Combined Effect of \textit{d}-Limonene Pretreatment and Temperature on the Rat Skin Permeation of Lipophilic and Hydrophilic Drugs

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The combined effect of \textit{d}-limonene and temperature on the skin permeation of lipophilic and hydrophilic penetrants has been investigated in rats \textit{in vitro}. Prednisolone was used as a lipophilic penetrant, and glucose and isoniazid were used as hydrophilic ones, respectively. When the skin was pretreated with 30\% ethanol without \textit{d}-limonene, the steady state permeability coefficient \((P)\) of every penetrant through the skin was difficult to calculate because of very low permeability. On the other hand, the cumulative amount of each penetrant increased with an increase in temperature when the skin was pretreated with 1.5\% \textit{d}-limonene in 30\% ethanol. The Arrhenius plots of \(P\) values for glucose and isoniazid showed a linear relationship, and the activation energies of skin permeation were estimated to be 87.6 and 66.5 kJ/mol, respectively. When prednisolone was used as a penetrant, however, the Arrhenius plot of \(P\) values exhibited a convex curvature. This may suggest that the combined use of \textit{d}-limonene and temperature effectively changes the barrier structure of the non-polar pathway in the stratum corneum, while no synergistic effect was observed on the polar pathway.

Key words skin permeation; \textit{d}-limonene; temperature effect; enhancement action; Arrhenius plot; percutaneous absorption

Studies on transdermal drug delivery have been performed by many investigators. However, in these studies the rate and the extent of skin permeation were generally low. Therefore, further improvement is required for the practical application of transdermal drug delivery. Recently, we found that terpenes (e.g. \textit{d}-limonene) remarkably enhanced the skin permeation of drugs\(^{1-6}\) when the terpenes coexisted with ethanol. A relatively large amount of ethanol as a co-solvent was required in order to produce a strong activity of \textit{d}-limonene. Too much amount of ethanol in the formulation, however, may cause severe damage to the skin. Thus, another method for improving the enhanced action of \textit{d}-limonene should be developed.

To date, little attention has been paid to the effect of temperature on the enhancement of the permeation of drugs. In the past, several studies have demonstrated that the percutaneous absorption of anti-inflammatory drugs was greatly promoted by increasing the temperature.\(^{7-9}\) Furthermore, the penetration rate of a series of alcohols and the water flux across the skin have been reported to be significantly enhanced with an increase in temperature.\(^{10,11}\) In general, routes of skin penetration were classified into two pathways, \textit{i.e.}, polar and non-polar pathways in the intercellular domain.\(^{12-15}\) However, the synergistic effect of permeation enhancers and temperature on the polar and nonpolar pathway has not yet been clarified. In a previous work,\(^{16}\) we reported the combined effect of \textit{d}-limonene and temperature on the percutaneous absorption of ketoprofen in rats \textit{in vivo} and \textit{in vitro}. As a result, the combined use of \textit{d}-limonene and temperature brought about a pronounced enhancement action on the percutaneous absorption of drugs.

In this study, the synergy of \textit{d}-limonene and temperature on skin permeation of lipophilic and hydrophilic drugs was studied in detail in rats \textit{in vitro} in order to clarify their combined effect on the polar and non-polar pathway of the stratum corneum.

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MATERIALS AND METHODS

Materials The \textit{d}-limonene was of extra pure reagent grade and was purchased from Tokyo Chemical Industries Co., Ltd., (Tokyo, Japan). Prednisolone was purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). D-Glucose (anhydrite) was purchased from Daiichi Pure Chemicals Co., Ltd., (Tokyo, Japan). Isoniazid (isonicotinic acid hydrazide) was purchased from Sigma Co. (St. Louis, MO., U.S.A.). Other chemicals used were of reagent grade.

