Comparative Analysis of Percutaneous Absorption Enhancement by *d*-Limonene and Oleic Acid Based on a Skin Diffusion Model

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Percutaneous absorption-enhancing effects of *d*-limonene and oleic acid were investigated using three model drugs with different lipophilicities in *in vitro* diffusion experiments with guinea pig skin. Pretreatment of the skin with *d*-limonene resulted in a large penetration enhancement for the lipophilic butylparaben (BP) and amphiphilic 6-mercaptopurine (6-MP) but had little effect on the hydrophilic mannitol (MT). Oleic acid caused a large effect only on 6-MP penetration. The penetration profiles were analyzed with a two-layer skin diffusion model consisting of stratum corneum with polar and nonpolar routes and viable epidermis plus dermis. Through curve-fitting, six parameters corresponding to drug diffusivity and partitioning in these three regions of the skin were obtained, and the mechanisms of enhancers were assessed in comparison with those of 1-geranylazacycloheptan-2-one (GACH) reported previously. Increased penetration was caused mainly by modification of the barrier property of the nonpolar route in the stratum corneum in all cases. In the nonpolar route, *d*-limonene increased mainly drug diffusivity, while GACH enhanced predominately drug partitioning. On the other hand, oleic acid moderately increased both parameters.

KEY WORDS: percutaneous absorption; percutaneous penetration enhancer; *d*-limonene; oleic acid; skin diffusion model.

INTRODUCTION

To study the mechanism of skin penetration enhancement (1–3), we have constructed a two-layer skin diffusion model consisting of stratum corneum with polar and nonpolar routes and viable dermis plus epidermis (4,5). This analysis is based on Fick's diffusion theory since drugs passively permeate through the skin. Together with numerical analysis, it enables us to evaluate drug absorption in terms of diffusivity and partitioning in each absorption route. The improved model can account for the relationship between the enhancer effects and drug lipophilicities, which was not possible with a homogeneous two-layer model (5).

We have already reported the enhancement mechanism of 1-geranylazacycloheptan-2-one (GACH) (5), which was developed as the most potent and safe enhancer among nine azacycloalkanone derivatives with alkyl or alkenyl (terpene) chains (6). Based on the diffusion model analysis, GACH was found to enhance the skin penetration of drugs by increasing their partitioning into the nonpolar route of the stratum corneum. In the present investigation, we compared the action mechanisms of two known absorption enhancers, *d*-limonene (7–10) and oleic acid (11–16), using the same analytical method. Mannitol (MT), 6-mercaptopurine (6-MP), and butylparaben (BP) were used as model drugs with highly hydrophilic (MT), amphiphilic (6-MP), and highly lipophilic (BP) properties, respectively.

MATERIALS AND METHODS

Materials

d-Limonene was purchased from Wako Pure Chemical Industry Ltd., Japan. Oleic acid (Nissan Extra Oleic 99) was kindly supplied by Nippon Oil & Fats Co., Ltd., Japan. Mannitol (MT), 6-mercaptopurine (6-MP), and butylparaben (BP) were obtained from Nacalai Tesque Inc. (MT, 6-MP) and Tokyo Chemical Industry (BP), Japan. Radiolabeled ^{14}C-MT and ^{14}C-BP were obtained from Daiichi Pure Chemicals, Japan, and ^{14}C-6-MP was purchased from Commissariat a L'Energie Atomique, France.

In Vitro Percutaneous Penetration Experiment

The skin diffusion experiment was carried out using a flow-through-type diffusion cell as described previously (6). After removal of hair with electric clippers and adherent adipose tissue, full-thickness dorsal skin of a male guinea pig (Hartley strain, weighing approx 250 g) was punched out into 3-cm-diameter disks (four pieces from each animal) and mounted on the diffusion cell with the epidermis side facing the donor cell (exposed area, 3.14 cm^2). This apparatus was kept at 37°C in a water bath throughout the experiment.

The mounted skin was pretreated with 0.2 mL of ethanol, dissolving various amounts of enhancers for 24 hr. In the control study, the skin was pretreated with only ethanol. After removal of residual ethanol from the skin surface by evaporation with a hair dryer, a 1-mL aliquot of drug solution (1 m*M*) with radiolabeled compound (18 kBq) was applied to the donor cell. The donor solution was not stirred during the experiment. The dermis side of the skin was continuously washed with saline containing streptomycin sulfate (50 mg/L; Sigma Chemical Co., MO) and penicillin G potassium salt (30 mg/L; Toyo Jozo, Japan) at a flow rate of 6 mL/hr and the receptor fluid was collected every 90 min for 24 hr. The donor cell was capped with a silicone stopper. At the end of the diffusion experiment, the drug remaining in the donor cell and in the skin was recovered as described previously (6).

Tape-stripping of the skin was repeated 15 times until the skin surface glittered. The skin excised and mounted on the diffusion cell was pretreated with saline for 24 hr. In this case, the drug was applied in the form of a saline solution to prevent the osmotic convective flow from donor to receptor. Other experimental procedures were the same as for the intact skin.

The radioactivities in the receptor fluid and the donor

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solution were determined by liquid scintillation counter (Aloka LSC-900, Japan). The skin sample was measured after solubilization with Soluene-350 (Packard Instruments, the Netherlands).

