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LIMONENE AND RELATED COMPOUNDS AS POTENTIAL SKIN PENETRATION PROMOTERS

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ABSTRACT

A transdermal therapeutic system can be developed by promoting the skin permeability of drugs with some materials. In this study, the promoting effect of terpenes present in essential oils on the percutaneous absorption of indomethacin from alcoholic hydrogels was investigated in rats *in vivo*. The enhancing activity of terpenes was expressed quantitatively as a quadratic function of lipophilicities of the terpenes, measured as a lipophilic index. Further increase in the significant level was observed when the molecular weight of terpenes was included in this function as the second factor. The synergism of ethanol with *d*-limonene, selected from among the other terpenes, on the promoting activity was statistically investigated. It was evident that both *d*-limonene and ethanol were important factors for promoting the percutaneous absorption of indomethacin. A significant synergism of *d*-limonene with ethanol was also observed. As a possible mechanism for enhancement action of *d*-limonene and ethanol, it was considered 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 with *d*-limonene in the skin. Thus, the permeation of indomethacin can be promoted due to its affinity with ethanol.

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). Barrier properties of the stratum corneum may be manipulated by using skin permeation enhancers. Therefore, the possibility of using many synthesized or naturally occurring compounds as effective enhancers has been investigated. Recently, we have investigated the promoting effect of mono-, sesqui- and diterpenes present in essential oils on the percutaneous absorption of nonsteroidal antiinflammatory drugs such as indomethacin and ketoprofen from the hydrogels in rats (3,4). Monoterpenes such as menthane, limonene, terpinene and terpinolene exhibited a

TABLE 1. Effect of terpenes on the percutaneous absorption of indomethacin from hydrogels in rats.

Terpenes	Plasma concentration (mg/l) 2 h	4 h	6 h	8 h	*AUC* (mg.h/l)
Monoterpenes					
cis-p-menthane	3.2±0.7	11.5±0.8	20.0±0.5	27.9±1.8	97.3±5.8
trans-p-menthane	3.4±0.6	15.1±4.3	27.7±9.7	34.0±8.4	126.4±37.6
d-limonene	5.9±1.8	15.6±3.6	21.9±4.1	29.5±5.5	113.4±23.3
l-limonene	2.9±2.8	12.0±4.7	24.3±3.9	31.2±5.1	109.6±27.9
dl-limonene	3.6±1.3	14.8±2.9	26.9±5.1	31.6±5.1	122.2±23.7
α-terpinene	2.9±1.0	8.3±1.9	11.6±3.5	16.8±6.1	62.4±18.9
terpinolene	5.4±2.3	19.8±5.3	32.6±5.3	46.4±2.7	162.0±28.5
Sesquiterpenes					
α-caryophyllene	-	1.6±0.8	3.0±1.4	6.0±2.2	15.7±7.2
longifolene	-	0.8±0.1	1.8±0.4	3.2±0.9	8.3±1.7
farnesol	-	-	8.9±0.5	13.9±1.6	34.1±2.8
nerolidol	-	-	0.4±0.3	1.5±0.8	3.3±2.8
Diterpenes					
phytol	-	6.8±2.5	11.9±3.4	13.9±3.7	55.5±11.8
isophytol	-	2.4±1.4	6.1±3.5	11.8±3.8	29.1±10.6
geranyl-linalool	1.1±0.2	6.2±1.5	11.1±1.5	15.0±1.5	51.7±7.6
Control					
(without terpene)	-	-	-	-	-

Each hydrogel contains 1% indomethacin, 1% terpene and 50% ethanol. Each value represents the mean±*SD* (*n*=3-5). - indicates that the plasma concentration fell under the limit of detection (< 0.5 mg/l).

pronounced activity on the percutaneous absorption of these drugs under the coexistence of ethanol. A possible mechanism of action for *d*-limonene, which is the main component of orange or lemon oils, was investigated in detail, since this compound is considered to have a pragmatic merit (5).

