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Research paper

Effects of cinnamene enhancers on transdermal delivery of ligustrazine hydrochloride

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Abstract

Cinnamene compounds, cinnamic acid, cinnamaldehyde and cinnamic alcohol, were employed as enhancers. The effects and mechanisms of penetration promoters on the *in vitro* percutaneous absorption of ligustrazine hydrochloride across hairless porcine dorsal skin were investigated. Transdermal fluxes of ligustrazine hydrochloride through porcine skin were determined *in vitro* by Franz-type diffusion cells. The results indicated that the penetration flux of ligustrazine hydrochloride by cinnamic acid was the greatest. Significant statistical differences ($P < 0.05$) were found between cinnamic acid and other promoters. Fourier transform-infrared (FT-IR) were carried out to analyze the effects of enhancers on the biophysical properties of the stratum corneum and the permeation enhancement mechanisms. FT-IR results revealed that the changes of peak shift and peak area due to C–H stretching vibrations in the stratum corneum lipids were associated with the selected enhancers. All of them could perturb and extract the stratum corneum lipids to different extent. Morphological changes of the skin treated with enhancers were monitored by a scanning electron microscope. It was demonstrated that the extraction of the stratum corneum lipids by the enhancers led to the disruption of stratum corneum and the desquamation of stratum corneum flake. Apparent density was newly proposed to estimate the desquamated extent of stratum corneum flake. Correlation analysis revealed that there was a linear relationship between apparent density and decrease in peak area. The results showed that the permeation enhancement mechanisms of cinnamene were pleiotropic ones, including disordering the lipids, extracting the lipids and competitive hydrogen bonding between cinnamene enhancers and amides of ceramide head groups in stratum corneum.
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Keywords: Cinnamene enhancers; Permeation mechanism; Transdermal delivery; Ligustrazine hydrochloride

1. Introduction

Ligustrazine hydrochloride (LH) has been developed as venous administered formulation. As it has short biological half-life ($t_{1/2} = 0.167$ h), it needs to be administered frequently resulting in variable absorption profiles and cumulative toxicosis [1–3]. The undesirable side effects of LH administered via venous can be offset by using the transdermal route, which avoids hepatic first-pass metabolism, variable absorption profiles and cumulative toxicosis resulting from LH administration. Success of the transdermal route depends on the ability of drugs to permeate the skin at a rate and in amounts sufficient to attain effective plasma concentrations [4].

However, the stratum corneum (SC), the outermost layer, is the principal rate limitation to percutaneous delivery. The horny layer is composed of keratin-rich cells embedded in multiple lipid bilayers, which mainly includes ceramides, cholesterol and free fatty acids [5,6]. It is widely accepted that intercellular lipid domain is the main pathway for permeation of most drugs through the SC [7,8].

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Both physical methods (iontophoresis and phonophoresis) and chemical method (penetration enhancers) are used to promote the delivery limitation of drugs across skin. Of these methods, penetration promoters are effective and convenient. Penetration promoters enhance the drug flux by modifying the organization of the intercellular lipids, such as changing fluidity and extracting lipids.

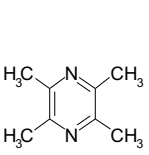
It is reported that cinnamic acid permeates through hairless rat skin [9,10] and human skin easily [11]. Cinnamaldehyde is used as accelerant in combination with ethanol and propylene glycol to promote the penetration of 5-FU [12]. However, there is no report that cinnamic alcohol is used as penetration promoters.

The purpose of the present investigation is to evaluate the influence and permeation mechanisms of cinnamic acid, cinnamaldehyde and cinnamic alcohol on transdermal delivery of LH, and the relationship between the functional group of enhancers and the permeation ratio of LH. A concept, apparent density (AD), is newly proposed to estimate the desquamated extent of SC flake [13,14].

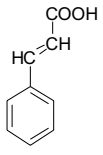
2. Materials and methods

2.1. Materials

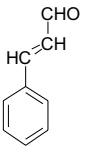
The factory attached to China Pharmaceutical University (Nanjing, China) presented polyvinyl alcohol (PVA). Sodium carboxymethyl cellulose (CMC-Na) was obtained from Tianjin Bodi Chemical Company (Tianjin, China). Ligustrazine hydrochloride (purity > 98.5%) was bought from Guangdong Shuangbai Pharmaceutical Corporation (Guangzhou, China). Cinnamic acid (99.2%), cinnamaldehyde (98.7%) and cinnamic alcohol (98.3%) were purchased from Wuhan Yuancheng Technology Development Co. Ltd. Potassium bromide was provided by Jasco (USA). Azone was purchased from Shanghai Qidi Chemical Corporation (Shanghai, China).



