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Combined effect of *d*-limonene and temperature on the skin permeation of ketoprofen

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Abstract

The effect of temperature on the enhancement action of *d*-limonene on percutaneous absorption of ketoprofen (KPF) was investigated in rats in vivo and in vitro. The apparent penetration rate (R_p) of KPF absorbed from alcoholic hydrogels in rats was estimated based on a pharmacokinetic model, which was derived on the assumption of a constant penetration rate through the skin. The R_p value increased sigmoidally with increase in the temperature applied, i.e., the R_p value was almost constant at the lower temperatures, however, it increased abruptly at the critical temperature (T_c) of applied heat. The T_c value was significantly lowered by increasing the content of *d*-limonene in the hydrogel. The steady-state permeability coefficients (P) of KPF through the skin was determined by employing two-chamber diffusion cells in which the excised rat abdominal skin was mounted. The combined effect of *d*-limonene and temperature on the P value was also clarified. The Arrhenius plots of P values showed a linear relationship when the skin was pretreated with 30% ethanol without *d*-limonene. The activation energy of permeation of KPF through the skin was estimated to be 6.49 kJ/mol. When the skin was pretreated with 1.5% *d*-limonene in 30% ethanol, the Arrhenius plots of P exhibited a convex curvature. Morphological changes of the skin surface were also observed microscopically. The application of heat, along with the synergistic effect of *d*-limonene, may effectively change the dense barrier structure of the stratum corneum.

Key words: Percutaneous absorption; *d*-Limonene; Temperature effect; Enhancement action; Arrhenius plot

1. Introduction

Transdermal drug delivery has been recognized as a desirable route for the administration of many types of drugs. Many reports are available concerning the basic investigation of absorption promoters in order to improve the permeability of drugs through the skin. Recently, we have demonstrated the promoting effects of *d*-limonene and related compounds on the percutaneous absorption of antiinflammatory drugs such as indomethacin (Okabe et al., 1989; Kikuchi et al., 1992), ketoprofen (Okabe et al., 1990), diclofenac sodium (Obata et al., 1990, 1991), and diclofenac (Obata et al., 1993). However, further improvement is required for practical application of transdermal drug delivery systems.

Up to now, little attention has been paid to the applicability of temperature to the enhancement

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of percutaneous absorption of drugs. Stoughton and Fritsch (1963) reported that the percutaneous absorption of aspirin was promoted by an increase in temperature. Blank et al. (1967) determined the penetration rate of a series of alcohols over the temperature range 5–50°C. Sasaki et al. (1986, 1987) demonstrated that the percutaneous absorption of anti-inflammatory drugs was greatly enhanced with increasing temperature. Golden et al. (1987) investigated the effect of temperature on the in vitro water flux across the skin. The structural changes in the stratum corneum lipids were estimated by an increase in temperature.

In this study, the effect of temperature on the enhancing action of *d*-limonene was studied in rats, in vivo and in vitro. Ketoprofen (KPF) was used as a model drug.

2. Materials and methods

2.1. Materials

The *d*-limonene was of extra pure reagent grade and was purchased from Tokyo Chemical Industries Co., Ltd, Tokyo, Japan. KPF was purchased from Sigma Co. (St. Louis, MO, U.S.A.). The carboxyvinyl polymer, marketed as 'Hiviswako 105' ®, cholesterol CII-testWako® and phospholipids B-testWako® were purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan. Other chemicals used were of reagent grade.

