Effect of *d*-Limonene on the Amounts of Ethanol and Indomethacin Penetrated from Aqueous Gel Ointments to Rat Skin

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The amounts of *d*-limonene, ethanol and indomethacin (IMC) which were transferred from aqueous gel ointments to the skin were determined in rats. The concentration of IMC in the skin correlated well with the plasma concentration of IMC percutaneously absorbed from the gel ointment. The increase of *d*-limonene concentration in the gel ointments was directly proportional to the accumulation of ethanol in the skin. The amount of ethanol in the skin was closely associated with the percutaneous absorption of IMC. As a possible mechanism for enhancement action of *d*-limonene and ethanol, it was considerd that, at first, *d*-limonene penetrates into the skin under coexistence with ethanol and may change the barrier structure of the stratum corneum. The transfer of ethanol to the skin is thereby enhanced under the coexistence of *d*-limonene in the skin. Thus, the permeation of IMC can be promoted due to its affinity with ethanol.

Keywords percutaneous absorption; *d*-limonene; ethanol; indomethacin; accumulation; skin tissue; binary enhancer system

Introduction

The transdermal route for drug administration is limited by the barrier properties of the stratum corneum.[1] The intercellular region has been identified as the major route of transdermal penetration of drug molecules.[2] For the percutaneous absorption of drugs, it is important to improve the permeation of drugs across the skin. Barrier properties of the stratum corneum may be manipulated by using skin permeation enhancers. Therefore, the possibility of using many sythesized or naturally occurring compounds as effective enhancers has been investigated. Recently, we found that *d*-limonene present in lemon or orange oils remarkably enhanced the percutaneous absorption of indomethacin (IMC),[3] ketoprofen[4] and diclofenac sodium[5] under the coexistence of ethanol. Also, the promoting activity of terpenes for the percutaneous absorption of IMC was quantitatively expressed as a function of the lipophilicity and the molecular weight of terpenes.[6] Synergism of *d*-limonene with ethanol was also significantly observed in the enhancement of the percutaneous absorption of IMC.[6] Essential oils in citrus fruits such as lemon or orange have been widely used as flavoring substances for perfumes, foodstuffs and medicines. The biological safety of these compounds has been well documented.[7] In this study, in order to elucidate in detail the synergism of *d*-limonene with ethanol on the transdermal absorption of IMC, we determined the concentration of *d*-limonene, ethanol and IMC which penetrated into the skin from a gel ointment in rats *in vivo*. A possible mechanism of action for this binary enhancer system was discussed.

Materials and Methods

Materials *d*-Limonene used in this study, extra pure reagent grade, was purchased from Tokyo Kasei Industrial Co., Ltd. IMC was purchased from Sigma Co., Ltd. Carboxyvinyl polymer, marketed as Hiviswako 105, was supplied by Wako Pure Chemical Industries, Ltd. Other chemicals were of reagent grade.

Preparation of Gel Ointments The gel ointments were prepared as follows: IMC (1 g) was dissolved in ethanol (50 g) with *d*-limonene (1—3 g). Separately, carboxyvinyl polymer (2 g) and triethanolamine (2.5 g) were dissolved in distilled water. Both were well mixed and the total amount of the mixture was adjusted to 100 g by the amount of distilled water. The resulting gel ointment was stored at room temperature for 24 h under air-tight conditions prior to use.

Animal Experiment Male Wistar rats weighing 180—200 g were used. After anesthetization with urethane saline solution (25%; 3 ml/kg i.p.), the rats were secured on their backs and the hair on the abdominal skin was gently removed with an electric clipper. Glass cells (16 mm inner diameter, 10 mm height) were attached to the shaved skin with cyanoacrylate-type adhesives. The gel ointment (1.5 g) under test was applied and the glass cell was covered with Parafilm (American Can Company) to prevent evaporation of volatile components in the gel ointment.

Determination of IMC, *d*-Limonene and Ethanol in the Skin At 2 and 8 h after application, the rats were sacrificed with overdosing of ether inhalation, and the gel ointment remaining on the skin surface was wiped off completely (50 times) by using absorbent cotton wetted with distilled water. The application part of the skin was excised with scissors and the skin tissue was cut into small pieces in ethanol (10 ml). The weight of skin was measured beforehand. The samples were shaken at 32 °C for 24 h in order to extract IMC from the skin. After centrifugation, the upper layer was filtered with a disposable filter unit (Gelman Science, Ltd., Ekikuro-Disk 3CR). The filtrate (100 μl) was taken and thoroughly mixed with methanol (250 μl) containing an appropriate amount of *p*-hydroxybenzoic acid *n*-hexylester as an internal standard. The concentration of IMC in the mixture was determined using an HPLC method (Shimadzu Corp., LC-3A): Ultraviolet detection at 254 nm was employed; the column (YMC Pack A-302 S-5 120A ODS, 4.5 × 150 mm, Yamamura Chemical Lab.) was eluted at room temperature with a mobile phase consisting of a mixture of 0.1% phosphoric acid and methanol (25 : 75 in volume ratio); the flow rate was 1 ml/min. Under this condition, the limit of detection of IMC in the skin was less than 20 μg/g. The amount of *d*-limonene in the skin at 2 h after application of the gel ointments was determined using a GC method (Shimadzu Corp., GC-7A): The filtrate described above (1.5 μl) was again used as the sample solution; a hydrogen flame ionization detector was employed; the column (3 mm diameter × 3 m length) was packed with Chromosorb WHP coated with 15% polyester FF (Shimadzu Corp.); the column and injection part temperatures were kept at 105 and 250 °C, respectively; nitrogen was used as a carrier gas; the flow pressures of nitrogen, air and hydrogen were kept at 1.8, 0.5 and 1 kg/cm^2, respectively. The limit of detection of *d*-limonene in the skin was less than 0.05 μl/g. In order to determine the amount of ethanol in the skin, exactly the same experiments described above were performed. The skin was excised at 2 h after application of the gel ointment and ethanol in the skin was extracted with butyl alcohol in the same way as *d*-limonene. The amount of ethanol in the sample solution was determined by the GC method under the same conditions described above. The limit of detection of ethanol in the skin was less than 0.03 μl/g.

