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Infrared spectroscopic study of lipid interaction in stratum corneum treated with transdermal absorption enhancers

Yasuko Obata [a,*], Shunichi Utsumi [a], Hiroshi Watanabe [a], Masafumi Suda [a], Yoshihiro Tokudome [b], Makoto Otsuka [b], Kozo Takayama [a]

[a] Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo, 142-8501, Japan
[b] Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Musahino University, Shinnmachi 1-1-20, Nishitoukyou-shi, Tokyo, 202-0023, Japan

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ABSTRACT

To develop a transdermal drug delivery system, it is necessary to search for effective modulators to act as permeation enhancers and evaluate its mechanisms of action. It has been suggested that attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) is a useful tool for evaluating the functional group interaction of the intercellular lipids in the stratum corneum. The purpose of this study is elucidation of the effect of the transdermal permeation enhancers on the intercellular lipid in hairless rat stratum corneum using the ATR-FTIR. Firstly, to confirm the frequencies related to the intercellular lipid in stratum corneum, CH_2 asymmetric and symmetric vibrations were clearly related to the intercellular lipids. In intact stratum corneum, the blue shift of CH_2 asymmetric and symmetric stretching vibrations begins at about 40 °C and remarkable change is induced at 50 °C. The administration of L-menthol causes disorder of the intercellular lipids in stratum corneum similar to that of heat application. The disordering of intercellular lipid lattices in stratum corneum induced by the L-menthol might be related to the enhancing effect of L-menthol. The results provide information for the development of novel transdermal drug delivery systems.

1. Introduction

Transdermal drug delivery systems have many advantages compared with the conventional administration routes for drugs such as intravenous injection or oral administration. However, it is difficult to deliver sufficient amounts of drugs for treatment of disease to the circulation via the skin because of the barrier function of the stratum corneum. Previously, we have reported that L-menthol ((1*R*,2*S*,5*R*)-menthol) and related compounds enhanced the skin permeation of various drugs *in vitro* and *in vivo* (Obata et al., 1990). Moreover, the enhancing mechanisms of L-menthol related to increasing the drug partition and diffusion parameters have been clarified (Obata et al., 2006). The active site of those enhancers is thought to be intercellular lipids as confirmed using laser scanning confocal microscopy (Obata et al., 2006). To develop an effective transdermal delivery system, it is important to clarify the mode of action of enhancers, not only from a macroscopic point of view including reconstruction of formulations, but also from the aspect of the molecular interactions between enhancers and components of stratum corneum.

The stratum corneum of the skin is a distinctive two-compartment system, consisting of corneocytes embedded in the intercellular lipid matrix, which are responsible for the barrier to dehydration and invasion of foreign substances. The intercellular lipids in the stratum corneum form two kinds of lamellar structures with repeat distances of approximately 6 and 13 nm, and two kinds of hydrocarbon chain packing consisting of hexagonal and orthorhombic hydrocarbon chain packing (Bouwstra et al., 1992; Ohta et al., 2001). It is considered that the lamellar structure is one of the key factors controlling drug permeation of the skin. To develop transdermal drug delivery systems, it is necessary to investigate the microstructure of intercellular lipids in the stratum corneum in detail and to search for effective modulators to act as permeation enhancers. Recently, the lipid organization and microstructure of the stratum corneum had been investigated using various techniques including synchrotron X-ray scattering (Bouwstra et al., 1992; Ohta et al., 2001; Hatta et al., 2006), differential scanning calorimetry (Al-Saidan et al., 1998), and Fourier transform infrared spectroscopy (FTIR) (Jain et al., 2002; Vaddi et al., 2002; Tokudome and Sugibayashi, 2003). Among them, FTIR is considered the most powerful tool for determining the molecular vibrations of the materials in the stratum corneum at the functional group level. Because of the complex composition of stratum corneum including lipids, proteins, minerals, and amino acids, many infrared absorption bands appear in the spectrum obtained

* Corresponding author. Tel.: +81 3 5498 5783; fax: +81 3 5498 5783.
E-mail address: obata@hoshi.ac.jp (Y. Obata).