2.2. In vivo absorption study

KPF hydrogels for the in vivo study were prepared as previously reported (Okabe et al., 1990). Briefly, KPF (2%) and *d*-limonene (0–1.5%) were dissolved in ethanol. Separately, carboxyvinyl polymer (2%) and triethanolamine (2.5%) were dissolved in purified water. Both solutions were then mixed well to produce hydrogels. The final concentration of ethanol was fixed at 30%. The hydrogels produced were kept for at least 24 h prior to use. Male Wistar rats weighing 180–200 g were used. After anesthetization with carbamic acid ethyl ester solution (25%; 4 ml/kg; i.p.), the rats were secured on their back and the hair on the abdominal skin was gently removed with an electric animal clipper. An absorption cell with a water jacket (available application area, 2.0 cm^2; height, 3 cm) was attached on the shaved skin with cyanoacrylate type adhesives, and then the hydrogel ointments (1.5 g) containing 0–1.5% *d*-limonene were applied under occlusive conditions. The absorption experiments were performed over 8 h at 24–40°C. The temperature of the absorption cell was kept constant during the experiments by the circulation of water at constant temperature into the cell. Blood samples (500 μl) were taken periodically via the jugular vein until 8 h after application. The concentration of KPF in the plasma was determined in the same way as previously reported (Okabe et al., 1990) using HPLC apparatus (Model LC-3A, Shimadzu Corp., Kyoto, Japan) equipped with a variable wavelength UV monitor (Model SPD-6A, Shimadzu Corp., Kyoto, Japan).

2.3. In vitro 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 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 30% ethanol-PBS (pH 7.0) solution containing 1.5% *d*-limonene and the receiver cell was filled with PBS (pH 7.0). Both cells were stirred by a magnetic stirrer for 2 h. After the pretreatment, the solution of donor and receiver cells was removed and washed several times with PBS. The KPF suspended in a 30% ethanol solution was then applied to the donor cell. The receiver cell was again filled with PBS. The drug in the medium was kept in a suspended condition throughout the experiment. In vitro permeation was performed at 20–40°C. At appropriate intervals, aliquots (20 μl) were withdrawn from the receiver cell and analyzed by HPLC.

2.4. Determination of drug solubility

A KPF suspension (including excess amounts of KPF and 30% ethanol) was placed in a water bath (20–40°C) for 24 h with stirring using a magnetic stirrer. The sample was then centrifuged and the upper layer was filtered through a 0.45 μm membrane filter (Gelman Science Japan Ltd, Tokyo, Japan). The concentration of KPF was determined spectrophotometrically at 254 nm using a U-best 30 spectrophotometer (Japan Spectroscopic Co., Ltd, Tokyo, Japan).

2.5. Morphological study of the skin surface

The skin excised from male WBN hairless rats weighing 180–200 g was mounted in the two-chamber diffusion cells as described above, and a 30% ethanol solution containing 1.5% *d*-limonene was introduced into the donor cell. The receiver cell was filled with PBS (pH 7.0). Both cells were stirred by a magnetic stirrer for 2 h at 20–40°C. The skin surface was washed with PBS and pre-fixed with 2% glutaraldehyde for 2 h. After being soaked in 7.5% saccharose buffer solution for 24 h, the skin was again fixed with a 1% osmic acid solution for 2 h. The skin was then dehydrated using a graded series of acetone solutions (60–100%). After drying at the critical point using a critical point dryer (Model HCP-2, Hitachi Ltd, Tokyo, Japan), the skin surface was coated with gold and examined by a scanning electron photomicroscope (Model JSM-T200 Jeol Ltd, Tokyo, Japan).

2.6. Determination of skin lipids leached

The pretreatment of the skin with a 30% ethanol solution containing 1.5% *d*-limonene was performed in exactly the same way as described above in the permeation study. Ceramides leached in the donor solution during the pretreatment were determined according to the method of Lauter and Trams (1962). Cholesterol and phospholipids leached in the donor cell were measured by cholesterol CII-testWako® and phospholipids B-testWako® (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

3. Results and discussion

3.1. Combined effect of d-limonene and temperature on the in vivo percutaneous absorption of KPF

Fig. 1 shows the combined effect of *d*-limonene and temperature on the absorption profiles of KPF through the skin from hydrogels in rats in vivo. When *d*-limonene was not included in the hydrogel, the percutaneous absorption of KPF