PROMOTING EFFECT OF TERPENES

The effect of different kinds of terpenes on the percutaneous absorption of indomethacin was investigated in rats, *in vivo* (3,6). Results are given in Table 1. The absorption of indomethacin was remarkably enhanced by the addition of hydrocarbons of cyclic monoterpenes such as menthane, limonene, terpinene and terpinolene (3). On the other hand, no significant promoting effect was observed when monoterpenes containing hydroxyl or carbonyl groups, or ether oxygen were employed (e.g., menthol, menthone or cineole). We also found very strong activity of hydrocarbons such as *d*-limonene on the percutaneous absorption of ketoprofen (4). Barry and Williams reported that 1,8-cineole was the most effective on *in vitro* permeation of 5-fluorouracil through human cadaver skin, while the activity of hydrocarbons was poor (7,8). Eucalyptus oil and camphor

promote the flux of nicotine through excised hairless mouse skin (9). Terpineol and acetyl terpineol promote *in vitro* diffusion of prednisolone through hairless mouse skin (10). Lately we found *l*-menthol to be the most effective absorption promoter among cyclic monoterpenes on the percutaneous absorption of diclofenac sodium from gel ointments in rats (11). Although significant promoting activity was observed when using sesqui- and diterpenes (Table 1), the promoting effect of these compounds was rather poor, when compared with the hydrocarbon monoterpenes.

PHYSICOCHEMICAL PROPERTY OF TERPENES

The lipophilicities of all compounds were measured as a lipophilic index (*LI*) (12). It will be seen that terpenes with relatively high *LI* values exhibited absorption promoting effects, whereas too strong lipophilicity led to the lower promoting activities. Thus, the optimal lipophilicity of the terpenes may exist for their promoting activities. In order to know the quantitative relationship, the area under the curve of plasma concentration of indomethacin versus the application time of the hydrogel for up to 8 h (*AUC*) was calculated as an index for evaluating the absorption of indomethacin. A quadratic equation for the *AUC* values listed in Table 1 was obtained as a function of *LI*.

$$AUC = -649(\pm 273)\,LI^2 + 222(\pm 119)LI + 90.1(\pm 23.5) \quad (1)$$

$$n = 14,\ r = 0.79,\ F_0 = 9.13\ (p<0.01)$$

where r is multiple correlation coefficient, and F_0 is a ratio of mean square regression to mean square residual. Each monomial in Eqn 1 was considered to be significant with significantly high F_0 value, though the r value was not sufficient. When a molecular weight (*MW*) of terpenes was included in Eqn 1 as the second factor, further increase in significant levels was observed.

$$AUC = -474(\pm 288)LI^2 + 174(\pm 114)LI - 8.98(\pm 7.95)\sqrt{MW} + 205(\pm 104) \quad (2)$$

$$n = 14,\ r = 0.86,\ F_0 = 9.25\ (p<0.01)$$

Results predicted by Eqns 1 and 2 are shown in Fig. 1. The optimal value of *LI* was calculated to be 0.17 from Eqn 1 and 0.18 from Eqn 2. It has been generally understood in analogous compounds that the diffusion coefficient of molecules is inversely proportional to the square or cube root of their molecular weights. This may suggest the smaller molecule could easily permeate into the skin. Accordingly, the terpenes with a small molecular weight and an adequate lipophilicity might be desirable for exhibiting promoting activities.

In order to estimate the thermodynamic nature of indomethacin in the hydrogel, the effect of terpenes on the solubility of indomethacin and the partition coefficient of indomethacin between octanol and the buffer solution (pH 7.2) were determined. As for the solubility, a 70% ethanol-water solution was employed instead of the hydrogel, because an accurate determination of solubility in the gel was difficult due to its high viscosity. In addition, a relatively high concentration of ethanol was required in order to dissolve all of the terpenes. Since the solubility and partition

