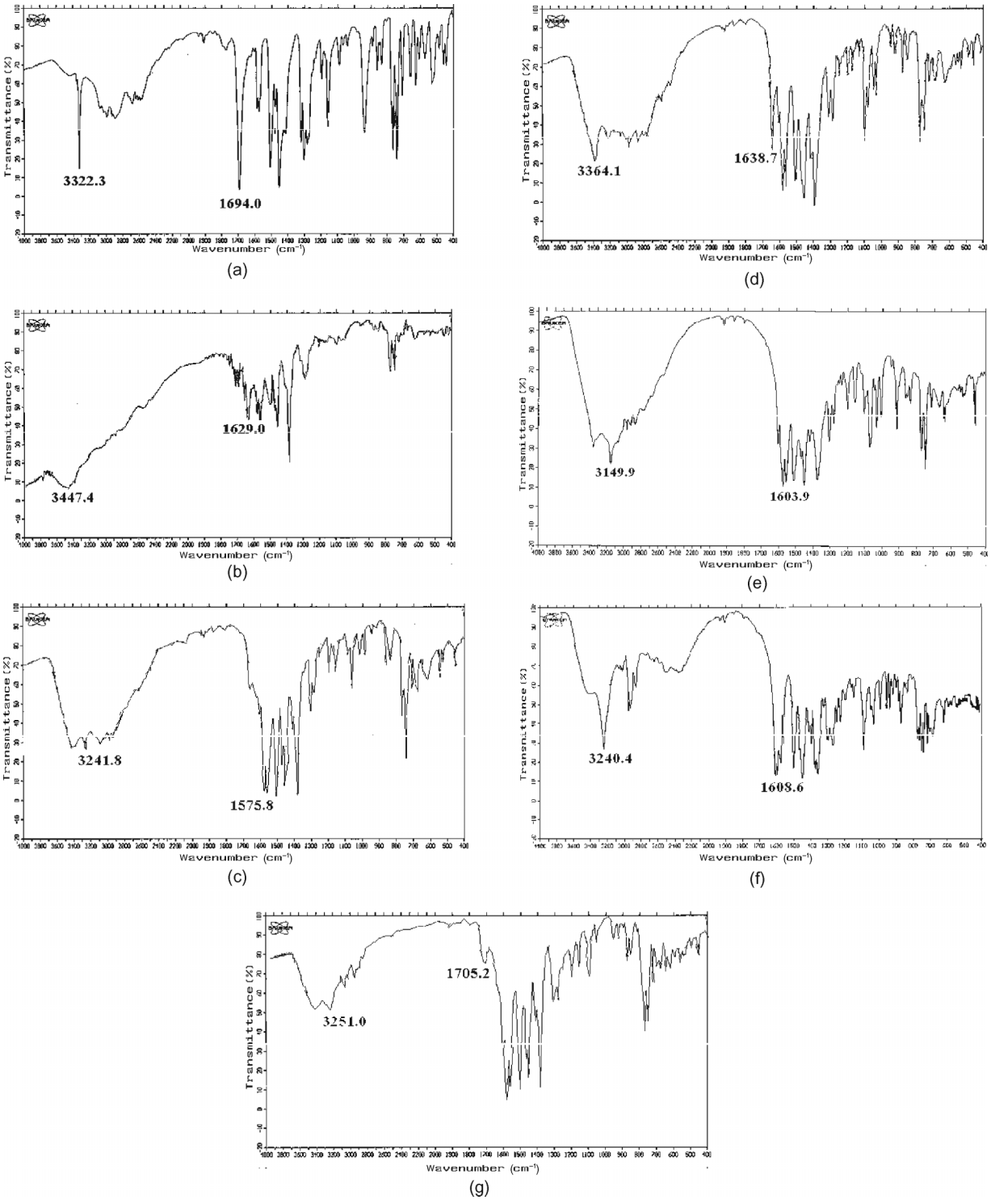

Figure 4. FTIR spectra of DA and its salts. (a) DA, (b) D-DETA, (c) D-EA, (d) D-DEA, (e) D-TEA, (f) D-HEPP, and (g) D-K.