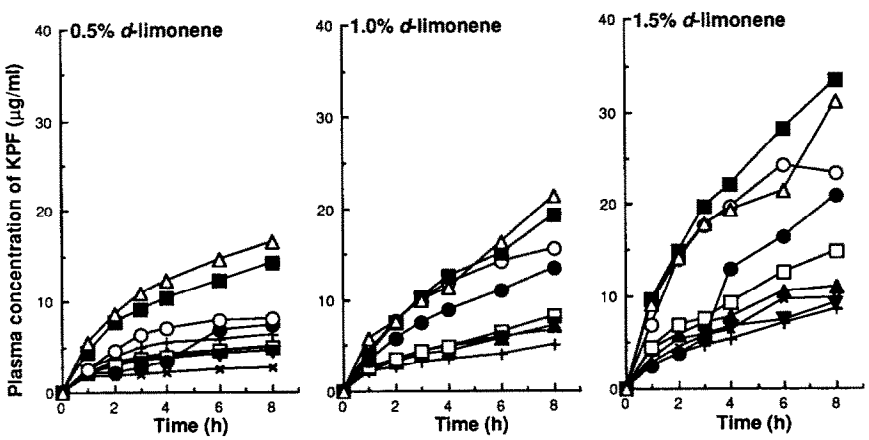

Fig. 1. Combined effect of *d*-limonene and temperature on the percutaneous absorption of KPF in rats. Each point is the mean of 4–6 determinations. (×) 24°C, (▼) 26°C, (+) 28°C, (▲) 30°C, (□) 32°C, (●) 34°C, (○) 36°C, (■) 38°C, (△) 40°C.

was hardly observed. On the other hand, the plasma concentration of KPF was significantly increased when 0.5–1.5% of *d*-limonene was formulated in the hydrogels. Furthermore, the promoting action of *d*-limonene was enhanced significantly by an increase in the applied heat. Thus, the synergistic action of *d*-limonene and temperature can be anticipated on percutaneous absorption in vivo. The pharmacokinetic parameters of KPF percutaneously absorbed from the hydrogels were estimated in order to elucidate the synergy between *d*-limonene and temperature. The pharmacokinetics of percutaneous absorption has been widely discussed and several effective models have been developed for understanding the absorption behavior of drugs through the skin (Naito and Tsai, 1981; Naito et al., 1985). In this study, we employed a simple model on the assumption of a constant penetration rate through the skin after the initial induction period (lag time) (Takayama and Nagai, 1991). The plasma concentration of KPF can be given as follows;

$$C = \frac{R_p}{V_1 k_{10}} \cdot \left\{ 1 + \frac{\beta - k_{10}}{\alpha - \beta} e^{-\alpha(t - t_L)} + \frac{k_{10} - \alpha}{\alpha - \beta} e^{-\beta(t - t_L)} \right\}$$

where C is the plasma concentration, R_p denotes the apparent penetration rate, t is time, t_L represents the lag time, α and β are hybrid first-order rate constants, V_1 denotes the distribution volume of the central compartment and k_{10} is the elimination rate constant from the central compartment. The previously reported pharmacokinetic parameters of KPF after i.v. administration were employed in order to estimate the R_p and t_L values (Takayama and Nagai, 1991). Fig. 2 shows the R_p values of KPF percutaneously absorbed from hydrogels including 0–1.5% *d*-limonene as a function of temperature. When the hydrogel not including *d*-limonene was applied, the effect of temperature on the R_p values was not clearly observed due to the very low penetration of KPF through the skin. On the other hand, the R_p values increased sigmoidally as a function of temperature in the cases of the hydrogels containing 0.5–1.5% *d*-limonene. Specifically, the

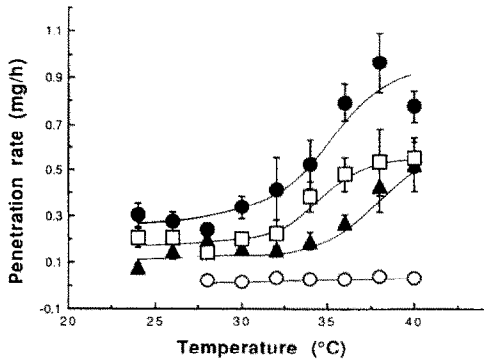

Fig. 2. Relationship between penetration rate and temperature on the percutaneous absorption of KPF in rats. Each point is the mean ± S.D. of 4–6 determinations. (○) Without *d*-limonene, (▲) 0.5% *d*-limonene, (□) 1.0% *d*-limonene, (●) 1.5% *d*-limonene.

