Regular Article

Effect of Nerolidol and/or Levulinic Acid on the Thermotropic Behavior of Lipid Lamellar Structures in the Stratum Corneum

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Received June 26, 2016; accepted September 19, 2016

Permeation enhancers are required to deliver drugs through the skin efficiently and maintain effective blood concentrations. Studies of the barrier function of the stratum corneum using l-menthol, a monocyclic monoterpenes widely used in medicines and foods, have revealed an interaction between characteristic intercellular lipid structures in the stratum corneum and permeation enhancers. The variety of permeation enhancers that can be used to contribute to transdermal delivery systems beyond l-menthol is increasing. In this study, we focused on nerolidol and levulinic acid and investigated their influence on stratum corneum lipid structures. Nerolidol, a sesquiterpene, has been reported to enhance the permeation of various drugs. Levulinic acid is reported to enhance the permeability of buprenorphine and is used as a component of the buprenorphine patch. Synchrotron X-ray diffraction and attenuated total reflectance Fourier transform IR spectroscopy measurements revealed that nerolidol disturbs the rigidly arranged lipid structure and increases lipid fluidity. Levulinic acid had a smaller effect on stratum corneum lipid structures, but did increase lipid fluidity when co-administered with nerolidol or heat. We found that nerolidol has an effect on stratum corneum lipid structures similar to that of l-menthol, and levulinic acid had an effect similar to that of oleic acid.

Key words  nerolidol; levulinic acid; stratum corneum; synchrotron X-ray diffraction; attenuated total reflectance Fourier transform IR spectroscopy

Chemical penetration enhancers are useful in transdermal delivery systems. These enhancers act on cellular lipid structures in the stratum corneum, reversibly decreasing its barrier function and increasing drug permeability. Many types of enhancers have been investigated, including alcohols, pyrrolidones, surfactants, fatty acids, and terpenes. The modes of action of penetration enhancers include increasing lipid partitioning or diffusion, disturbing lipid structure, and extracting lipids. Chemical absorption enhancers must not only have high permeation effects but also a margin of safety for administration to live organisms, and thus many compounds are restricted from practical use. Based on their safety and efficacy, terpenes have been studied extensively as permeation enhancers. Terpenes increase the diffusion of drugs in the intercellular lipids of the stratum corneum, and fatty acids increase the distribution of drugs to the surface. Combining enhancers with different actions can have a synergistic effect on drug permeability. The compatibility of the principal agent and the effect of permeation enhancers on drug permeability has been investigated.

Intercellular lipid structures in the stratum corneum of skin act as a barrier to drug permeability. These lipids have been shown to form hexagonal and orthorhombic structures. The observation of hydrocarbon chain packing by wide-angle X-ray diffraction has revealed hexagonal packing with a lattice distance of approximately 0.42 nm and orthorhombic packing with lattice distances of approximately 0.42 and 0.37 nm. Although characteristic structures have been identified, the mechanism of action of permeation enhancers on stratum corneum lipids remains unclear.

L-Menthol monocyclic monoterpenes have been studied as permeation enhancers. They are commonly used in medicines and food, and their efficacy and safety have been established. However, the detailed mechanism by which they increase the drug permeability of the skin has not been elucidated. Increasing the range of available permeation enhancers and elucidating their mechanisms will facilitate the development of improved formulations for transdermal delivery patches.

In this study, we focused on two permeation enhancers: nerolidol and levulinic acid. Nerolidol, a straight sesquiterpene (molecular structure: C15H26O), has promoting effects on various drugs and is one of the main components of neroli, which is used for aromatherapy. Levulinic acid, a keto fatty acid (molecular structure: C5H8O3), is used in buprenorphine tape and in cosmetics. These two permeation enhancers are considered effective and safe. In our study, we used 20% (w/w) isopropanol as the vehicle for enhancer preparations because it is hypoallergenic.

Nerolidol and levulinic acid were used as model permeation enhancers to elucidate the mechanism of thermal change in the intercellular lipid structures of the stratum corneum.

Experimental

Materials  Nerolidol was purchased from Tokyo Chemical Industry (Tokyo, Japan). Levulinic acid was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other chemicals used were of reagent grade.