the peak intensity of D-K and D-DETA was markedly reduced. These changes were assigned to the ν (COO^-) vibrations in the infrared spectra of the salts of DA, indicating that the carboxyl group of DA had been converted to a carboxylate anion. In addition, there was a substantial difference in the N–H or O–H stretching region. DA has a strong narrow signal around 3322.3 cm^{-1}; however, D-DETA, D-EA, D-DEA, D-TEA, D-HEPP, and D-K had very weak peaks in the stretching vibration region, and these signals were around 3447.4, 3241.8, 3364.1, 3149.9,

3240.4, and 3251.0 cm^{-1}, respectively, suggesting that the intermolecular hydrogen bonds of the DA salts might have shifted the N–H or O–H stretching bands. These results also indicate a change in the structure of DA due to the formation of salts.

In vitro permeation of DA and D-K

When cumulative amounts permeating as a function of the enhancer concentration were compared at 8 hours, a wide fluctuation was apparent. At 5%, 10%, and 20% *l*-menthol levels (w/w), the cumulative transport of DA was 0.62 ± 0.01 μmol/cm^2, 1.13 ± 0.06 μmol/cm^2, and 0.92 ± 0.01 μmol/cm^2, and the 10% level was significantly higher than that of the control group, which was 0.90 ± 0.04 μmol/cm^2 ($P < 0.05$). Accordingly, the 10% level was selected to perform subsequent experiments.

At 0%, 5%, 10%, and 20% concentrations of *l*-menthol, the cumulative amounts of D-K that permeated were 0.91 ± 0.15 μmol/cm^2, 0.64 ± 0.07 μmol/cm^2, 1.85 ± 0.22 μmol/cm^2, 1.80 ± 0.09 μmol/cm^2, respectively, and both the 10% and 20% levels were higher than the control ($P < 0.05$), whereas no significant difference was observed between the two groups ($P > 0.05$), so the 10% level was selected. The results show that although in all cases the greatest enhancement of the skin transport occurs in the presence of the menthol, there is no direct linear relationship between the *l*-menthol concentration and the permeation rate.

The effects of MET and *O*-acylmenthol, which were equal to the molar concentrations of *l*-menthol selected for DA and D-K on the percutaneous permeation parameters of DA and D-K (C_s, J_s, P, and ER) through rat skin, are presented in Table 2. The control values for DA were determined to be 24.41 μmol/mL for the C_s, $1.38 \pm 0.25 \times 10^{-1}$ μmol/cm^2/h for the J_s, and $5.67 \pm 0.58 \times 10^{-3}$ cm/h for P. Nearly all the evaluated *O*-acylmenthol had no effect on the percutaneous permeation of DA ($P > 0.05$), except M-OA, M-ACE, and M-PRO relative to the control. M-OA provided the highest increase in Q (1.44 ± 0.093 μmol/cm^2), followed by M-ACE (1.31 ± 0.12 μmol/cm^2) and M-PRO (1.20 ± 0.11 μmol/cm^2).

The control percutaneous permeation parameters for D-K were 0.25 μmol/mL for the C_s, $1.86 \pm 0.29 \times 10^{-1}$ μmol/cm^2/h for the J_s, 3.28 hours for the lag-time, and $7.39 \pm 1.16 \times 10^{-1}$ cm/h for P. In Figure 5, the enhancers that had effects on the percutaneous permeation of D-K are M-LA (2.51 ± 0.41 μmol/cm^2), followed by M-TET (2.26 ± 0.076 μmol/cm^2), M-HEP (1.56 ± 0.098 μmol/cm^2), M-OA (1.58 ± 0.25 μmol/cm^2), MET (1.30 ± 0.06 μmol/cm^2), and M-SA (1.23 ± 0.08 μmol/cm^2).

According to the penetration results of DA and D-K enhanced by *O*-acylmenthol, M-OA had the best promoting activity on DA, whereas M-LA, M-TET, and

Table 2. Permeation parameters of DA and D-K through rat abdominal skin.