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from the stratum corneum. However, numerous databases have already been established from which to determine the origin of each vibration. Thus, we can readily assign the particular vibration derived from the functional group in which we are interested. Furthermore, infrared spectrometry is a probe-free and noninvasive technique for measurement of the stratum corneum. Its use ensures that the experimentally obtained vibrations directly reflect the characteristics of the stratum corneum. Because of those advantages, the vibrations derived from lipids are a good index from which to evaluate the microstructure of the lamellar lipid constructs in the intercellular space in the stratum corneum. This is important because the active sites of lipophilic transdermal permeation enhancers are thought to be intercellular lipids. Using attenuated total reflection (ATR)-FTIR, it is thought to be possible to observe the lipid organization in the stratum corneum not only on the surface, but also in deeper regions in the stratum corneum. In general, changes in the absorbance frequency reflect changes in the conformational arrangement of functional groups. It might be possible to predict the changes in the intercellular lipids in the stratum corneum induced by the treatment with skin permeation enhancers.

In this study, we focused on the phase transition of intercellular lipids in the stratum corneum as a function of temperature and elucidated the effect of L-menthol on functional group interaction by thermal scanning ATR-FTIR. We also attempted to determine quantitatively any changes in lamellar lipid constructs caused by absorption enhancers.

2. Materials and methods

2.1. Materials

L-Menthol (99%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Chloroform was of a certified grade. Other chemicals used were of reagent grade.

2.2. Preparation of stratum corneum sheet from hairless rat skin

The stratum corneum was separated from the excised abdominal regions of hairless rats (strain HWY/Slc, 12 weeks old, Sankyo Labo Service, Tokyo, Japan) by digestion with 0.1% (w/w) trypsin in phosphate-buffered saline (pH 7.4) at 37 °C for 24 h. The separated stratum corneum was rinsed in purified water and dried *in vacuo*. The stratum corneum was incubated in purified water, 40% (w/w) ethanol, permeation enhancer solution (0.5% (w/w) or 1.0% (w/w) L-menthol in 40% (w/w) ethanol) for 2 h at ambient temperature and dried under a stream of nitrogen until it reached an acceptable predetermined weight (125% of weight prior to treatment). Procedures involving animals and their care complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals of Hoshi University.

2.3. Lipid extraction

Lipids were extracted from the hairless rat stratum corneum using the method of Folch (Swartzendruber et al., 1987). Briefly, the stratum corneum was incubated in chloroform/methanol mixtures (1:2, 1:1, 2:1, v/v) for 2 h, respectively. The organic solvent was evaporated under a stream of nitrogen, and subsequently the extracted lipids were stored in desiccators.

2.4. ATR-FTIR measurement

Fourier transform infrared spectroscopic measurements were performed using an FTIR 4100 type A spectrophotometer (JASCO International, Tokyo, Japan) with an ATR accessory "Specac MKII Golden Gate" (JASCO International). All spectra were obtained as an average of 45 scans recorded between 4000 cm^{-1} and 1000 cm^{-1} at 4 cm^{-1} resolution. The stratum corneum sheets were placed on a sample stage. Spectra were routinely acquired at every 1 °C increment from 30 °C to 120 °C.

2.5. Statistical analysis

Student's *t*-test was employed for evaluation of the results. A $p < 0.05$ was considered as significant.

3. Results

3.1. Assignment of infrared absorption of hairless rat stratum corneum

Hairless rats are frequently used as a model to evaluate skin permeation of drugs in early stages of development of transdermal formulation. In this study, stratum corneum sheets obtained from hairless rats were used as a model stratum corneum. The infrared spectra of the stratum corneum was expected to have various bands, CH_2 stretching vibration, CH_2 scissoring vibration, and amide vibration, derived from intercellular lipids and keratin in corneocytes. The infrared spectra of intact stratum corneum (Fig. 1(A)), delipidated stratum corneum (Fig. 1(B)), and extracted lipids (Fig. 1(C)) were determined at 30 °C. In intact stratum corneum, the absorbance frequencies derived from the CH_2 asymmetric stretching vibration (~2920 cm^{-1}), the CH_2 symmetric stretching vibration (~2850 cm^{-1}), and amide I (~1650 cm^{-1}) and II (~1550 cm^{-1}) bonds were observed. On the other hand, the spectral absorbance bands from CH_2 stretching vibrations dramatically decreased in delipidated stratum corneum as shown in Fig. 1(B). However, the absorbance derived from amide I and II bonds was not changed. Furthermore, in the infrared spectra of extracted lipids, amide I and II vibrations dramatically decreased compared with those of intact stratum corneum. Thus, asymmetric and symmetric CH_2 vibrations are important elements for the evaluation of change in intercellular lipids. Those results suggested that CH_2 asymmetric and symmetric vibrations were derived from methylene groups on the acyl side chain of intercellular lipids (ceramides and fatty acids) in stratum corneum and the amide I and II vibrations belonged to the amide bonds of the keratin in corneocytes.