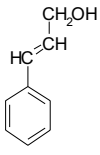
Ligustrazine



Cinnamic acid



Cinnamaldehyde



Cinnamic alcohol

2.2. Preparation of matrix

A solvent casting process prepared PVA matrix, containing ligustrazine hydrochloride. Briefly, 7.0 g of PVA and 3.0 g of CMC-Na (7:3) were dipped in 150 ml water overnight and dissolved in the condition of heating with vigorous stirring. About 400 mg LH was added to this polymer solution. This above-combined drug solution was poured onto a glass plate and the solvent was allowed to evaporate off at room temperature for four days. The matrix was removed from the plate. Then, 3.14 cm^2 pieces of matrix were cut from the membrane and thickness (0.198 ± 0.01 mm), weight (0.17 ± 0.01 g) and moisture ($9.05 \pm 0.21\%$) were measured accurately. The drug content of matrix was 1 mg/cm^2.

2.3. HPLC analysis of samples

Twenty microliters from each vial was injected into the HPLC system, equipped with a pre-packed ODS column (ZOBAX, 5 μm, 4×250 mm, Agilent). The HPLC system (Agilent 1100 series) consisted of an auto sampler and UV-detector. The quantification of LH was performed by integration of peak detected at 294 nm. The samples were chromatographed using a mobile phase consisting of methanol and water (6:4) at a flow rate of 0.8 ml/min. A calibration curve (peak area vs. drug concentration) was constructed by running standard LH (base) solutions in methanol and water (6:4) for every series of chromatographed samples. Calibration curves were linear over the range 0.31–31μg/ml.

2.4. Data analysis

As a result of the sampling of large volumes from the receiver solution, the receiver solution was constantly being diluted. Taking this process into account, the cumulative drug permeation (Q_t) was calculated from the following equation: $Q_t = V_r C_t + \sum_{i=0}^{t-1} V_S C_i$ where C_i is the drug concentration of the receiver solution at each sampling time, C_t is the drug concentration of the sample in the receiver, and V_r and V_s are the volumes of the receiver solution and the sampling solution, respectively. Data were expressed as the cumulative LH permeation per unit of skin surface area, Q_t/S ($S = 3.14$ cm^2). The steady-state fluxes ($J_{ss(4–12h)}$) were calculated by the following equation at steady state (between 4 and 12 h): $J_{ss(4–12)} = \Delta Q_t/(\Delta T.S)$.

Apparent permeability coefficients (K_p) were calculated according to the equation:

$$K_p = J_{ss(4-12\ h)}/C_d$$

where C_d is the drug content in the matrix (1.0 mg/cm^2), while assuming that under sink conditions the drug content in the receiver was negligible compared with the drug in the donor.

2.5. Preparation of skin samples for in vitro studies

Porcine (about 30 kg) was obtained from the animal holding unit in Southern Medical University (Guangzhou, China). Porcine skin was used in the study as a model membrane owing to its more morphological and physiological similarities with human skin [15,16]. Some reports [17–21] showed that the percutaneous flux of drugs with various polarities through pig skin ranged from 0 to 2 times that through human skin. Hair on the back area of the

porcine was removed by trimming with a clipper. Care was taken not to damage the SC. The hair-free back skin was excised with a surgical blade and a pair of scissors and then the adhering subcutaneous fat, tissue and capillaries were removed. The skin was cut into $1.0 \times 3.14\ cm^2$ samples for permeation studies. They were wrapped between aluminum foils and stored at −20 °C prior to use.

2.6. Preparation of SC for FT-IR

The SC was separated from the epidermis by heat treatment at 60 °C for 60 s and digested with 0.25% trypsin solution for 24 h at 37 °C. The SC was then washed with ultra-pure water, dried between filter papers and placed in a vacuum oven at 22 °C overnight for complete dehydration, then dipped in 3% enhancers for 6 h and dried for FT-IR. The dehydrated SC treated with no enhancers added was the control. This experiment was repeated three times. The FT-IR spectral figures were achieved from Jasco FT/IR-480 spectrometer. Jasco spectra manager recorded the peak area and shift. Data were treated with SPSS 10.0.