	DA				D-K			
Enhancer	C_s (μmol/mL)	J_s (μmol/cm^2/h) × 10	P (cm/h) × 10^3	ER[a]	C_s (μmol/mL)	J_s (μmol/cm^2/h) × 10	P (cm/h) × 10	ER[a]
Control	24.41	1.38 ± 0.25	5.67 ± 0.58	1.00	0.25	1.86 ± 0.29	7.39 ± 1.16	1.00
10%-M	44.50	1.86 ± 0.12*	4.18 ± 0.16	1.19	0.61	2.97 ± 0.51*	4.86 ± 0.89	2.03
MET	26.89	1.64 ± 0.21	6.11 ± 0.39	1.27	0.18	2.04 ± 0.02	11.12 ± 0.96*	1.43
M-ACE	28.85	1.82 ± 0.14*	6.32 ± 0.27	1.49	0.23	0.49 ± 0.07	2.16 ± 0.31	0.27
M-PRO	29.14	1.84 ± 0.11*	6.33 ± 0.22	1.36	0.18	0.83 ± 0.21	4.70 ± 1.18	0.49
M-BUT	35.35	0.14 ± 0.05	0.39 ± 0.07	0.14	0.51	0.88 ± 0.17	1.72 ± 0.33	0.97
M-PEN	30.81	1.63 ± 0.4	5.30 ± 1.29	1.32	0.24	1.20 ± 0.24	5.05 ± 1.02	0.68
M-HEX	33.67	1.30 ± 0.09	3.85 ± 0.27	1.25	0.29	0.97 ± 0.11	3.33 ± 0.37	0.57
M-HEP	32.08	0.51 ± 0.06	1.55 ± 0.20	0.42	0.12	2.65 ± 0.21*	21.12 ± 1.68*	1.71
M-OCT	41.86	0.82 ± 0.05	1.95 ± 0.11	0.63	0.10	1.24 ± 0.06	11.82 ± 0.56*	0.82
M-NON	34.38	0.82 ± 0.11	2.37 ± 0.31	0.50	0.11	1.24 ± 0.07	11.76 ± 0.67*	0.71
M-DEC	30.14	1.72 ± 0.35	5.72 ± 1.14	1.27	0.13	1.39 ± 0.16	10.52 ± 1.21	0.89
M-DOD	25.95	1.08 ± 0.11	4.15 ± 0.41	0.74	1.28	1.07 ± 0.23	0.83 ± 0.18	0.77
M-TET	22.27	1.25 ± 0.10	5.61 ± 0.44	0.86	0.25	3.44 ± 0.09*	13.98 ± 38*	2.48
M-PAL	24.32	1.34 ± 0.28	5.52 ± 1.57	1.06	0.14	1.14 ± 0.17	7.88 ± 1.98	0.77
M-STE	28.79	1.70 ± 0.36	5.51 ± 0.93	1.09	16.12	1.90 ± 0.29	0.12 ± 0.018	1.19
M-LA	50.41	0.06 ± 0.01	0.01 ± 0.001	0.03	54.92	3.98 ± 0.97*	0.072 ± 0.018	2.75
M-CA	28.54	1.11 ± 0.16	3.88 ± 0.55	0.73	0.36	1.15 ± 0.21	3.17 ± 0.54	0.79
M-SA	21.08	0.93 ± 0.17	4.42 ± 0.81	0.72	0.54	1.50 ± 0.17	2.76 ± 0.32	1.35
M-OA	22.91	1.96 ± 0.06*	8.57 ± 0.21*	1.51	0.53	1.62 ± 0.22	3.08 ± 0.42	1.74

The receiver phase used an identical pH 7.4 PBS and donor phases consisted of IPM; IPM/menthol (10:1) (w/w) and equivalent molar *O*-acylmenthol derivatives with *l*-menthol in IPM. Data are given as average ± SE ($n = 4$). *Value is significantly different from control group ($P < 0.05$). [a]ER is the enhancement ratio calculated as follows: ER = Q (with enhancer)/Q (without enhancer).