Sample Preparation  The stratum corneum was separated from the abdominal region of a hairless mouse (Hos-HR-1, 7 weeks old, Sankyo Labo Service, Tokyo, Japan) using 0.1% (w/w) trypsin in phosphate buffer solution (pH 7.4) at 37°C for 4 h. The separated stratum corneum was rinsed in purified water and dried in vacuo for 24 h. The dried stratum corneum was incubated in purified water and permeation enhancer solution for 2 h at 32°C. The permeation enhancer solutions included 0.5 and 1.0% (w/w) nerolidol and 3.0 and 9.0% (w/w)
levulinic acid, all in 20% (w/w) isopropanol. The weight of the stratum corneum was adjusted to 125% of the dried weight using nitrogen gas. All 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.

**Synchrotron X-Ray Diffraction Measurements**
Wide-angle X-ray scattering measurements were performed at the BL40B2 Structural Biology II Beamline at SPring-8 (Hyogo, Japan). The wavelength (λ) of the X-ray beam was approximately 0.151 nm, and the sample-to-detector distance was approximately 540 mm. The reciprocal spacing, \[ S = \frac{1}{d} = \frac{2\lambda}{\sin(2\theta/2)}, \] where \( 2\theta \) is the scattering angle and \( d \) is the lattice distance, was calibrated using the lattice spacing of a plum¬bum stearate crystal at room temperature (r.t.). Capillary tubes with a diameter of 1 mm containing the prepared stratum corneum were sealed using a gas burner and placed in the sample holder of the X-ray diffractometer.

**Attenuated Total Reflectance Fourier Transform IR Spectroscopy (ATR-FTIR) Measurements**
ATR-FTIR measurements were conducted using an FTIR-4200 spectrometer fitted with ATR PRO (JASCO International, Tokyo, Japan). All spectra were obtained as an average of 32 scans recorded from 4000 to 1000 cm\(^{-1}\) at 2 cm\(^{-1}\) resolution. The stratum corneum sheets were placed on a sample stage. Spectra were acquired in temperatures ranging from 20 to 100°C in 5°C increments.

**Statistical Analysis**
The results were evaluated using Student’s \( t \)-test, and differences were considered significant at \( p<0.05 \).

**Results and Discussion**
**Effect of Permeation Enhancers on Synchrotron X-Ray Diffraction Profiles**
The hairless mouse stratum corneum was analyzed by synchrotron X-ray diffraction. Diffractions at 2.40 and at 2.66 nm\(^{-1}\) were observed. The former is derived from hexagonal and orthorhombic structures, and the latter is derived from only orthorhombic structures.\(^{15,23,25}\) Figure 1 presents the synchrotron X-ray diffraction results for hairless mouse stratum corneum treated with water (W), 3.0% (w/w) levulinic acid (L3), 1.0% (w/w) nerolidol + 3.0% (w/w) levulinic acid (N1L3), and 1.0% (w/w) nerolidol (N1) at 20°C. The profile of L3 was nearly identical to that of W. By contrast, the stratum corneum treated with nerolidol (N1, N1L3) exhibited a significant decrease in intensity, and gel lipids occurred near 2.2 nm\(^{-1}.\)\(^{23}\) The orthorhombic and hexagonal structures of the intercellular lipids appear to have been strongly affected by nerolidol but not by levulinic acid.

**Changes in X-Ray Profiles as a Function of Temperature**
Synchrotron X-ray diffraction of stratum corneum treated with permeation enhancers was performed at various temperatures (Fig. 2). Between 20 and 40°C, the intensity ratio near 2.40 nm\(^{-1}\) of stratum corneum treated with water decreased dramatically with increasing temperature. The intensity ratio then increased gradually as the temperature rose from 40 to 60°C, then decreased as the temperature rose above 60°C. This result indicates that the transition from the orthorhombic structure to the high-temperature hexagonal structure occurred near 40°C.\(^{19}\) The behavior of L3 was nearly identical to that of W. By contrast, the profiles of stratum corneum treated with nerolidol (N1, N1L3) were different. N1 only exhibited decreasing behavior, and N1L3 exhibited intermediate behavior between that of N1 and W.

The changes in lateral hydrocarbon chain packing near 2.4 nm\(^{-1}\) indicated characteristic behavior as a function of temperature (Fig. 3). The lattice distance of the stratum corneum treated with water gradually increased as the temperature rose from 20 to 40°C. From 40 to 50°C, the lattice distance decreased, and then as the temperature rose above 50°C the lattice distance increased again. These behaviors indicate thermally induced disordering of lateral packing structures in the 20 to 40°C range. The behavior between 40 and 60°C indicated the formation of a high-temperature hexagonal structure and shrinking of the lattice distance. Above 60°C,
the high-temperature hexagonal structures gradually became disordered, and the lattice distance increased again as a function of temperature. The lattice distance profile of L3 was nearly identical to that of W. By contrast, the behavior of the stratum corneum treated with nerolidol (N1, N1L3) differed from that of the stratum corneum treated with water. N1 exhibited increasing lattice distance only, whereas the behavior of N1L3 was intermediate between that of N1 and W. These results suggest that nerolidol treatment strongly affected the orthorhombic and hexagonal structures, whereas levulinic acid had little effect.