2.7. In vitro skin permeation studies

The Franz-type diffusion cell was used to investigate in vitro skin penetration kinetics at 32 °C. Firstly, 3% enhancer solution was coated in a cut porcine skin. A $3.14\ cm^2$ patch attached to the porcine skin was placed between the donor and receptor compartment. The receptor compartment contained 7.0 ml of water. The diffusion surface was $3.14\ cm^2$. All of the receiver solution was withdrawn at predetermined time (1, 2, 3, 4, 6, 8, 10, 12, 14, 18 and 24 h), and replaced with an equivalent volume of water. Using the described HPLC method in HPLC analysis of sample, the samples were assayed for LH. The cumulative corrections were made to determine the total amount permeated of LH at each time interval. This experiment was repeated three times with three cells at a time. The receptor chambers were thermostated at 32 °C and the solution in the receptor chambers was stirred continuously at 300 rpm.

2.8. Preparation of skin for SEM

The appropriate skin size ($1 * 1\ cm^2$) was cut and dipped in the 3% enhancer solution for 6 h, washed 6× with ethanol and stored overnight in a freezer at 2.5% glutaraldehyde. The skin with no enhancer solution was control. The image from Hitachi S-3005N SEM was turned into *tiff* format firstly and opened with ImageTool software. An area was chosen optionally and the number of SC flakes was counted in the area, which was repeated at least 6×. The apparent density (AD) of desquamated SC was calculated according to the equation: $AD = \frac{N}{A}$ (where N is the number of SC flakes and A is the chosen area). Data were analyzed with SPSS 10.0.

3. Results

3.1. Infrared spectroscopy studies

FT-IR spectral shifts of porcine SC treated with enhancers are indicated in Fig. 1. Infrared absorption spectra are displayed in Fig. 2. The results showed that the SC treated with promoters produced a higher shift in asymmetric and symmetric C–H vibration peak positions. The peak shifts by azone, cinnamic acid, cinnamaldehyde and cinnamic alcohol in asymmetric and symmetric C–H vibration absorption were 1.90 and 0.96, 4.82 and 2.89, 5.79 and 2.89, 7.72 and $5.78\ cm^{-1}$, in comparison with that of control. The v_a (CH_2) peak shift by cinnamene compounds was higher than that of azone. And there were significant differences ($P < 0.05$) between both promoters. The

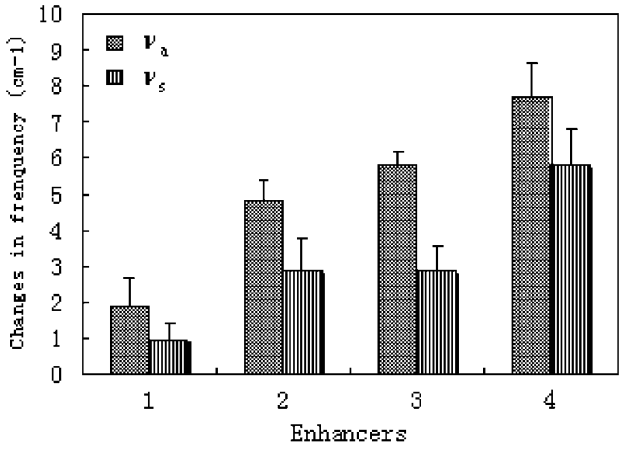


Fig. 1. FT-IR changes in CH_2 C–H stretching vibration frequencies treated with enhancers such as azone (1), cinnamic acid (2), cinnamaldehyde (3) and cinnamic alcohol (4) ($n = 3$, means ± SD): (▨) express v_a (CH_2); (▥) express v_s (CH_2).