Determination of the Solubility of IMC The solubility of IMC was determined in ethanol or *d*-limonene. An excess amount of IMC (1 g) was added to 10 ml each of ethanol and *d*-limonene. The mixture was thoroughly agitated with a magnetic stirrer for 24 h at 30 °C. After centrifugation, the supernatant was filtered with a disposable filter unit (Gelman Science, Ltd., Ekikuro-Disk 13CR). Ethanol and *d*-limonene in the filtrates (100 μl) were evaporated at 70 °C and the residues were dissolved in methanol. The concentration of IMC was then determined using a spectrophotometer (Jasco, Ubest-30). The mean value of 3 determinations was used as the solubility of IMC.

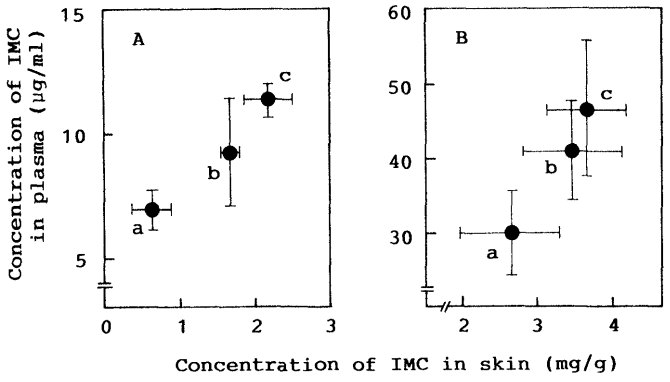

Fig. 1. Relationship between Concentrations of IMC Observed in Skin and in Plasma at 2 h (A) and 8 h (B) after Application of Gel Ointments Containing 1—3% of *d*-Limonene

a, 1% *d*-limonene; b, 2% *d*-limonene; c, 3% *d*-limonene. The concentration of IMC in the skin is represented as the mean ± S.D. ($n=3$). The plasma concentration of IMC is represented as the mean ± S.D. ($n=5$), which was cited from a previous investigation.[6]

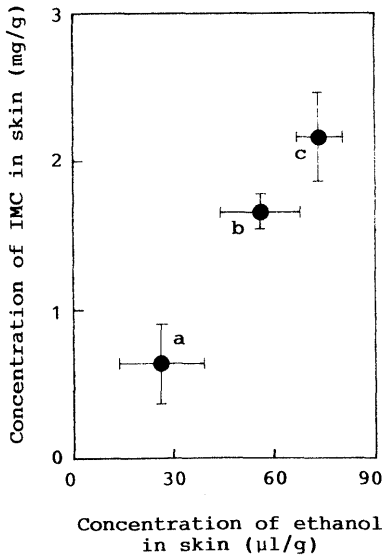

Fig. 2. Relationship between Concentrations of Ethanol and IMC Accumulated in Skin at 2 h after Application of Gel Ointments Containing 1—3% of *d*-Limonene

a, 1% *d*-limonene; b, 2% *d*-limonene; c, 3% *d*-limonene. Each point represents the mean ± S.D. ($n=3$).

Results and Discussion

Figure 1 shows the relationship between IMC concentrations observed in the skin and in plasma at 2 and 8 h after application of the gel ointments containing 1—3% of *d*-limonene. In both skin and plasma, the concentration of IMC increased linearly as a function of *d*-limonene in the gel ointments. The plasma concentration of IMC was nearly proportional to the amount of IMC accumulated in the skin, suggesting that the rate-determining step on the absorption of IMC was the penetration process from the gel ointment to the skin rather than the process from the skin to the blood circulation. The effect of differing concentrations of *d*-limonene formulated in the gel ointment on the accumulation of ethanol in the skin was investigated. Since ethanol and *d*-limonene have high volatility, values measured at the initial stage (2 h) after application were mutually compared. Figure 2 shows the relationship between the amounts of ethanol and IMC accumulated in the skin at 2 h after application of the gel ointments containing 1—3% of *d*-limonene. Increasing the concentration of *d*-limonene in the gel ointments was directly proportional to the transfer of ethanol to the skin. Furthermore, the amount of ethanol accumulated in the skin was closely associated with that of IMC. The solubility of IMC in ethanol (26.1 g/l at 30 °C) was approximately 40 times greater than that in *d*-limonene (0.644 g/l at 30 °C). Therefore, the penetration of IMC from the gel ointment to the skin might be promoted with an increasing amount of ethanol accumulated in the skin. Although the amount of *d*-limonene accumulated in the skin was measured similarly to IMC and ethanol, the evaluation of the data was rather difficult due to its large deviation (data not shown).

Based on the result described above, a possible mechanism for the enhancement action of *d*-limonene and ethanol may be explained as follows: At first, *d*-limonene penetrates into the skin under the coexistence of ethanol and may change the barrier structure of the skin; the transfer of ethanol to the skin is thereby accelerated under the coexistence of *d*-limonene in the skin; thus, the permeation of IMC could be promoted due to its affinity for ethanol. In the future, much work should be done to elucidate the precise mechanism of enhancement of the binary enhancer system consisting of *d*-limonene and ethanol.

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