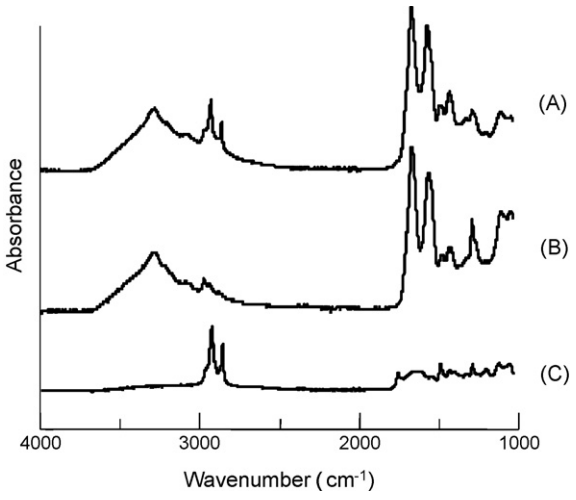

Fig. 1. The infrared spectrum of the hairless rat stratum corneum. (A) Intact stratum corneum, (B) delipidated stratum corneum, and (C) extracted lipid.

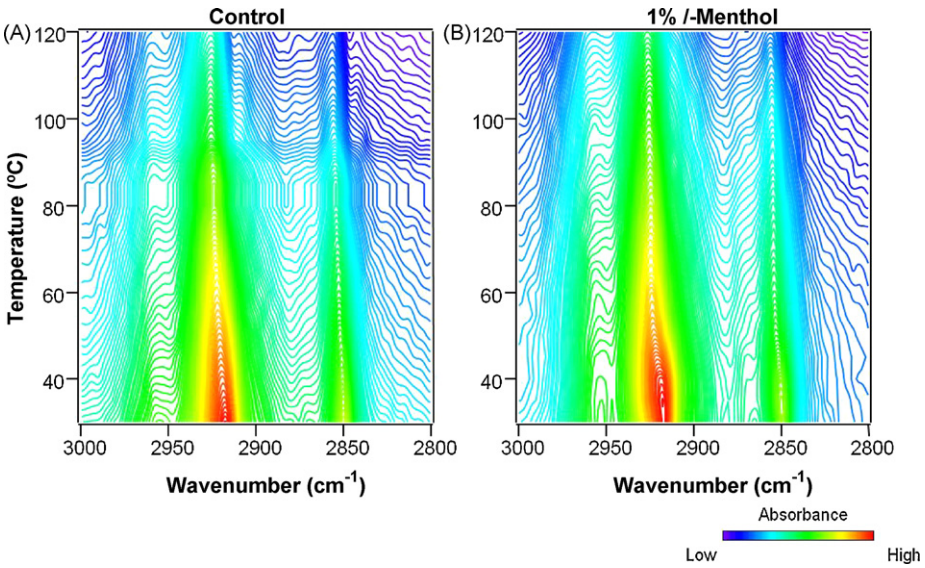

Fig. 2. The contour map of thermotropic response of the CH_2 asymmetric and symmetric frequencies in the intercellular lipids in stratum corneum. (A) Intact stratum corneum and (B) stratum corneum treated with 1.0% L-menthol for 2 h.