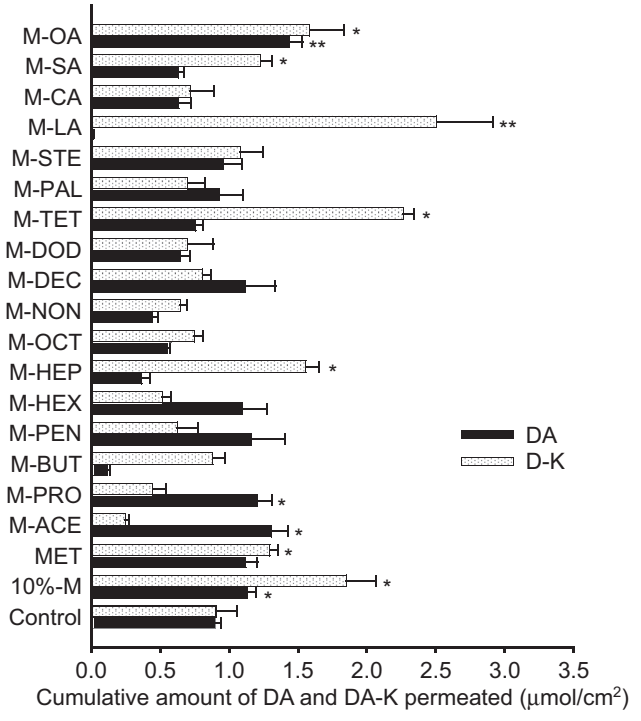

Figure 5. Cumulative amount (average ± SE; $n = 4$) of drugs passing through rat skin, the amount of MET or *O*-acylmenthol is equal to the molar concentration of *l*-menthol in the profile. *Value is significantly different from that of control group ($P < 0.05$); **Value is significantly different from menthol group ($P < 0.05$).

M-HEP had the best enhancing effects on D-K. Therefore, the above four *O*-acylmenthol derivatives were chosen to enhance the permeation of organic diclofenac salts, and the concentration of selected *O*-acylmenthol during the subsequent experiments was at the same molar concentration as that used in promoting DA and D-K.

In vitro permeation of DA salts

The effects of ion-pair formation with organic amines on the skin permeation of DA were examined by using a suspension in IPM of the organic diclofenac complexes. Diethylamine produced the highest increase in Q (4.02 ± 0.49 μmol/cm^2); a 4.5-fold increase in the flux was observed. However, the Q obtained using amines with hydroxyl groups as counterions were significantly lower than that of diclofenac free acid.

Almost all the evaluated *O*-acylmenthol had no promoting effects on the percutaneous permeation of D-DETA ($P > 0.05$), except M-TET, compared with D-DETA in IPM. It is exciting that M-TET produced a 9.74-fold increase in the Q compared with DA in IPM ($P < 0.01$). As far as D-EA was concerned, M-TET and M-OA provided 5.84- and 1.72-fold increase in the Q compared with D-EA in IPM, respectively ($P < 0.05$). In the case of D-DEA, M-OA produced the highest increase in Q (0.98 ± 0.11 μmol/cm^2), with an ER_2 of 2.69-fold, followed by M-TET (0.72 ± 0.14 μmol/cm^2), with an ER_2 of 2.01-fold. M-HEP and M-LA had no enhancing activity. All the evaluated *O*-acylmenthol had no enhancing effect on the percutaneous permeation of D-TEA and D-HEPP, the above results were presented in Table 3.

Discussion

Our previous reports[4,5] have shown when using IPM as vehicle, the enhancing activity of *O*-acylmenthol derivatives for each of the model drugs was relatively low (ER values <5). Similar results were also observed in this study, which could be attributed to the fact that IPM also has an enhancing effect, just as many literature reported[27–29]. We selected IPM as the nonaqueous vehicle mainly because it is known to have a low dielectric constant[30]. It is interesting to note that the changes in permeability of DA and D-K in the IPM were only minor, which is surprising regarding the differences in thermodynamic activity and the fact that DA was present in the nonionic and ionic forms. However, this is consistent with Vávrová who reported a minor difference in the permeation of adefovir from pH 3.4 to 7.8[31], which may partially be explained by the buffering capacity of the skin, that is, the ability of the skin to buffer applied acids or bases and resist any potential damage for a certain period of time[32].