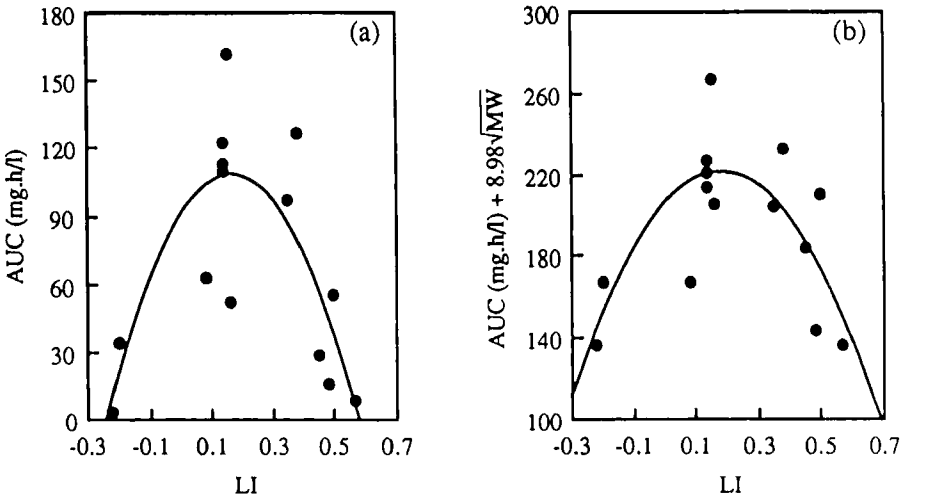

FIGURE 1. Relationship between AUC and physicochemical parameters of terpenes. a) *AUC* was represented as a function of *LI*. b) *AUC* was represented as a function of *LI* and *MW*.

coefficient of indomethacin were hardly affected by the addition of terpenes, the promoting activities observed in several terpenes could arise from direct effects of these compounds on the barrier properties of the skin.

SYNERGISM OF LIMONENE AND ETHANOL

The combined effect of *d*-limonene and ethanol on the promoting activity was investigated in detail. *d*-Limonene was selected from among the other terpenes for pragmatic reasons. It is readily available as the main component in orange or lemon oils, and its toxicity to the skin is considered to be low. On the other hand, the promoting effect of ethanol on the percutaneous absorption of drugs has been reported in the literature (13,14). Fig. 2 shows the effect of differing concentrations of *d*-limonene and ethanol on the percutaneous absorption of indomethacin in rats. When the concentration of *d*-limonene was low in the ointment, plasma levels of indomethacin increased proportionally with an increase in the amount of ethanol. Especially at the higher concentrations of *d*-limonene, a remarkable increase in the plasma level of indomethacin was observed due to the change in ethanol concentration from 30 to 40%. However, any further increase in ethanol concentration (from 40 to 50%) resulted in only a slight enhancement of the percutaneous absorption of indomethacin. The *AUC* values of indomethacin were again calculated from Fig. 2 and visually represented as a function of *d*-limonene and ethanol concentrations as shown in Fig. 3. It will be seen that at least 2% *d*-limonene and 40% ethanol were required to obtain conspicuous promoting effects. In order to elucidate the synergism of *d*-limonene and ethanol statistically, an ANOVA technique was applied to the *AUC* values. As a result, it was evident that *d*-limonene and ethanol were very important factors for enhancing the *AUC* values.

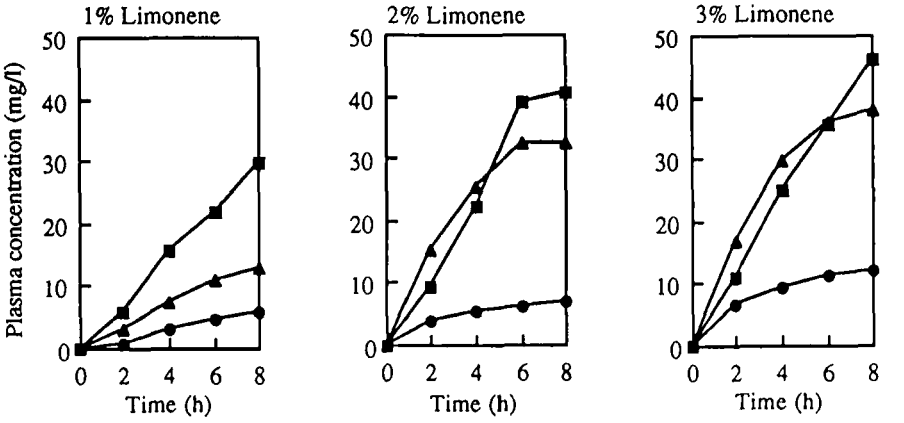

FIGURE 2. Effect of differing concentrations of *d*-limonene and ethanol on the percutaneous absorption of indomethacin from hydrogels in rats.
Concentration of ethanol: ●, 30%; ▲, 40%; ■, 50%. Each point represent the mean (n=5).