R_p values hardly changed at the lower temperatures but increased abruptly at the critical temperature (T_c) as the applied heat was increased. The T_c value was lowered significantly by an increase in the *d*-limonene concentration formulated in the hydrogels.

3.2. Combined effect of d-limonene and temperature on in vitro permeation of KPF

d-Limonene (1.5% in 30% ethanol-buffer solution) was applied to the donor side as a pretreatment solution for 2 h at various temperatures. The results are shown in Fig. 3. The cumulative amount of KPF that permeated through the skin, which was pretreated with *d*-limonene, increased considerably and the lag time tended to be short

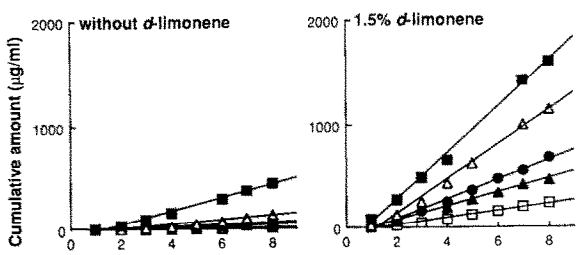

Fig. 3. Combined effect of *d*-limonene and temperature on the percutaneous absorption of KPF in vitro. Each point is the mean of 3 determinations. (□) 20°C, (▲) 25°C, (●) 30°C, (△) 35°C, (■) 40°C.

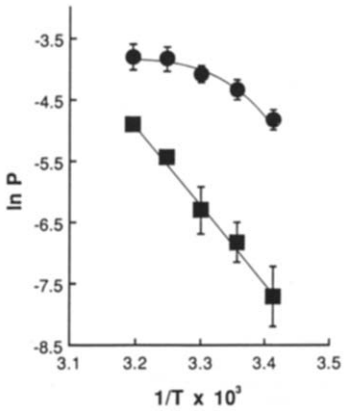

Fig. 4. Arrhenius plots of the steady-state permeability coefficient of KPF. Each point is the mean ± S.D. of 3 determinations. (■) Without *d*-limonene, (●) 1.5% *d*-limonene.

with an increase in temperature. On the other hand, the skin permeation of KPF was not significantly promoted when the skin was pretreated with just 30% ethanol, except for permeation at 40°C. The steady-state permeability coefficient (P) of KPF was determined from the solubility of KPF in the donor solution (30% ethanol-buffer solution) and the slope shown in Fig. 3. Fig. 4 shows Arrhenius plots of the P values. A linear relationship was observed when the skin was pretreated with 30% ethanol without *d*-limonene. The activation energy of permeation of KPF through the skin was estimated to be 6.49 kJ/mol. This value coincided well with those reported in the literature (Blank et al., 1967; Golden et al., 1987). When the skin was pretreated with 1.5% *d*-limonene in 30% ethanol, the Arrhenius plots of the P values exhibited a convex curvature, suggesting that the structure of the skin was altered with the increase in temperature. In general, the P value was expressed as a function of the diffusion coefficient through the skin, the partition coefficient between the skin surface layer and the donor solution, and the effective length of diffusion. Okabe et al. (1990) reported that the solubility of KPF in ethanol-buffer solution and the partition coefficient of KPF between octanol and water were barely affected by the addition of *d*-limonene. Assuming that the effective length of diffusion is constant, the pronounced increase in the P values with increasing temperature was mainly attributed to the enlarge-