Similarly to our previous reports[4,5], *O*-acylmenthol also produced significant promoting activity on the penetration of DA and its salts in this study. M-HEP, which had a significant enhancing effect on the penetration of D-K, did not promote the permeation of the other salts of DA. This phenomenon might be attributed to the high water solubility of D-K. M-TET, with a C14 alkyl chain that was a little different from the literature where C10–12 is the optimal chain length[33], had significant effects on D-K, D-DETA, D-EA, and D-DEA, and no matter whether the chain length was decreased or increased, the promoting effects were reduced. M-TET may act via a mechanism proposed in a previous report[5]. M-TET with a six-membered ring group and a C14 alkyl chain has some structure similarity with ceramides, having a relatively small polar head and two long, straight, and saturated hydrophobic chains[34], are the main constituent of the SC and offer resistance to chemicals. So, M-TET is capable of inserting its hydrophobic chains between the hydrophobic chains of the SC lipids to disturb the lipid packing, produce lateral fluidization of the lamellae, and reduce the skin barrier resistance. Of course, more

Table 3. Permeation parameters of DA and its salts through rat skin.

Permeants	Enhancers	C_s (μmol/mL)	J_s (μmol/cm^2/h) × 10	P (cm/h) × 10^2	Q (μmol/cm^2)	$^aER_1$	$^bER_2$
DA	Control	24.41	1.38 ± 0.25	0.57 ± 0.058	0.90 ± 0.04	1.00	—
D-DETA	Control	4.22	6.23 ± 0.82*	14.77 ± 1.95*	4.02 ± 0.49*	4.47	1.00
	M-HEP	24.69	1.61 ± 0.12	0.65 ± 0.048	1.32 ± 0.17	—	0.33
	M-TET	15.83	14.92 ± 1.09**	9.42 ± 0.69	8.77 ± 0.81**	—	2.18
	M-OA	2.47	5.32 ± 0.88	21.50 ± 3.55**	3.10 ± 0.52	—	0.77
	M-LA	49.85	6.82 ± 0.87	1.37 ± 0.17	3.90 ± 0.47	—	0.97
D-EA	Control	0.21	0.19 ± 0.04	9.37 ± 1.83*	0.13 ± 0.03	0.14	1.00
	M-HEP	1.05	0.18 ± 0.02	1.68 ± 0.19	0.15 ± 0.01	—	1.10
	M-TET	2.39	1.14 ± 0.25**	4.77 ± 1.06	0.76 ± 0.14**	—	5.84
	M-OA	0.35	0.41 ± 0.06**	11.61 ± 1.78	0.22 ± 0.04**	—	1.72
	M-LA	42.51	0.22 ± 0.06	0.05 ± 0.013	0.13 ± 0.03	—	0.96
D-DEA	Control	0.22	0.51 ± 0.07	22.40 ± 3.22*	0.36 ± 0.05	0.40	1.00
	M-HEP	1.66	0.12 ± 0.04	0.71 ± 0.25	0.11 ± 0.02	—	0.28
	M-TET	1.03	1.13 ± 0.21**	11.05 ± 2.09	0.72 ± 0.14**	—	2.01
	M-OA	0.31	1.43 ± 0.14**	46.77 ± 4.73**	0.97 ± 0.11**	—	2.69
	M-LA	44.29	0.13 ± 0.01	0.029 ± 0.002	0.10 ± 0.01	—	0.27
D-TEA	Control	0.30	0.74 ± 0.05	24.77 ± 1.84*	0.52 ± 0.03	0.58	1.00
	M-HEP	0.42	0.21 ± 0.05	5.04 ± 1.16	0.18 ± 0.03	—	0.35
	M-TET	0.53	0.21 ± 0.04	4.07 ± 0.83	0.20 ± 0.01	—	0.39
	M-OA	0.22	0.32 ± 0.05	15.11 ± 2.42	0.38 ± 0.01	—	0.73
	M-LA	34.91	0.036 ± 0.003	0.01 ± 0.001	0.05 ± 0.003	—	0.06
D-HEPP	Control	1.27	0.97 ± 0.08	7.67 ± 0.64*	0.62 ± 0.05	0.69	1.00
	M-HEP	3.79	0.13 ± 0.02	0.35 ± 0.06	0.09 ± 0.01	—	0.15
	M-TET	3.17	0.86 ± 0.21	2.71 ± 0.67	0.49 ± 0.11	—	0.80
	M-OA	1.52	0.92 ± 0.06	6.01 ± 0.38	0.58 ± 0.02	—	0.93
	M-LA	29.51	0.07 ± 0.01	0.024 ± 0.005	0.05 ± 0.001	—	0.06