3.2. Thermotropic behavior of infrared absorption of hairless rat stratum corneum

Figs. 2A and 3A show the thermotropic behavior of the CH_2 asymmetric and symmetric stretching vibrations of the intercellular lipid in intact stratum corneum. The asymmetric and symmetric CH_2 stretching vibrations showed a blue shift and the full-width at half maximum was gradually increased as a function of temperature. The change in those parameters indicates that the vibration of the hydrocarbon chain of ceramide and fatty acids increased. As shown in Figs. 2A and 3A, the blue shift of asymmetric and symmetric CH_2 stretching vibrations began at about 40 °C and remarkable change was induced by 50 °C. The increase in the full-width at half maximum of the infrared absorption profile indicates a deviation of the vibration of particular functional groups. Those phenomena indicated that the rigid microstructure constructs of intercellular lipids became disordered according to increase in temperature. This might be related to phase transition of intercellular lipids. The change in peak position of absorbance frequency is shown in Fig. 3. Those findings suggested that the disorder of intercellular lipid in the stratum corneum was induced from 40 °C by heat application. However, the phase transition observed at 71 °C in hairless mouse stratum corneum was considered lower in hairless rat stratum corneum, about 60 °C shown in Fig. 3. It is speculated that the difference in phase transition temperature is caused by differences in the components of intercellular lipids. From these findings, it is suggested that the change in CH_2 stretching vibration reflected in the phase behavior of microstructure is a good parameter for measuring the disorder of intercellular lipids in the stratum corneum.

3.3. Effect of L-menthol on the infrared adsorption characteristics of intercellular lipids

To elucidate the effect of L-menthol on the intercellular lipids in the stratum corneum, we firstly measured the infrared spectrum of L-menthol at 30 °C. CH_2 asymmetric and symmetric stretching vibrations (~2925 cm^{-1} and ~2845 cm^{-1}, respectively) and CH_3 asymmetric and symmetric stretching vibrations (~2955 cm^{-1} and ~2869 cm^{-1}, respectively) were appeared in the ATR-FTIR spectrum of the L-menthol. The asymmetric and symmetric CH_3 vibrations in the L-menthol spectrum were quite strong compared with absorption from intercellular lipids in the stratum corneum.

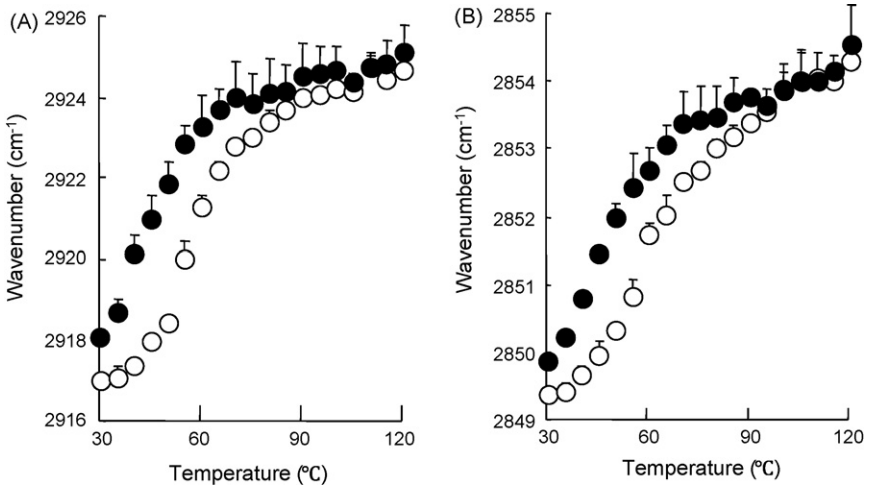

Fig. 3. The effect of L-menthol on the change in peak wavenumber of asymmetric (A) and symmetric (B) CH_2 absorption identified by curve fitting. Each point represents the mean ± S.D. (n = 3). (○) control and (●) 1.0% L-menthol treated.

Table 1
The asymmetric and symmetric CH_2 stretching frequencies of intercellular lipids in the stratum corneum at 30 °C.

	Asymmetric stretching	Symmetric stretching
Control	2916.836 ± 0.209	2849.286 ± 0.105
40% ethanol	2917.053 ± 0.125	2849.410 ± 0.141
0.5% L-menthol	2917.546 ± 0.385**	2849.532 ± 0.092*
1.0% L-menthol	2917.984 ± 0.175**	2849.831 ± 0.126**

Each value represents the mean ± S.D. ($n = 3$).
* $p < 0.05$.
** $p < 0.01$.