Determination of the Effect of Permeation Enhancers on Lipid Fluidity by ATR-FTIR Functional group interaction of intercellular lipids were evaluated using ATR-FTIR. This technique can reveal lipid organization in the stratum corneum not only at the surface, but also in deeper regions. In general, changes in absorbance frequency reflect changes in the conformational arrangement of functional groups and increased lipid fluidity. The CH$_2$ asymmetric and symmetric stretching vibrations are derived from intercellular lipids, whereas the amide I and amide II absorptions are derived from keratin. Therefore, we focused on the CH$_2$ asymmetric stretching vibrations of intercellular lipids in the stratum corneum, the IR spectrum was investigated at 20 and 100°C (Table 1). At 20°C, W and L3 exhibited identical wavenumbers, whereas N1 exhibited a blue shift, and N1L3 was further blue shifted. At 100°C, L3 exhibited the same wavenumber as N1 and N1L3. The lipid fluidity of L3 and W were similar at 20°C but differed at 100°C. The lipid fluidity of L3 was comparable to that of N1 and N1L3. These results suggest that nerolidol increased lipid fluidity by disturbing the rigidly arranged lipid structures. By contrast, levulinic acid had little influence on lipid structure. The combination of nerolidol and levulinic acid exhibited synergistic activity.

Evaluation of Wavenumber Frequency as a Function of Temperature To elucidate the effect of permeation enhancers on intercellular lipids in the stratum corneum, the IR spectrum was investigated at 20 and 100°C (Table 1). At 20°C, W and L3 exhibited identical wavenumbers, whereas N1 exhibited a blue shift, and N1L3 was further blue shifted. At 100°C, L3 exhibited the same wavenumber as N1 and N1L3. The lipid fluidity of L3 and W were similar at 20°C but differed at 100°C. The lipid fluidity of L3 was comparable to that of N1 and N1L3. These results suggest that nerolidol increased lipid fluidity by disturbing the rigidly arranged lipid structures. By contrast, levulinic acid had little influence on lipid structure. The combination of nerolidol and levulinic acid exhibited synergistic activity. To further examine lipid fluidity, the frequency of W was compared with that of N1 or L3 at each temperature. To quantify the effects of the enhancers, we defined a Δν value as follows:

$$\Delta\nu = \left[ \frac{\text{Peak value of N1 or L3 at specified temperature}}{-\text{Peak value of W at the same temperature}} \right]$$
L3 was shifted to a significantly higher frequency compared to W at temperatures above 65°C.

**Effect of Heat Application and Administration of Enhancers on the Shift in Frequency**

To elucidate the effects of permeation enhancers on intercellular lipids in the stratum corneum in detail, different concentrations of individual enhancers were compared. First, the effect of permeation enhancer concentration on lipid fluidity in thermal profiles was investigated. The thermal profiles of W, 0.5% (w/w) nerolidol (N0.5) and 1.0% (w/w) nerolidol (N1) were compared (Fig. 6). The blue shift of peak position in the thermal profiles indicates concentration-dependent changes; the peak position of N1 was higher than that of N0.5 at 20°C. The thermal profiles of W and L were compared in an identical manner (Fig. 7). Levulinic acid concentrations of 3.0% (w/w) (L3) and 9.0% (w/w) (L9) were used. Levulinic acid exhibited the same behavior as W and did not exhibit concentration-dependent changes.

Next, the relationship between lipid fluidity and heat application was investigated. To clarify the effect of temperature corresponding to heat application, the W value was fitted to a logistic function as follows:

$$ Y = \frac{A_1 - A_2}{1 + (x / x_0)^p} $$

where Y is the peak wavenumber and x is the applied temperature. For W, $A_1$ is 2916.9, $A_2$ is 2925.6, $x_0$ is 64.0, and $p$ is 5.4. For L3, $A_1$ is 2916.9, $A_2$ is 2926.9, $x_0$ is 60.5, and $p$ is 7.6. The regression coefficient was 0.998 for W and 0.997 for L3. The peak wavenumber obtained by nerolidol at 20°C was then extrapolated to W. The temperature was 53.1°C for N0.5 and 62.1°C for N1 (Fig. 8). The peak wavenumber obtained by W at 100°C was then extrapolated to L3. The corresponding temperature of W was 72.7°C (Fig. 9). This result indicates that nerolidol disorders lipid structures at physiological temperatures. Administration of nerolidol without heat application resulted in the same lipid fluidity as that obtained by high temperature heat application. By contrast, L3 at low temperature exhibited similar behavior to W, and at high temperature, L3 exhibited higher lipid fluidity than W. This indicates that levulinic acid may increase lipid fluidity in disordered lipid structures at high temperatures.