Identical receiver phase pH 7.4 PBS and donor phases consisting of equivalent molar *O*-acylmenthol with that in permeation of DA or D-K in IPM were used. Data are given as average ± SE (n = 4). *Value is significantly different from DA in IPM (P < 0.05). **Value is significantly different from DA salts in IPM (P < 0.05). $^aER_1$ is calculated as follows: ER_1 = Q (DA salts in IPM)/Q (DA in IPM). $^bER_2$ is calculated as follows: ER_2 = Q (DA salts with enhancer)/Q (DA salts without enhancer).

detailed investigations are required to prove this hypothesis.

The addition of M-OA had a greater enhancing effect on the transdermal permeation of DA with a lower water solubility compared with its salts and this finding agreed well with our previous report[4]. So, M-OA enhances the permeation of lipophilic drugs (DA) across the skin mainly by affecting the nonpolar pathway, which is the esterfied fatty acid-rich pathway within the SC that contributes to diffusional resistance. However, the reason why M-OA also had an enhancing effect on D-K, D-EA, and D-DEA, which are much more hydrophilic than DA, is unknown. M-LA, in particular, was the most effective enhancer in promoting the permeation of the DA salt having a highest water solubility (D-K). Nevertheless, for the relatively lipophilic compounds with a low water solubility, including DA and its salts, partition into the 'hydrated' SC was made difficult—with a reduction in the permeation capacity through the skin (ER < 1) by M-LA. In a previous work[4], the effect of M-LA was attributable to the α-hydroxyl moiety that has hygroscopic properties and exerts a skin hydration effect, which produces an increase in partition into the skin of highly water-soluble compounds (D-K) and in the permeation capacity through the skin.

The extent of penetration of topically applied drugs into underlying tissues is principally determined by (i) the ability of the drug to partition into or dissolve in intercellular lipids, (ii) diffusion across the structural barrier of the epidermis, and (iii) clearance by a combination of the underlying dermal blood supply into the systemic circulation and transport into other tissues[35]. Therefore, an ideal drug should be soluble in water and at the same time have an affinity for its lipid surroundings. As the organic salt of DA contains both an organic cation and anion, a residual degree of hydrophobicity could be present, despite its ionic character. For the diclofenac salts in our study, the ratio of the solubility in *n*-octanol and in water, which can be considered as an approximate partition coefficient, is higher than that expected for these ionic compounds, and this also demonstrates a moderate affinity of the salt for a lipid phase.

In the solution of IPM, organic diclofenac salts can exist only as ion pairs, due to the low dielectric constant of IPM, which prevents ionic dissociation. Charged compounds do not readily penetrate the SC; but when ion pairs are formed, they can be partitioned into lipid domains of the horny layer, as charges are mutually neutralized. To our surprise, the steady-state fluxes through rat skin of DA were not significantly increased but reduced to some extent after the ion-pair formation with amine cations except diethylamine. There may be several explanations for this, such as the reduction in partition coefficient (i.e., the ratio between the solubility of the drug in the membrane and the surrounding medium) or a change in the nature of the permeant species. However, the main reason might be attributed to the reduction in the diffusion coefficient (D) (i.e., the speed with which the permeant can move across a medium, according to its size and shape) due to hydrogen (H)-bonding. Comparison between diethylamine and diethanolamine shows an increase in hydrophilicity as a consequence of the two hydroxyl groups at the end of the ethyl chains that make the diethanolamine cation more hydrophilic than diethylamine. The hydrophobicity of the organic counterions improves the partition coefficient of the ion pairs, which may partly account for the reduced flux of D-DEA compared with D-DETA, and may also partly account for the significant reduction in the permeation rate of D-EA, D-TEA, and D-HEPP compared with that of D-DETA, notwithstanding the fact that the hydrophilicity is not the determinant factor. As the presence of hydroxyl groups in the cations of the diclofenac salts is not sufficient to improve the solubility in water of a particular salt compared with the alkylamine salt (D-DETA).