The infrared spectra of intercellular lipids in stratum corneum treated with L-menthol indicated less absorption by asymmetric and symmetric CH_3 vibrations. Therefore, the presence of L-menthol crystal in stratum corneum can be neglected under this experimental condition.

When 40% ethanol was used as a solvent for L-menthol, an insignificant blue shift was observed as shown in Table 1. From these findings, it is suggested that the effect of ethanol to increase the vibration of lipids was negligible under these experimental conditions. In a study dealing with the skin permeation of ionic molecules, at lower ethanol concentrations (~40%) slight changes in asymmetric and symmetric CH_2 stretching vibrations might occur (Kurihara-Bergstrom et al., 1990). Moreover, the lipid lamellar structure was only slightly affected by application of 40% ethanol in synchrotron X-ray diffraction studies (unpublished data). On the other hand, the asymmetric and symmetric CH_2 stretching vibrations showed a significant blue shift after L-menthol treatment. In stratum corneum treated with 1.0% L-menthol in 40% ethanol, the asymmetric CH_2 stretching vibration was shifted from 2916.8 cm^{-1} to 2918.0 cm^{-1}, and symmetric stretching vibration was shifted from 2849.3 cm^{-1} to 2849.8 cm^{-1} at 30 °C. The effect of 1.0% L-menthol on the both CH_2 stretching vibrations was shown in Figs. 2 and 3. In controls, absorption is observed at ~2920 cm^{-1} and gradually decreased according to increases in temperature. In stratum corneum treated with 1.0% L-menthol in 40% ethanol, the change in absorption at ~2920 cm^{-1} showed irregularity. As shown in Fig. 3, the wavenumber for asymmetric and symmetric CH_2 stretching vibrations of stratum corneum treated with 1.0% L-menthol in 40% ethanol was greater than that of control particularly at the physiological temperature. On the other hand, at higher temperatures, the blue shift of asymmetric and symmetric CH_2 stretching vibrations reached a steady state similar to the control (Fig. 3). It is suggested that the microstructure of intercellular lipids in the stratum corneum was strongly disturbed at lower temperature and the phase transition became unclear by the L-menthol treatment.

4. Discussion

The light of ATR-FTIR can attain approximately 1 μm in depth at specific area. Moreover, the distribution of components thought to be heterogeneous in stratum corneum. However, in this study, we can focus on the behavior of lipids. Thus, the change in infrared spectra derived from lipids at the surface of stratum corneum gave us the useful information of intercellular lipids affected by administration of absorption enhancers. As shown in Fig. 1, the CH_2 asymmetric and symmetric stretching vibrations characterized the infrared spectrum of the intercellular lipid in the stratum corneum. The previous studies, the asymmetric (~2920 cm^{-1}) and symmetric (~2850 cm^{-1}) CH_2 stretching vibrations have been commonly used as parameters for evaluation of lipid acyl chains (Narishetty and Panchagnula, 2004; Rerek et al., 2005; Gooris and Bouwstra, 2007). Until now, it has not been clear which vibration is more important for evaluation of molecular interactions between lipids and other materials. Thus, we have evaluated both asymmetric and symmetric CH_2 vibrations in this study.