Skin Permeation Study Full-thickness abdominal skin was excised from male Wistar rats weighing 180—200 g, whose hair had been previously removed with an electric animal clipper. The excised skin was used as a permeation membrane for the \textit{in vitro} study. Two-chamber diffusion cells (available diffusion area, 0.785 cm\(^2\); volume of each half-cell, 3.0 ml) with a water jacket were employed. The donor cell was filled with a pretreatment solution composed of a 30\% ethanol—phosphate buffer solution (pH 7.0) containing 0 or 1.5\% \textit{d}-limonene, and the receiver cell was filled with phosphate buffer solution (pH 7.0). Both cells were stirred by a magnetic stirrer for 2 h. After pretreatment, the solution of donor and receiver cells was removed and washed several times with phosphate buffer solution. Drugs suspended in a 30\% ethanol—phosphate buffer solution were then applied to the donor cell. The receiver cell was again filled with phosphate buffer solution. The drugs in the media were kept in a suspended condition throughout the experiment. \textit{In vitro} permeation was performed at 20—40°C. At appropriate intervals, aliquots (20 \(\mu\)l) were withdrawn from the receiver cell.

Determination of Drugs Concentration Prednisolone: The sample solution (20 \(\mu\)l) in the skin permeation study was thoroughly mixed with methanol (200 \(\mu\)l) containing 1 \(\mu\)g/ml methyl p-hydroxybenzoate as an internal standard. These mixtures were filtered using a disposable filter unit (Eikiruro-Disk 3CR, Gelman Science Japan, Ltd., Tokyo, NII-Electronic Library Service
Japan), and the concentration of prednisolone in the filtrate was determined using an HPLC apparatus (Model LC-3A, Shimadzu Corp., Kyoto, Japan). Ultraviolet detection (Model SPD-6A, Shimadzu Corp., Kyoto, Japan) at 254 nm was employed; the column (4.6 mm x 150 mm) was packed with A-302 S-S 120A ODS (YMC Co., Ltd., Tokyo, Japan); elution was at room temperature with a mobile phase composed of water and methanol (42:58); and the flow rate was 1 ml/min.

Glucose: The concentration of 20 μl aliquots was determined with the Glucose B-test Wako® (Wako Pure Chemical Industries, Ltd., Osaka, Japan) spectrophotometrically at 505 nm using a U-best 30 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

Isoniazid: The sample solution (20 μl) was thoroughly mixed with methanol (200 μl) containing 0.5 μl/ml phthalic acid di-n-butyl ester as an internal standard. These mixtures were filtered using a disposable filter unit, and the concentration of isoniazid in the filtrate was determined using HPLC in the same way described above. Working conditions for isoniazid determination were as follows: ultraviolet detection at 254 nm was employed; the column (4.6 mm x 100 mm) was packed with A-301 S-S 120A ODS (YMC Co., Ltd., Tokyo, Japan); elution was at 60 °C, with a mobile phase (4.4 g of dioctyl sodium sulfosuccinate—sodium salt, 600 ml of methanol and 400 ml of water) which was adjusted to pH 2.5 with 2 N sulfuric acid; and the flow rate was 2 ml/min.

**Solubility Determination** A prednisolone, glucose and isoniazid suspension (including excess amounts of each drug in the 30% ethanol-phosphate buffer solution) was placed in a water bath (20—40 °C) for 24 h with stirring using magnetic stirrer. The sample was then placed in and the upper layer was filtered through a 0.45 μm membrane filter (Gelman Science Japan, Ltd., Tokyo, Japan). The concentrations of prednisolone and isoniazid were determined by the HPLC method described above. The concentration of glucose in the suspension was determined spectrophotometrically with the Glucose B-test Wako®.

**Determination of Apparent Partition Coefficients** Prednisolone (0.5 mg) was dissolved in 100 ml of 30% ethanol-phosphate buffer solution (pH 7.0) containing 0 or 1.5% d-limonene as a solvent. Each of these solutions (1 ml) was agitated with the same volume of isopropyl myristate in a water bath (20—40 °C) for 24 h. Then, the samples were placed in a static condition to separate the two layers. The apparent partition coefficients were determined as the ratio of drug concentration in the isopropyl myristate and the 30% ethanol-phosphate buffer solution. The concentration of prednisolone in both layers was measured by the HPLC method described above.

**RESULTS AND DISCUSSION**

Prednisolone was chosen as a suitable model penetrant which will permeate through the non-polar pathway in skin. Figure 1a shows the effect of pretreatment of the skin with d-limonene and temperature on the permeation of prednisolone. When d-limonene was not included in the pretreatment solution, no permeation was observed at 20—30 °C. At the higher temperature (35—40 °C), a small amount of prednisolone permeated through the skin. On the other hand, the cumulative amount of prednisolone significantly increased, and the lag time tended to be short with elevated applied heat when the skin was pretreated with d-limonene. Such permeation profiles were similar to those obtained with ketoprofen, as reported previously.¹⁶

Glucose and isoniazid were used as hydrophilic penetrants which penetrate across the polar pathway in skin. The combined effects of d-limonene and temperature on the permeation of glucose and isoniazid are shown in Figs. 1b and 1c, respectively. These hydrophilic penetrants barely permeated through skin pretreated with the 30% ethanol-phosphate buffer solution without d-limonene, even though high temperature was applied. However, when the skin was pretreated with d-limonene, the skin permeation enhanced considerably with an increase in temperature.