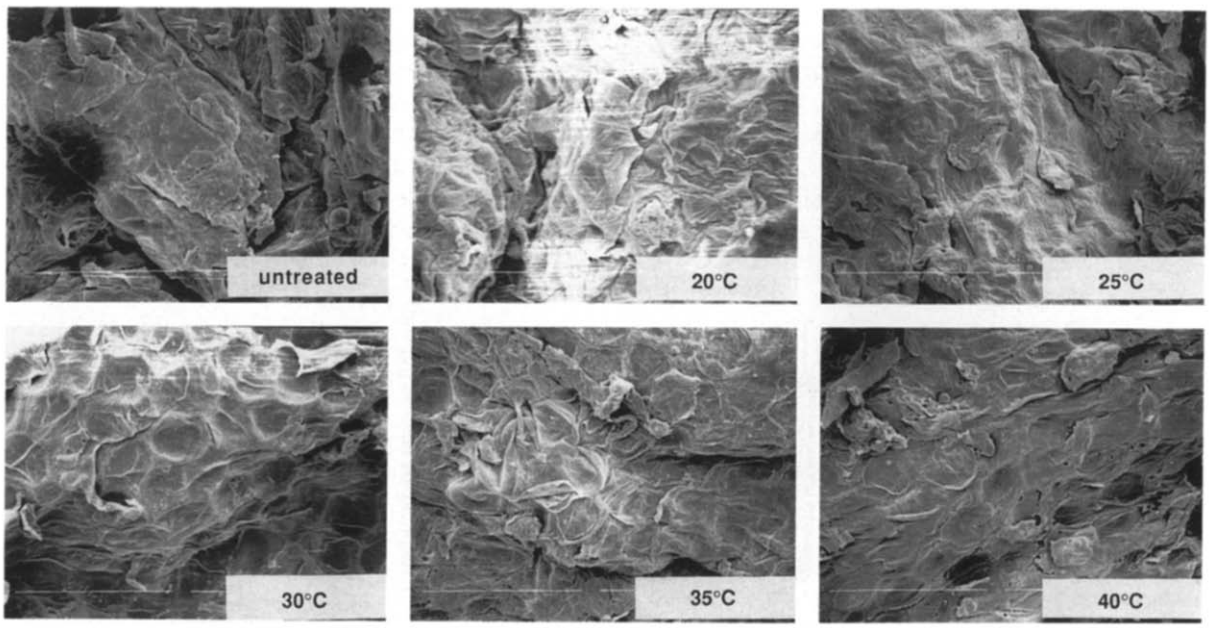

Fig. 5. Scanning electron micrographs of hairless rat skin pretreated with 30% ethanol for 2 h at various temperatures. Magnification, ×500.

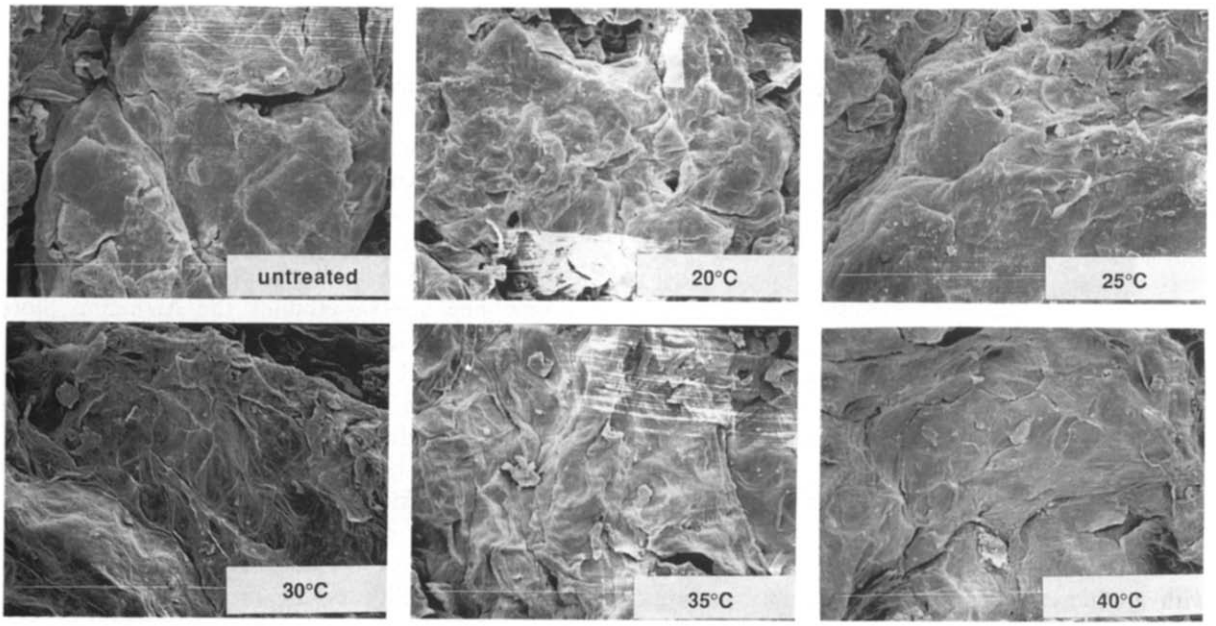

Fig. 6. Scanning electron micrographs of hairless rat skin pretreated with 1.5% *d*-limonene in 30% ethanol for 2 h at various temperatures. Magnification, ×500.

