Effect of Pretreatment of Skin with Cyclic Monoterpenes on Permeation of Diclofenac in Hairless Rat

Yasuako Obata,* Kozo Takayama, Yoshie Maitani, Yoshiharu Machida and Tsuneji Nagai

Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan.

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The promoting effect of d-limonene and l-menthol on the percutaneous absorption of nonionized and ionized diclofenac (DF) was investigated employing a pretreatment method. After the pretreatment of hairless rat skin with an ethanol buffer solution containing terpenes, the permeation study was performed in vitro. The permeability coefficients of nonionized (Pn) and ionized diclofenac (P) were calculated under the assumption that the total flux was composed of individual fluxes of nonionized and ionized DF, in the case of pretreated DF. In the case of pretreated DF, the Pn and P values were increased dramatically. However, the promoting magnitude was not affected by an extension of the pretreatment period. In contrast, when the skin was pretreated with l-menthol, the Pn and P values increased gradually as the pretreatment period increased. Based on the measurement of the solubility of terpenes in the pretreatment solution, the difference in promoting efficiency could arise from the difference in thermodynamic activity of the terpenes.

Keywords nonionized diclofenac; ionized diclofenac; terpene pretreatment; d-limonene; l-menthol; ethanol

Introduction

Transdermal drug delivery is now one of the most attractive routes for the application of drugs and is being watched with keen interest. However, the barrierability of the stratum corneum to foreign substances is the most difficult problem to overcome. An attempt to seek absorption enhancers which act quickly and reversibly to the skin is important for the development of transdermal drug delivery systems.

In previous works,1,2) we have reported the promoting effect of terpenes on the percutaneous absorption of diclofenac sodium (DFS) in vivo. Furthermore, we have investigated the effect of ethanol on skin permeation of nonionized and ionized diclofenac (DF), considering the ionization degree of DF in the donor solution.3) In this study, the effect of terpenes such as d-limonene and l-menthol on the skin permeation of nonionized and ionized DF was investigated employing a pretreatment method in hairless rat skin in vitro. The difference in promoting efficiency of these terpenes was discussed.

Materials and Methods

Materials DFS was generously supplied by SS Pharmaceutical Co., Ltd. DF was obtained by recrystallization of DFS in an acidic medium (0.1N HCl solution). d-Limonene and l-menthol were of extra pure reagent grade and were purchased from Tokyo Chemical Industries Co., Ltd. Other chemicals were of a reagent grade.

Preparation of a Donor Solution Using Pretreatment of Skin and Using Permeation Study A mixture of terpene (1 or 2%), ethanol (40%) and McIlvaine buffer (pH 4 and 6) was used as a pretreatment donor solution. DFS suspended McIlvaine buffer (pH 4 and 6) was used as a donor solution for a permeation study.

Pretreatment of Skin with Terpenes Two-chamber diffusion cells (available diffusion area is 0.785 cm², each half-cell volume is 3.0 ml) with a water jacket (37°C) were used.4) Full-thickness abdominal skin was excised from male hairless rats (WBN rat, body weight 160—180 g, 8 weeks old) immediately after sacrifice, and mounted in the cells. The donor cell was filled with a pretreatment solution containing terpene and ethanol. The receiver cell was filled with a pH 7.2 phosphate buffer. Both cells were stirred by a magnetic stirrer during pretreatment and the permeation experiment.

Skin Permeation Study After the pretreatment (1—5 h), the solution of both donor and receiver cells were removed. The donor cell was rinsed by McIlvaine buffer without DFS several times, and the receiver cell was rinsed by pH 7.2 phosphate buffer. After rinsing, a permeation experiment was started immediately. Excess amounts of DF (about 10-fold of solubility) were added to the McIlvaine buffer. The ionic strength of each donor solution was made constant (0.5 m) using KCl and the pH value of the drug suspension was measured again following saturation. The drug suspension was transferred to the donor cell. The receiver cell was filled with pH 7.2 phosphate buffer. Every 1 h, 0.02 ml samples were taken from the receiver solution and replaced by the same volume of fresh buffer to maintain a constant volume. The concentration of DF in the samples was determined using HPLC.

Determination of Drug Concentration The sample solution (0.02 ml) in the skin permeation study was thoroughly mixed with methanol (0.2 ml) containing an appropriate amount of p-hydroxybenzoic acid n-hexyl ester as an internal standard. The mixture was filtered using a disposable filter unit (Gelman Science Japan, Ltd., Elkaido-Disk 3CR). The DF in the samples was determined using HPLC apparatus (Model 655, Hitachi Ltd.) equipped with a variable wavelength UV monitor. The column was a YMC Packed A-302 S-5 120A ODS 4.6 x 150 mm (Yamamura Chemical Laboratories Co., Ltd.). Elution was done at room temperature with a mobile phase consisting of 0.1% aqueous phosphoric acid—methanol (1:4 in volume) and the flow rate was 1.0 ml/min. The column effluent was monitored at 283 nm.