The steady state flux (J) and permeability coefficient (P) of each penetrant were determined from the slope shown in Fig. 1, the available diffusion area and the solubility in the donor solution (30% ethanol-phosphate buffer solution). The results are summarized in Table I. The permeation parameters of ketoprofen determined in the previous study¹⁶ are also listed in Table I. Isoniazid showed the highest flux among the penetrants due to its high solubility in the donor solution and its small molecular weight. In lipophilic drugs, the permeability of ketoprofen was greater than that of prednisolone. The P values of prednisolone and ketoprofen increased linearly with increasing temperature at the range of 20—30 °C. However, the incremental increase in P values declined

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*Fig. 1. Combined Effect of d-Limonene and Temperature on the Skin Permeation of Prednisolone (a), Glucose (b) and Isoniazid (c)*

*Each point is the mean ± S.D. of 3 determinations. ○, 40 °C; ▲, 35 °C; □, 30 °C; ●, 25 °C; △, 20 °C.*
at the higher temperature (30—40 °C), suggesting that the temperature sensitivity of drug permeation through the non-polar pathway might be lowered at the higher temperature when the skin was pretreated with d-limonene. On the other hand, the $P$ values of hydrophilic penetrants such as glucose and isoniazid increased exponentially as a function of applied temperature at the range of 20—40 °C. These results suggest that the combined use of d-limonene and temperature may differently affect the non-polar and polar pathways in the skin.

In order to clarify the combined effect of d-limonene and temperature on the non-polar and polar pathway, the Arrhenius equation was applied to the $P$ value of each penetrant. The results are shown in Fig. 2. In the case of prednisolone, the Arrhenius plots of $P$ values exhibited a convex curvature (Fig. 2a). Exactly the same result has been observed with ketoprofen, although a good linearity of the Arrhenius plots was obtained when d-limonene was not included in the pretreatment solution.  

The $P$ value is expressed as a function of the diffusion coefficient through the skin, the partition coefficient between the skin surface and the donor solution, and the effective length of diffusion. The apparent partition coefficient of prednisolone between isopropyl myristate and 30% ethanol–phosphate buffer solution was not greatly changed as a function of temperature (0.105 at 20 °C and 0.129 at 40 °C). Furthermore, the partition coefficient of prednisolone was barely affected by the addition of d-limonene (0.104 at 20 °C and 0.128 at 40 °C). Assuming that the effective length of diffusion is constant, the increase in $P$ values with increasing temperature can be attributed mainly to the enlargement of the diffusion coefficient of prednisolone through the skin. This may suggest that the barrier structure of the non-polar pathway in the skin is altered with increasing temperature when the skin is pretreated with d-limonene in 30% ethanol–phosphate buffer solution. On the other hand, a good linearity of the Arrhenius plots was observed in the case of hydrophilic penetrants such as glucose (Fig. 2b) and isoniazid (Fig. 2c): the activation energies of skin permeation were estimated to be 87.6 and 66.5 kJ/mol, respectively. The low activation energy of isoniazid compared with that of glucose is a good reflection of the difference in permeation profiles of these penetrants.

Figure 3 shows the proposed mechanism of the combined effect of d-limonene and temperature on the permeation route of the stratum corneum. When d-
limonene was not included in the pretreatment solution, the structures of both the polar and non-polar pathways were hardly altered, even though higher temperature was applied to the skin. In contrast, the pretreatment of skin with d-limonene brought about a significant increase in drug permeation through the skin. The dense barrier structure of the non-polar pathway in the stratum corneum was altered by the combined use of d-limonene and temperature, and such alteration became greater as a function of increasing temperature. On the other hand, the barrier function of the polar pathway remained at constant over the range of 20—40°C, though the polar route was significantly altered by pretreatment with d-limonene.

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