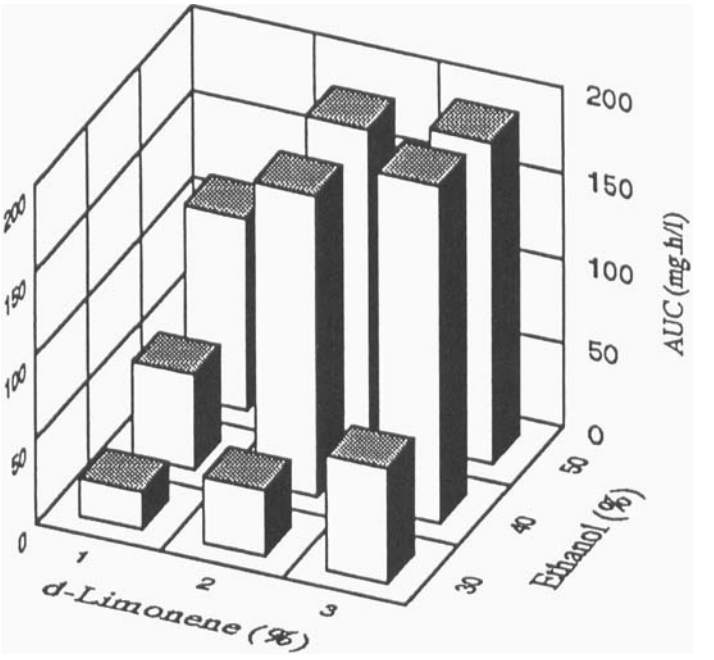

FIGURE 3. Effect of differing concentration of *d*-limonene and ethanol on *AUC* of indomethacin. Each bar represents the mean (n=5).

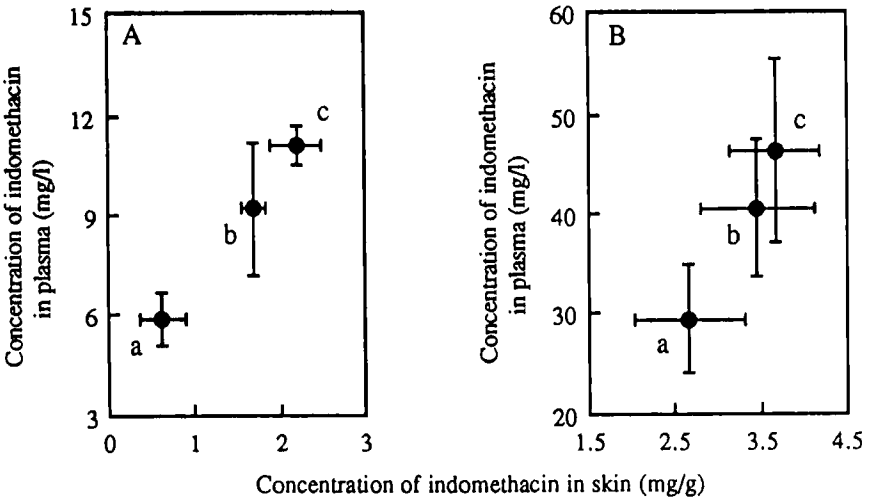

FIGURE 4. Relationship between concentrations of indomethacin observed in skin and in plasma at 2 h (A) and 8 h (B) after application of hydrogels containing 1% indomethacin and 50% ethanol in rats.
a, 1% *d*-limonene; b, 2% *d*-limonene; c, 3% *d*-limonene. The concentration in the skin is represented as the mean±*SD* (n=3). The plasma concentration is represented as the mean±*SD* (n=5).

Although the value of F_0 in the interaction term was somewhat low, the synergism of *d*-limonene and ethanol on percutaneous absorption was significantly observed.

MECHANISM OF PROMOTING ACTION

Fig. 4 shows the relationship between indomethacin concentrations observed in the skin and in plasma at 2 and 8 h after application of the hydrogels containing 1% indomethacin, 1-3% *d*-limonene and 50% ethanol. In both skin and plasma, the concentration of indomethacin increased linearly as a function of *d*-limonene in the hydrogels. The plasma concentration of indomethacin was nearly proportional to the amount of indomethacin accumulated in the skin, suggesting that the rate-limiting step on the absorption of indomethacin was the penetration process from the hydrogel 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 hydrogel 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. Fig. 5 shows the relationship between the amounts of ethanol and indomethacin accumulated in the skin at 2 h after application of the hydrogels. Increasing the concentration of *d*-limonene in the hydrogels 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 indomethacin. The solubility of indomethacin in ethanol at 30°C (26.1