4.1. Thermotropic behavior of infrared absorption of lipids in hairless rat stratum corneum

In the development of transdermal delivery systems, lipid organization at physiological temperatures is considered to be important. However, the lipids show several forms depending on the surrounding temperature. It might be possible to understand the characteristics of lipids inclusively by measurement of their characteristics at a wide range of temperatures. Previous study using differential scanning calorimetry, indicated that the phase transitions of intercellular lipids in hairless mouse stratum corneum occurred at 39 °C, 51 °C, and 71 °C (Hatta et al., 2006). Furthermore, the change in lamellar structure of intercellular lipids was consistent with the phase behavior investigated by a synchrotron X-ray diffraction study using hairless mouse stratum corneum (Hatta et al., 2006). The intercellular lipids in the stratum corneum of hairless rats are considered to form a rigid lamellar structure and hydrocarbon chain packing below 39 °C. At 39 °C, the orthorhombic hydrocarbon chain packing disappears and is transposed to high-temperature hexagonal hydrocarbon chain packing at higher temperatures. The disappearance of long lamellar structure occurred at 51 °C and changed to a liquid crystalline phase of hexagonal hydrocarbon chain packing. Subsequently, the short lamellar structure melts at 71 °C. Al-Saidan et al. reported that the thermotropic behavior of the hairless rat stratum corneum was similar to that of hairless mouse. Therefore, those phase transitions of hairless mouse stratum corneum might support the phase behavior in hairless rat stratum corneum. The change in asymmetric and symmetric CH_2 vibration frequencies by the heat application was very small. Nevertheless, a clear tendency was observed in infrared absorption by lipids with increasing temperature. Transdermal drug delivery increased by heat application has been seen *in vitro* (Akomeah et al., 2004). Moreover, clinically, the effect of heat on the transdermal delivery of fentanyl has been reported (Shomaker et al., 2000). Those findings suggested that an increase in lipid acyl chain vibration facilitated an increase in drug permeation. In general, the asymmetric and symmetric CH_2 stretching vibrations blue shift reflects the increasing disorder of the intercellular lipids in the stratum corneum. Small changes in the asymmetric and symmetric CH_2 stretching vibrations might have a profound effect on the conformation of lipids in the stratum corneum influencing drug permeation via the skin. Further study is necessary to clarify those phenomena.

4.2. Effect of L-menthol on the infrared adsorption characteristics of intercellular lipids

To elucidate the interaction between intercellular lipids in stratum corneum and skin permeation enhancers was useful for the development of the effective transdermal drug delivery systems. As shown in Fig. 3 and Table 1, the wavenumber for the CH_2 asymmetric and symmetric stretching vibrations were significantly increased by the treatment of L-menthol. This indicates that L-menthol strongly induced disorder of lipid lamellar structures at physiological temperatures. The orthorhombic hydrocarbon chain packing was dominant below 39 °C in stratum corneum; thus, it is supposed that L-menthol causes orthorhombic packing below physiological temperature. Focused on the results at 30 °C, the blue shift of the asymmetric and symmetric CH_2 stretching vibration frequencies was evaluated according to temperature. $\Delta\nu$ value was defined as Eq. (1) to quantify the effect of L-menthol:

$$\Delta\nu = (\text{Peak value at specified temperature}) - (\text{Peak value at } 30\ ^\circ\text{C}) \quad (1)$$

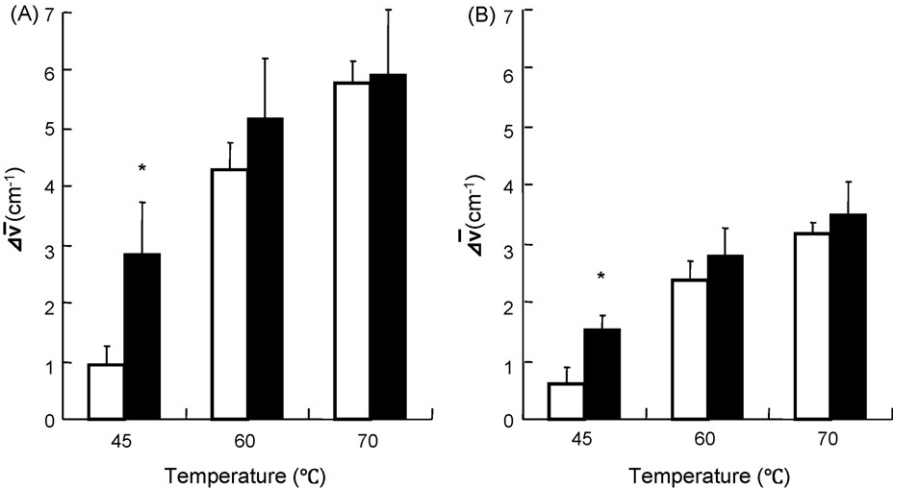

Fig. 4. Effect of L-menthol on the change in wavenumber of the asymmetric (A) and symmetric (B) CH_2 stretching frequencies of intercellular lipids in the stratum corneum. The open column represents intact stratum corneum and the filled column represents stratum corneum treated with 1.0% L-menthol in 40% ethanol. Each column represents the mean ± S.D. ($n = 3$). $^*p < 0.05$.