The orthorhombic and hexagonal structures were not strongly affected by levulinic acid, indicating that levulinic acid has a weak effect on lipid fluidity at low temperatures. At higher temperatures, levulinic acid increased lipid fluidity compared with the water treatment. In addition, in the case of co-administration of 1% nerolidol and levulinic acid, lipid fluidity increased in the 20–40°C range compared with 1%
nerolidol alone. In higher temperatures, the rigidly arranged intercellular lipid structures were strongly disturbed by heat application. On the other hand, in case of co-administration of 1% nerolidol and levulinic acid, nerolidol strongly disturbed the rigidly arranged intercellular lipid structures. Levulinic acid may have an effect on the intercellular lipids in the disturbed states, and the phenomena could be synergistic. Above 40°C, when intercellular lipid structures are disturbed, the effect of heat application on lipid fluidity may be stronger than the effect of enhancers.

Previous studies using synchrotron X-ray diffraction have shown that \( \text{-menthol, a cyclic monoterpene (molecular structure: C}_{10}\text{H}_{20}\text{O}), acts strongly on the orthorhombic and hexagonal structures in the stratum corneum of the skin, and induces liquid crystallization of lipid structure.}^{23} \) In addition, an ATR-FTIR study showed that the fluidity of intercellular lipids increased with the application of \(-menthol, and indicated that the heat application effect occurred at 20°C.\)\(^{24} \) These results are similar to the results reported here with nerolidol. Oleic acid, a long chain monounsaturated free acid (molecular structure: \( \text{C}_{18}\text{H}_{34}\text{O}_{2} \)), does not affect the orthorhombic and hexagonal structures. In addition, the lipid fluidity of stratum corneum treated with oleic acid was same as the stratum corneum treated with water at low temperatures, and shifted higher than the stratum corneum treated with water at high temperatures.\(^{27} \) These results were similar to the thermal profiles of the stratum corneum treated with levulinic acid that are reported here.

**Conclusion**

Synchrotron X-ray diffraction revealed that nerolidol, a sesquiterpene, acts on lipid structures and prevents the formation of high-temperature hexagonal structures with increasing temperature. Furthermore, ATR-FTIR analysis indicated that the effect of nerolidol on increasing lipid fluidity in the stratum corneum is comparable to that of heat application. By contrast, levulinic acid, a short-chain fatty acid, did not act on the lipid structures. However, levulinic acid increased lipid fluidity at high temperature or upon co-administration with nerolidol. These conditions both result in disordered lipid structure, the former from heat application and the latter from the permeation enhancer. Therefore, levulinic acid increases lipid fluidity in the context of disturbed lipid structure. Despite differences in chemical structure, the action of nerolidol is similar to that of \(-menthol,\)\(^{23} \) and the action of levulinic acid is similar to that of oleic acid.\(^{27} \) The combination of nerolidol and levulinic acid may simultaneously minimize the disturbance of lipid structures and increase permeability.

Previous research has shown a relationship between the concentrations of various permeation enhancers and the permeation of diclofenac sodium.\(^{14} \) The rank order of enhancement effect of various permeation enhancers for diclofenac sodium differed depending on the concentration. The interaction between the concentration of enhancers and the permeation rate of the active ingredients is complex. Therefore, additive interactions should be also considered for transdermal therapeutic systems. Further studies should be needed to elucidate the mode of action of enhancements and their interaction with ingredients. These studies would be beneficial for optimizing the enhancement effect and lowering dermal irritation.

**Acknowledgments**

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan-Supported Program for the Strategic Research Foundation at Private Universities, 2014–2018, S1411019. The synchrotron X-ray scattering experiments were performed at BL6A at the Photon Factory under approval of the Photon Factory Advisory Committee (2014G137, 2016G146) and BL40B2 at SPring-8 with the approval of the SPring-8 Proposal Review Committee (2015A1139, 2015B1099, 2016A1209).
Conflict of Interest  The authors declare no conflict of interest.

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