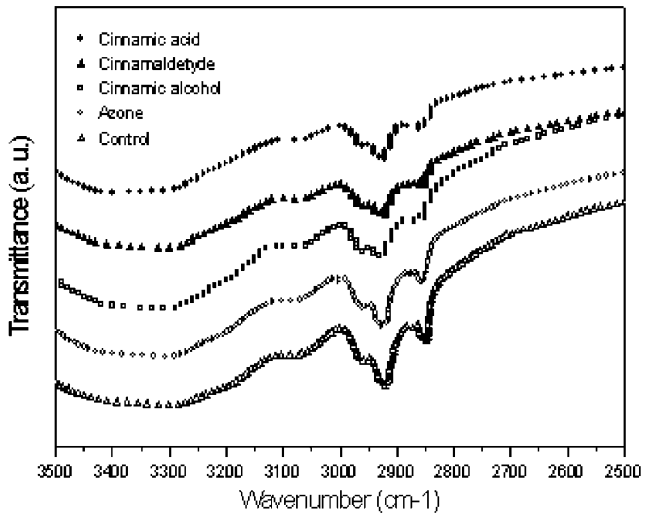


Fig. 2. Infrared absorption spectra of porcine SC treated with various enhancers. Control was SC treated with no enhancer ethanol solution.

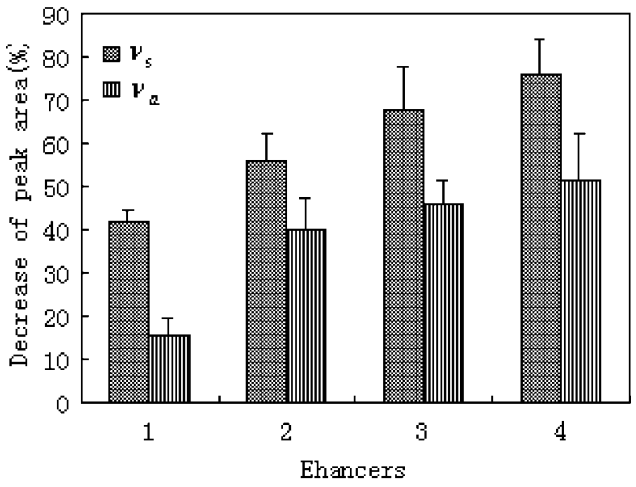


Fig. 3. Decrease in peak area of ν_a (CH_2) and ν_s (CH_2) stretching vibration of porcine SC ($n = 3$, mean ± SD) treated with azone (1), cinnamaldehyde (2), cinnamic alcohol (3) and cinnamic acid (4). (▩) express ν_s (CH_2); (▥) express ν_a (CH_2).

increasing trend in ν_a (CH_2) shift of enhancers was azone < cinnamic acid < cinnamaldehyde < cinnamic alcohol. As for ν_s (CH_2) peak shift, no difference ($P > 0.05$) between cinnamic acid and cinnamaldehyde was found. The ν_s (CH_2) peak shift by cinnamic alcohol was the highest among the enhancers. The increasing trend in ν_s (CH_2) peak shift was azone < cinnamic acid ≈ cinnamaldehyde < cinnamic alcohol.

The decreases in peak area of C–H stretching vibration could be found in Fig. 3. The peak area by cinnamic acid, cinnamaldehyde, cinnamic alcohol and azone compared with control decreased 75.7% and 51.5%, 55.88% and 39.99%, 67.52% and 50.80%, 40.63% and 15.38% in asymmetric and symmetric C–H stretching vibrations, respectively. The decreasing trend in peak area was cinnamic acid > cinnamic alcohol > cinnamaldehyde > azone. There were significant differences ($P < 0.05$) in the decrease in peak area between both enhancers.

3.2. In vitro permeation studies of LH

The transdermal parameters of LH affected by different enhancers across the excised hairless porcine skin are listed in Table 1. Cinnamic acid showed the greatest transdermal flux which was 1.70 and 1.67× in comparison with cinnamaldehyde and cinnamic alcohol. The flux by cinnamic acid was significantly higher than those of control and azone. There were statistically significant differences ($P < 0.05$) between cinnamic acid and any other enhancers. The fluxes by both of cinnamaldehyde and cinnamic alcohol were significantly higher ($P < 0.05$) than those by both of control and azone. However, there were no significant differences ($P > 0.05$) between cinnamaldehyde and cinnamic alcohol, between control and azone. The decreasing trend in enhancement permeation capacity of LH was cinnamic acid > cinnamic alcohol ≈ cinnamaldehyde > azone ≈ control (ethanol). The lag time by cinnamic acid was almost 1.41 and 1.04×, respectively, compared with cinnamaldehyde and cinnamic alcohol.