As shown in Fig. 4, the absorption was shifted significantly at 45 °C. This indicates that L-menthol significantly affected the rigid structure of lipids at physiological temperatures. At higher temperatures, the effect of L-menthol on the lipid lamellar structure was decreased.

Moreover, administration of L-menthol produced the same effect on the molecular vibration as heat application. Furthermore, equivalence between heat application and administration of L-menthol is discussed in comparison with the blue shift of infrared vibration. To clarify the effect of temperature corresponding to heat application, the control value of asymmetric and symmetric CH_2 stretching vibration was fitted to a logistic function. The equation is as follows:

$$y = \left(\frac{A_1 - A_2}{1 + (x/x_0)^p}\right) + A_2 \quad (2)$$

where y is the peak wavenumber and x is the applied temperature. In the case of the asymmetric and symmetric CH_2 stretching vibration, A_1 is 2916.6, A_2 is 2924.3, x_0 is 58.2, and p is 6.5. In the case of symmetric CH_2 stretching vibration, A_1 is 2849.0, A_2 is 2854.3, x_0 is 62.7, and p is 4.5. The regression coefficient is 0.995 in asymmetric stretching vibration and 0.994 in symmetric stretching vibration. The peak wavenumber obtained by each treatment at 30 °C was then extrapolated to the control. In Table 2, the corresponding temperature is summarized. When the stratum corneum was treated with 40% ethanol, the vibration state was improved by more than 6 °C. However, the corresponding temperature was still bellow the phase transition temperature, which might be observed around 39 °C (Hatta et al., 2006). On the other hand, the corresponding phase transition temperature was drastically increased by the application of 0.5% L-menthol. In the case of asymmetric vibrations, the corresponding temperature was above the phase transition. This indicates that L-menthol plays an important role in the change of intercellular lipid structure at a functional group level.

Table 2
The corresponding temperature required to influence stratum corneum obtained from pretreated stratum corneum at 30 °C.

	Asymmetric stretching	Symmetric stretching
40% ethanol	37.8	36.5
0.5% L-menthol	42.8	38.1
1.0% L-menthol	46.0	43.4

The phase transition caused dissolution of orthorhombic packing at 39 °C. The dissolution of lateral packing could be considered as a decrease in the resistance to diffusion of drugs. Thus, an increase in corresponding temperature indicates a decrease in phase transition temperature. When the concentration of L-menthol was increased to 1.0%, the corresponding temperature was also increased. The solubility of L-menthol in 40% ethanol is about 1.99% (Obata et al., 1993); thus, 1.0% L-menthol in 40% ethanol is below the solubility. We can speculate that the amount of L-menthol absorbed into the skin surface increases with increasing application concentration until the solubility depending on the thermodynamic activity of L-menthol in 40% ethanol solution. Thus, the contribution of L-menthol to the change in lateral packing of intercellular lipids will increase depending of the amount of L-menthol adsorbed into the skin.

In this study, we have succeeded to the quantitative evaluation of asymmetric and symmetric CH_2 stretching vibration changes with treatment by transdermal permeation enhancers. Moreover, we found that treatment with permeation enhancers, such as L-menthol, was quite similar to heat application at a functional group level. These results are considered to provide important fundamental information for developing novel transdermal delivery drug systems.

5. Conclusions

In conclusion, it is suggested that ATR-FTIR is a useful tool for evaluating the functional group interaction of the intercellular lipids in stratum corneum and to elucidate the mechanisms of action of transdermal permeation enhancers. L-Menthol disordered the rigid microstructure of the intercellular lipids in the stratum corneum to the same extent as heat application. The disorder of intercellular lipids in the stratum corneum induced by the L-menthol is related to the enhancing effect of L-menthol. Finally, these findings provide fundamental information for the development of novel transdermal drug delivery systems.

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