ment of the diffusion coefficient of KPF through the skin. This consideration was also supported by the fact that the lag time was shorted with increasing temperature. Thus, the dense barrier structure of skin could be altered by the combination of *d*-limonene and temperature.

3.3. Morphological changes of the skin surface

Morphological changes of the skin surface were observed microscopically. For the absorption and permeation studies, we employed Wistar rats, however, the skin surface of Wistar rats was covered with thick hair. Therefore, the abdominal skin excised from hairless rats was used for the morphological study. Fig. 5 and 6 show the results of microscopic photographs of the hairless rat skin surface pretreated with or without 1.5% *d*-limonene in 30% ethanol for 2 h. When the skin was pretreated with 30% ethanol without *d*-limonene, little change in the surface structure was observed with increase in temperature. How-

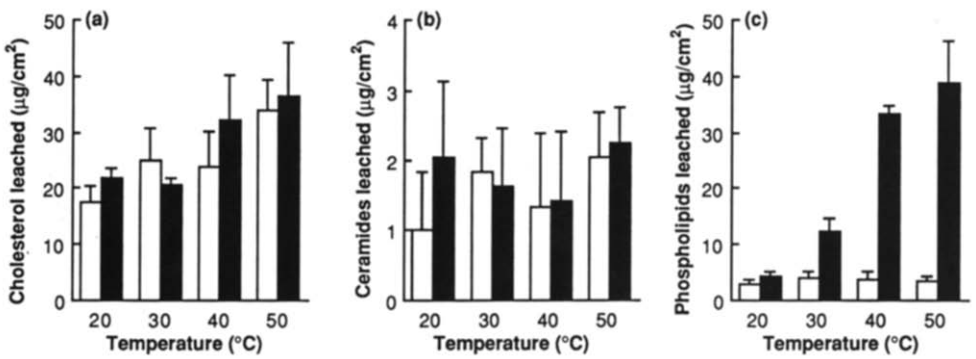

Fig. 7. Amount of (a) cholesterol, (b) ceramides and (c) phospholipids leached after pretreatment for 2 h at various temperatures. Each point is the mean ± S.D. of 3 determinations. (□) Without *d*-limonene, (■) 1.5% *d*-limonene.

ever, when the skin was pretreated with 1.5% *d*-limonene in 30% ethanol at high temperatures (30–40°C), significant changes in the intercellular regions were observed, i.e., the spaces between the cells were extended, and the shape of each cell was clarified with rising temperature.

Fig. 7 shows the amount of lipids leached into the donor side during pretreatment of the skin. The amount of cholesterol leached into the donor side tended to increase as the temperature increased, although the effect of *d*-limonene was not clearly observed. The amount of ceramides leached into the donor side was a fixed quantity within the temperature range 20–50°C. On the other hand, the amount of phospholipids leached into the donor side increased significantly when the skin was pretreated with *d*-limonene in 30% ethanol compared to the pretreatment with 30% ethanol alone. This may suggest that the dense barrier structure of the stratum corneum is partially relaxed by the pretreatment with *d*-limonene and ethanol at high temperature, and thereby small amounts of phospholipids that exist in the viable tissue under the stratum corneum are leached into the donor side, since no phospholipids exist in the stratum corneum (Yardley and Summerly, 1981). However, the ratio of phospholipid amounts leached into the donor side to those contained in the unit area of the skin was less than 9%.

In practice, no significant irritation response was macroscopically observed on the skin surface after the absorption experiment in vivo. We may conclude that the combined use of *d*-limonene and temperature brings about effective changes in the dense barrier structure of the stratum corneum and a pronounced enhancement action on the percutaneous absorption of drugs.

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