3.3. SEM studies

Morphological changes in the skin treated with enhancers were examined by SEM (Fig. 4). It was observed that a large number of flakes were desquamated from the intact porcine skin treated with the enhancers and the surface of the skin was very plain. But there was no desquamated SC flake in the intact skin of the control and a large number of wrinkles in the skin surface.

The important difference in these images is the exfoliated extent of SC flake. In order to describe the desquamated extent of SC, a new concept called apparent density (AD) was proposed. AD is expressed by the quantity of desquamated flakes in unit area, including the flakes desquamated partly.

The image of AD with different enhancers is displayed in Fig. 5. AD were 0.78, 2.62, 3.37 and 3.61 pieces/mm^2, respectively. The AD of cinnamic acid was bigger than those of cinnamaldehyde and cinnamic alcohol.

4. Discussion

4.1. Infrared spectroscopy studies

FT-IR had been used to investigate the biophysical changes of properties in the lipid bilayer. A representative FT-IR spectrum of porcine skin SC had absorption peak of C–H bending vibration and stretching vibration from the free fatty acid in lipids. The C–H bending vibration was much weaker than C–H stretching vibration [22]. Thus,

Table 1
Permeation parameters of enhancers through porcine skin to ligustrazine hydrochloride

Enhancers	J_{ss} (μg cm^{-2} h^{-1})	T_L (h)	K_p ($\times 10^5$, h^{-1})
Control	0.74 ± 0.05^{C}	3.29 ± 0.21^{a}	23.63 ± 1.59^{C}
Azone	0.74 ± 0.09^{C}	1.86 ± 0.41^{e}	27.20 ± 4.47^{C}
Cinnamic acid	9.01 ± 0.55^{A}	2.87 ± 0.15^{b}	286.99 ± 17.54^{A}
Cinnamaldehyde	5.33 ± 0.51^{B}	2.03 ± 0.21^{d}	173.43 ± 23.05^{B}
Cinnamic alcohol	5.45 ± 0.72^{B}	2.77 ± 0.04^{c}	169.59 ± 16.12^{B}

Data are expressed as means ± SD ($n = 3$); A, B, C, a, b, c, d and e expressed significant difference, $P < 0.05$. Control was ethanol solution.