Several models to predict permeation through human skin have been developed suggested that H-bonding played a significant role in diffusion through skin. It has been reported that H-bonding potentials within the permeant cause a decrease in D, suggesting that the permeants bind via these forces to the immobilized polar regions of the SC lipid and this leads to a negative effect on the D[36]. The intercellular space contains a complex array of lipids that are mainly composed of long chain fatty acids, ceramides, and cholesterol in roughly equimolar proportions[37]. The most obvious interaction would seem to be H-bonding between permeant and the –COOH of the fatty acids and the –OH or amide groups of the ceramides. Pugh et al.[38] showed that the presence of H-bonding groups on the permeant retarded the dermal penetration, and the number and type of pendant H-bonding groups was an important factor when considering potential candidates for transdermal delivery. The fluxes of the diclofenac salts containing a hydroxyl group were significantly lower than that of DA, suggesting that the H-bonding mechanism could be responsible for the retardation of permeation. In this study, the H-bonding between hydroxyl groups of cations and immobilized polar regions of the SC lipid could also play a major role in the global reduction in penetration compared with D-DETA. As some researchers[36,39] found hydroxyl groups had negative effects on the diffusion of permeants both across model membranes and human epidermis. However, more detailed information is required for further investigation.

Interestingly, D-EA with the lowest flux has the highest melting point (158.9°C, Table 1) among the organic diclofenac salts, which agreed well with a previous report[40] that showed that the lower the melting point, the greater the amount of drug transferred to the skin. Some researchers[41,42] also considered that as far as the effect of the permeant melting point on transdermal penetration was concerned, the lower the melting point of a substance, the greater its solubility in a given solvent, including skin lipids. Such behavior was attributed to the dependence of the melting point upon the sum of the energy required to disrupt the crystals[43].

In this study, all cations with hydroxyl groups had negative effects on the transdermal delivery of DA, the rank order of flux being as follows: D-HEPP > D-TEA > D-DEA > D-EA. Some researchers found that when alkylamines were employed as counterions, the amine counterions affected salicylate penetration through the human epidermis in the following order: tertiary > secondary > primary. The order was attributed to the higher stability of tertiary alkylamines compared with their primary or secondary counterparts, and it was believed that the higher the stability of the ion pair, the better its chance of penetrating through lipid membranes[12]. It has also been reported that the cation–anion network of hydrogen bonds of D-TEA containing tertiary alkanolamine in solid state could persist in solvents of low dielectric constant reducing the number of active sites available for solvent attack, and the three 2-hydroxyethyl arms surrounding the ammonium group could shield it from any intermolecular interaction[9].

It is obvious that ion-pair formation with counterions alone cannot enhance the penetration of DA in all cases. Rather, it is likely that the skin membrane flux reflects a combination of solvent, cation, and solute characteristics, such as the dielectric constant of the solvent, the hydrophobicity and shape of the cations, and the melting point and hydrogen bonding capacity of the permeants. The use of *O*-acylmenthol in combination is thus necessary to further increase the DA flux to provide better compliance for the patients undergoing clinical therapy.

Conclusions

From the results of this investigation, it is concluded that *O*-acylmenthol had the potential to enhance the penetration of DA and its salts, and temporary masking of the carboxylic group of DA by complexation with DETA is a useful means of modifying the lipophilicity of DA to maximize its percutaneous penetration. However, the cations with hydroxyl groups had a negative impact on the transdermal delivery of DA in IPM. M-LA and M-HEP, in particular, were effective enhancers in promoting the penetration of permeant having a high water solubility (D-K), and M-TET is extremely effective as far as the transdermal delivery of D-DETA is concerned. Thus, combined use of DETA with M-TET as revealed in this study is useful to develop future clinical transdermal therapeutic systems of DA.

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