Determination of the Solubility of Terpenes An excess amount of terpene was added to the mixture solution of ethanol—water, which was then incubated at 37°C for 24 h. After centrifugation, the lower layer was filtrated using a disposable filter unit (Gelman Science Japan, Ltd., Elkaido-Disk 3CR). The ethanol—water mixture was determined using a GC method (Shimadzu Corp., GC-7A), employing a hydrogen flame ionization detector. The column (3 mm diameter x 3 m length) was packed with Chromosorb WH coated with 15% polyester FF (Shimadzu Corp.); the column and injection part temperatures were kept at 150 and 250°C, respectively, in d-limonene determination, and at 180 and 250°C, respectively, in l-menthol determination. Nitrogen was used as the carrier gas; the flow rate of nitrogen and the pressure of air and hydrogen were kept at 40 ml/min, and 0.5 and 1 kg/cm², respectively.

Method of Data Analyses Permeability coefficients of nonionized and ionized forms of DF were determined separately in order to clarify the effect of cyclic monoterpenes on the skin permeation of DF. The following equation can be derived under the assumption that the total flux was composed of individual fluxes of the nonionized and ionized forms.5)

\[ J = P^n C^n + P^i C^i \]  

where \( P^n \) and \( P^i \) are the permeability coefficients of the nonionized and ionized forms, respectively. \( C^n \) and \( C^i \) are the concentration of the nonionized and ionized forms in the donor solution, respectively. These values can be obtained from Henderson-Hasselbalch's equation as follows:

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\[ C' = C^i - C' \]
\[ C' = \frac{C'}{1 + 10^{pK_a - pK_a}} \]

where \( C' \) is a total concentration of DF in the donor solution; DF was suspended in the donor solution; therefore, the total solubility of DF \( (S^i) \) was used as the \( C^i \) value for estimating \( C'^i \) and \( C' \) values (i.e., \( S^i \) and \( S'I \) values). Permeability coefficients of nonionized and ionized DF were determined by Eq. 1 using the experimental values at pH 4 and 6. The total solubility and the \( pK_a \) value of DF were cited from a previous investigation. The values used in this study are as follows: \( S^i = 8.34 \times 10^{-5} \text{m} \) at pH 4; \( S'I = 4.84 \times 10^{-4} \text{m} \) at pH 6; \( pK_a = 4.07 \).

Results and Discussion

\( d \)-Limonene (1% in 40% ethanol-buffer solution) was applied to the donor side as a pretreatment solution for various periods (1—5 h). The results are shown in Fig. 1. The permeability coefficient of nonionized and ionized DF \( (P^i \) and \( P' \) was dramatically enhanced by the pretreatment for 1 h (nearly 10-fold in \( P^i \) and 100-fold in \( P' \) compared with no pretreatment). However, no further increase of \( P^i \) and \( P' \) values was observed when the pretreatment period was extended. Only 1 h of pretreatment was necessary for attaining the maximum level of the promoting effect, suggesting that \( d \)-limonene distributes or attacks the skin surface very quickly, making it easily permeable to both nonionized and ionized DF.

Figure 2 shows the results of pretreatment with \( l \)-menthol (1% in 40% ethanol-buffer solution). \( P^i \) and \( P' \) values gradually increased with the extension of the pretreatment period with \( l \)-menthol. A relatively long pretreatment period was required to obtain a sufficient promoting activity with 1% \( l \)-menthol. This phenomenon was clearly different from the case involving the same experimental condition of \( d \)-limonene.

Figure 3 shows the solubility of terpenes in various concentrations of ethanol. The solubility of \( d \)-limonene in an ethanol–water solution was lower than that of \( l \)-menthol, and \( d \)-limonene could not be dissolved at a concentration of 1% in 40% ethanol. Therefore, the thermodynamic activity of \( d \)-limonene is thought to have already attained a maximum level in this condition. This may be the reason that the promoting efficiency of \( d \)-limonene quickly attains a maximum level. In fact, when the pretreatment was performed at 2% \( d \)-limonene, the promoting action was similar to the result observed at 1% (data not shown). On the other hand, \( l \)-menthol was completely dissolved at the concentration of 1% in 40% ethanol, suggesting that the thermodynamic activity of \( l \)-menthol was lower than the maximum level. When 2% \( l \)-menthol was used as the pretreatment solution, the promoting efficiency of \( l \)-menthol attained a maximum.
level in a short pretreatment period, as shown in Fig. 4. In this condition, the thermodynamic activity of 1-menthol was maximized, because the solubility of 1-menthol in the pretreatment solution was less than 2% (Fig. 3). Interestingly, the promoting efficiency by pretreatment with 2% 1-menthol was almost the same as that observed with 1% d-limonene. This may suggest that the effect of d-limonene on the barrier structure of the skin was essentially the same as that of 1-menthol. The difference in action between d-limonene and 1-menthol was considered to be brought about by the difference in solubility of these compounds in the pretreatment solution.

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