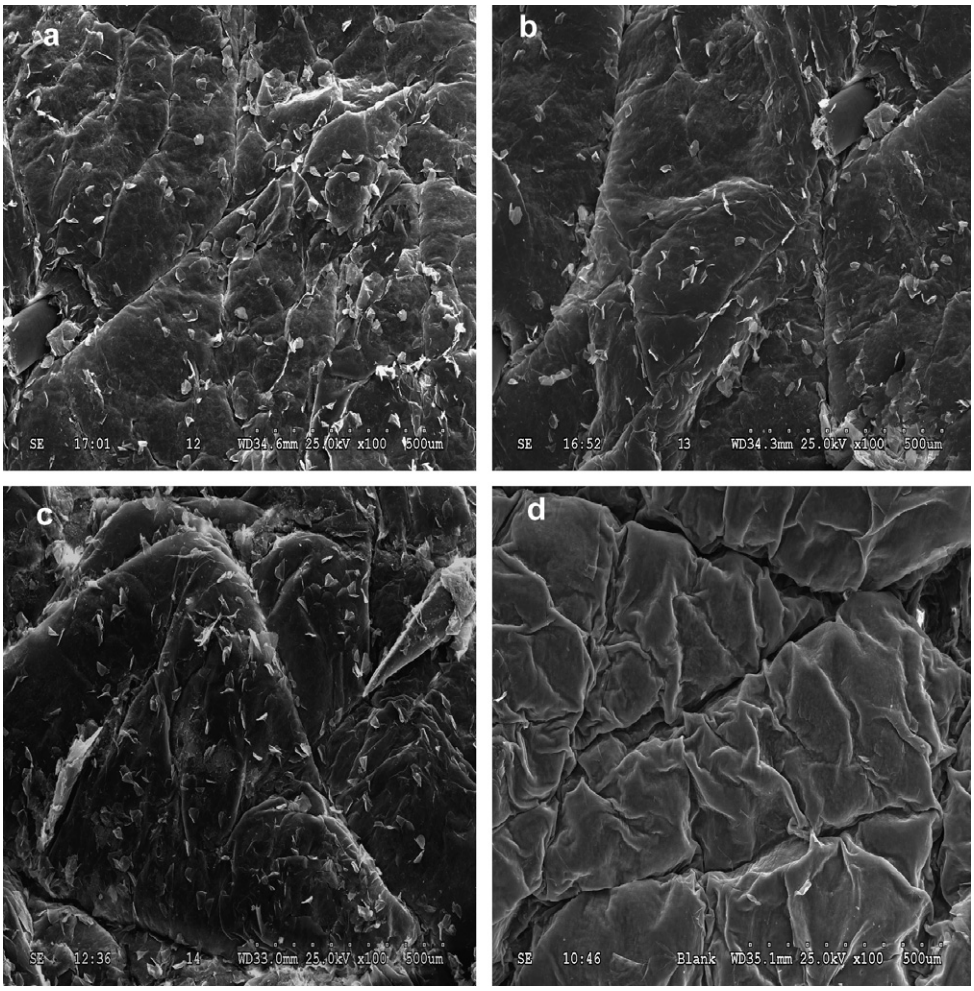


Fig. 4. Scanning electron microscope photographs treated with cinnamic alcohol (a), cinnamaldehyde (b), cinnamic acid (c) and blank (d).

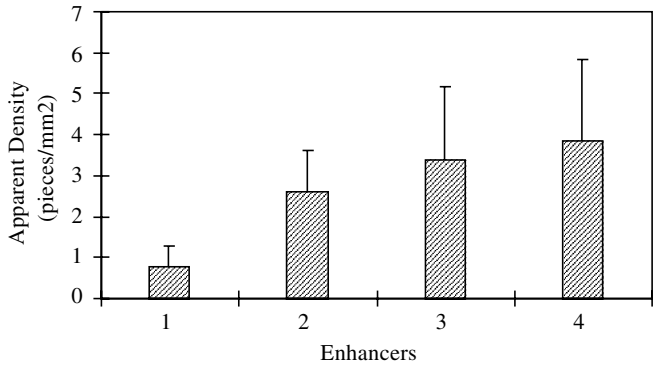


Fig. 5. Apparent density (pieces/mm^2) of the desquamated flake SC treated with azone (1), cinnamaldehyde (2), cinnamic alcohol (3) and cinnamic acid (4) ($n \geqslant 6$, mean $\pm$ SD).

the study of lipid biophysical changes stressed that the peaks near 2920 and 2850 cm^{-1} were produced by the asymmetric and symmetric C–H stretching absorption [23].

Casal and Mantsch had explained the shift in C–H stretching absorption in the molecular level [24]. The shift to higher frequency occurred when CH_2 groups along the alkyl chain of lipids changed from trans to gauche conformation, and indicated that the SC lipid was perturbed. The magnitude of the shift in C–H stretching vibration was directly related to the ratio of trans to gauche conformers in the alkyl chain. The higher the shifts were, the higher the ratio of trans to gauche there would be. It was demonstrated that the promoters causing the higher shift of C–H stretching vibration improved drug permeation [25–27]. The peak areas of the two C–H stretching vibration absorption bands were proportional to the amount of the SC lipids. In other words, any reduction of the SC lipids by enhancers would result in the decrease in peak areas and was beneficial to the permeation of drug [28–30].

The higher the shift of C–H stretching vibration produced, the stronger the ability to perturb the SC lipids. It can be concluded that the capacity of cinnamic alcohol to disorder the SC lipids was stronger than those of cinnamaldehyde and cinnamic acid. The increasing trend in disturbing lipid should be azone < cinnamic acid < cinnamaldehyde < cinnamic alcohol. The enhancement mechanism of cinnamic acid, cinnamaldehyde and cinnamic alcohol included disordering the SC lipids to different extent. The greater the decrease in peak area, the stronger

the activity to extract the SC lipids would become. From Fig. 3, the trend of capacity to extract the SC lipids was cinnamic acid > cinnamic alcohol > cinnamaldehyde > azone.