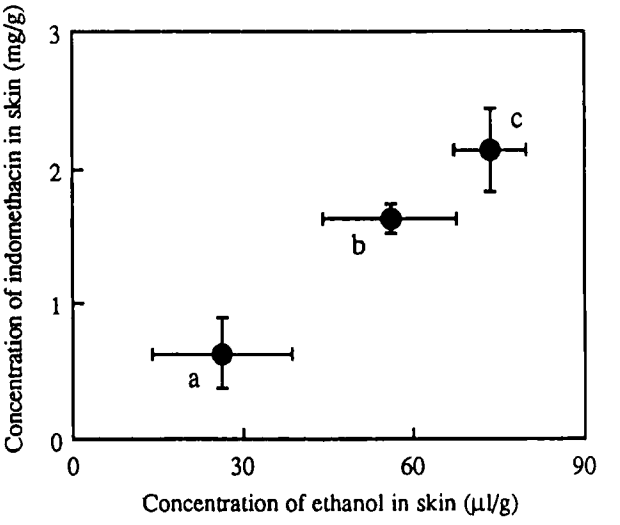

FIGURE 5. Relationship between concentrations of ethanol and indomethacin accumulated in skin at 2 h after application of hydrogels in rats. a, 1% *d*-limonene; b, 2% *d*-limonene; c, 3% *d*-limonene. Each point represents the mean±*SD* (n=3).

g/l) was approximately 40 times greater than that in *d*-limonene (0.644 g/l). Therefore, the penetration of indomethacin from the hydrogel 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 indomethacin and ethanol, the evaluation of the data was difficult due to its large deviation. 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 with ethanol and may change the barrier structure of the skin; the transfer of ethanol to the skin is thereby accelerated under the coexistence with *d*-limonene in the skin; thus, the permeation of indomethacin could be promoted due to its high affinity with ethanol.

Since the intercellular lipids of the stratum corneum provide the principal barrier to the percutaneous absorption (2), primary active site of cyclic monoterpenes is considered as being at the lipid bilayer in the intercellular region of the stratum corneum. The mechanism of promoting action of these terpenes was investigated, employing a phospholipid liposome composed of dipalmitoylphosphatidylcholine (DPPC), dicetyl phosphate (DCP) and cholesterol, as a simple model to represent the intercellular lipid structure in the stratum corneum. DCP gives a negative charge on the surface of liposome and protects a coagulation among the liposome particles. The molar ratio of DPPC, DCP and cholesterol was fixed to be 10:1:2. Structure change of lipid bilayer in the liposomes, which was brought about by the addition of terpenes, was monitored by means of DSC, ESR and fluorescence polarization measurements. As a result, enhancing activity of terpenes was correlated well with the transition temperature or transition enthalpy change, suggesting that terpenes, which have strong effect on the liposome membrane, bring about a strong enhancing action on the percutaneous absorption of indomethacin. From the measurements of ESR and fluorescence polarization,

fluidizing effect of terpenes on the lipid bilayer structure was observed when the liposome contained sufficient amount of cholesterol (≥20 mol%) as a membrane component. More hydrophobic compounds such as *d*-limonene among the terpenes showed stronger effect on the fluidity of the lipid bilayer.

CONCLUSION

Hydrocarbons of cyclic monoterpenes such as *d*-limonene exhibited a very strong activity on the percutaneous absorption of indomethacin under the coexistence of ethanol. The enhancing activities of terpenes were quantitatively predicted as a function of lipophilic index of these compounds. When a molecular weight of terpenes was included as the second factor in this function, further increase in significant levels was observed. It may suggest that the terpenes with a small molecular weight and an adequate lipophilicity are desirable for exhibiting promoting activities. The combined effect of *d*-limonene and ethanol was significantly observed, and 2% *d*-limonene and 40% ethanol was close to the optimal condition which will provide an excellent promoting effect and safety to the skin. A possible mechanism for the enhancement action was explained as follows: At first, *d*-limonene penetrates into the skin under the coexistence with ethanol and may change the barrier structure of the skin: the transfer of ethanol to the skin is thereby accelerated under the coexistence with *d*-limonene in the skin; thus, the permeation of indomethacin could be promoted due to its affinity with ethanol. Primary active site of terpenes was considered as being at the lipid bilayer in the intercellular region of the stratum corneum. More hydrophilic compounds such as *d*-limonene among the terpenes showed stronger effect on the fluidity of the lipid bilayer.

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