Considering the spectral shift and the decrease in peak area of C–H stretching vibration, it seemed that the penetration flux of LH by cinnamic alcohol should be much higher among the enhancers. In fact, the transdermal flux by cinnamic alcohol was lower than that of cinnamaldehyde while that of cinnamic acid was much higher than those of others. The results probably indicated that, besides the enhancement mechanism of perturbing and extracting the SC lipids, the other mechanism played an important role in transdermal absorption of LH.

4.2. In vitro permeation studies of LH

In SC, a large amount of ceramides was tightly arranged in the lipid bilayer because of the hydrogen bonding. It was the hydrogen bonding that made lipid bilayer strong and stable, and imparted the barrier trait to SC. The amide I group of one ceramide was connected by hydrogen bonding to the amide of another ceramide. The hydrogen bonding connection formed the network at the head of ceramide. The tight network was destroyed by the enhancers possessing functional group which can donate or accept hydrogen bonding. In this case the permeation rate of drug was promoted [31]. The molecular mechanism of the disruption was further explained as the preferential hydrogen bonding of oxygen-containing enhancers with ceramide head groups thereby breaking the lateral/transverse hydrogen bonding network of lipid bilayer [32]. Without cinnamenes, lipids were held together in lamellae by lateral and transverse hydrogen bonding. Cinnamenes broke transverse hydrogen bonding leading to widening of aqueous region near head groups thereby increasing diffusivity of polar molecules.

Cinnamic acid, cinnamaldehyde and cinnamic alcohol are aromatic compounds that have carboxy group, aldehyde group and hydroxy group in cinnamene, respectively. The only distinction is that different functional group is attached to the same skeleton. Cinnamic acid is the conjugated system, composed of cinnamene and carbonyl group, which strongly attracts the electron of O–H bond and weakens O–H bond. This makes it easier for cinnamic acid to form hydrogen bond with the amide I group easily. The hydrogen-bonding action between cinnamic acid and amides is stronger than that of amides themselves. Cinnamic acid has the greater activity to form hydrogen bond than cinnamic alcohol and cinnamaldehyde while the activity of cinnamic alcohol to form hydrogen bond is higher than that of cinnamaldehyde. So the theory of competitive hydrogen bond can explain the penetration flux order of LH. It seems that the penetration capacity of functional group attached to cinnamene is carboxyl > hydroxyl group > carbonyl group.

The interaction between the SC and penetration enhancers can vary the penetration enhancement and the lag time of drug. Therefore, the activity to promote the permeation and the lag time of LH through the skin were different from each other due to the multiple functional groups of these promoters.

It seemed that competitive hydrogen bond and extracting SC lipids played a main role in the permeation flux of LH, because the results of decrease in peak area and the activity of functional group conformed to the penetration flux of LH.

4.3. SEM studies

Fig. 4 illustrates that the skin area treated with various enhancers was larger than that of blank because the wrinkles of skin were opened. It was assumed that the larger skin area was one reason for raising the flux of LH.

The SC is composed of corneocytes enclosed by a continuous intercellular lipid domain. When the porcine skin was dipped in the enhancer solution, the intercellular lipid was probably dissolved and extracted by the solution, leading to the corneocytes separated from each other and desquamated from the intact SC. This is probably the reason that the SC flake desquamates from the intact SC.

The results demonstrated that the permeation mechanism of the enhancers contained extracting SC lipids. The extraction lipid action of cinnamic acid was stronger than those of cinnamaldehyde and cinnamic alcohol.

Correlation coefficient expresses the correlation extent of two variables. In the present work, we conducted correlation analysis between AD and decrease in peak area. The results showed that there was linear relationship between them. The correlation coefficient between AD and decrease in peak area (v_s) was 0.97 ($P < 0.05$), while it was 0.99 ($P < 0.01$) between AD and decrease in peak area (v_a). The correlation degree between AD and decrease in peak area (v_a) was much higher than that between AD and decrease in peak area (v_s). To some extent, we can conclude that AD can take the place of the decrease in peak area. SEM will probably be a new method to estimate the degree of extraction lipid.

5. Conclusion

The highest transdermal flux of LH by cinnamic acid was dependent on multiple factors such as disordering SC lipid, extracting SC lipid and competitive hydrogen-bond action. From the results of permeation studies, the mechanisms of competitive hydrogen bond and extracting SC lipids were stronger than the mechanism of disordering SC lipids. Therefore, the penetration mechanism of cinnamene was multiple ones.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2007.02.019.

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