Data Analyses

Analysis was carried out based on a two-layer skin diffusion model consisting of two serial layers, the stratum corneum layer and the underlying epidermis plus viable tissue layer. Parallel polar and nonpolar routes were also considered for the stratum corneum. The polar route in the stratum corneum was assumed to be filled with water (17,18). A well-stirred condition in the donor solution was assumed because the diffusion of drug in water is much faster than that in the skin. Sink conditions in the receptor phase were assumed, due to continuous washing of the dermal side. The Laplace transforms for the amount of drug appearing in the receptor across the intact and tape-stripped skins are expressed as follows (2,4):

$$\bar{Q}_{int} = Z_d X_0 (Z_{np}\sinh d_p + Z_{np}\sinh d_{np})/s/g(s) \quad (1)$$

$$\bar{Q}_{st} = Z_d X_0/s/k(s) \quad (2)$$

where s is the Laplace operator with respect to time and X_0 is the initially applied dose.

$$d_p = L_s(s/D_p)^{1/2} \quad (3)$$

$$d_{np} = L_s(s/D_{np})^{1/2} \quad (4)$$

$$d_d = L_d(s/D_d)^{1/2} \quad (5)$$

$$Z_p = K_p V_p/d_p \quad (6)$$

$$Z_{np} = K_{np} V_{np}/d_{np} \quad (7)$$

$$Z_d = K_d V_d/d_d \quad (8)$$

$$\begin{aligned} g(s) = {} & V_v(Z_p\cosh d_p\sinh d_{np}\sinh d_d + Z_{np}\sinh d_p\cosh d_{np} \\ & * \sinh d_d + Z_d\sinh d_p\sinh d_{np}\cosh d_d) \\ & + Z_p\{Z_p\sinh d_p\sinh d_{np}\sinh d_d + Z_{np}\sinh d_d \\ & * (\cosh d_p\cosh d_{np} - 1) + Z_d\cosh d_p\sinh d_{np}\cosh d_d\} \\ & + Z_{np}\{Z_{np}\sinh d_p\sinh d_{np}\sinh d_d + Z_p\sinh d_d \\ & * (\cosh d_p\cosh d_{np} - 1) + Z_d\sinh d_p\cosh d_{np}\cosh d_d\} \end{aligned} \quad (9)$$

$$k(s) = V_v\sinh d_d + Z_d\cosh d_d \quad (10)$$

where V_v is the volume of vehicle: D_i, K_i, and V_i (i = p, np, or d) are the diffusion coefficients in the i domain, the partition coefficient between the i domain and the vehicle, and the effective volume of the i domain for diffusion; and subscripts s, p, np, and d denote the stratum corneum, the polar route, the nonpolar route, and the second layer, respectively. V_i is obtained from the area (A), area fraction of the polar route (f), and diffusional pathlength (L_i) as

$$V_p = AfL_s \quad (11)$$

$$V_{np} = A(1 - f)L_s \quad (12)$$

$$V_d = AL_d \quad (13)$$

The penetration profiles obtained in this study were analyzed based on this model. Curve-fitting of Eq. (1) or (2) to penetration data was conducted using the nonlinear regression program MULTI(FILT) (19) on the mainframe computer M-382 at the Kyoto University Data Processing Center. Because of the difficulty in determining the real diffusional pathlength, we defined two parameters for drug diffusion and partitioning involving diffusional pathlength as follows:

$$D_i' = D_i/L_i^2 \quad (14)$$

$$K_i' = K_i V_i \quad (i = \text{p, np, or d}) \quad (15)$$

The six targeted hybrid parameters for each drug were determined according to the following procedures: First, D_d' and K_d' values were obtained from fitting Eq. (2) to a penetration profile through the tape-stripped skin. Second, the penetration profiles of MT at various doses of enhancers through the intact skin were analyzed using Eq. (1) under the assumption that MT, a highly hydrophilic compound, penetrated only the polar domain in the stratum corneum (K_{np} = 0). Since partitioning from the aqueous vehicle to the polar route (water channel) is considered to be one for all penetrants (20), the obtained parameters were treated as the common ones for all tested drugs at the same enhancer dose. On the other hand, diffusion parameters in the polar route of the tested drugs were corrected based on their molecular weights (21). Finally, parameters corresponding to the nonpolar route (D_{np}' and K_{np}') were estimated by fitting Eq. (1) to the corresponding penetration profiles with the four predetermined parameters obtained above.

RESULTS

Effect of Enhancers on Skin Penetration of Drugs in the Diffusion Experiment

The representative permeation profiles of amphiphilic 6-MP obtained in the diffusion experiment are illustrated in Fig. 1. Table I summarizes the amounts of drugs recovered in the receptor phase, the skin, and the donor phase at the end of the 24-hr diffusion experiment. Only higher doses of *d*-limonene (192, 288, 384 μmol) could improve the penetration of 6-MP through the intact skin, by about six to eight times. Thus, the effect of *d*-limonene on 6-MP penetration has a threshold. On the other hand, oleic acid showed penetration enhancement linearly up to 13-fold depending on its dose.

Results of the diffusion experiment for highly hydrophilic MT are also summarized in Table I. Oleic acid actually had no effect on MT irrespective of the preloading dose. *d*-Limonene also showed a little activity to improve MT permeation.

The permeation profiles of BP are illustrated in Fig. 2. *d*-Limonene enhanced BP penetration through the intact skin depending on its pretreatment doses. The penetration pattern obtained with the highest dose of *d*-limonene in intact skin is nearly the same as that obtained in stripped skin. Oleic acid did not change BP penetration profiles at the doses which caused a significant effect on 6-MP.

The remaining amounts of drugs in the skin at the end of the diffusion experiment are in proportion to their lipophilicities (Table I). *d*-Limonene and oleic acid did not change the remaining amounts, compared to the control (ethanol alone).

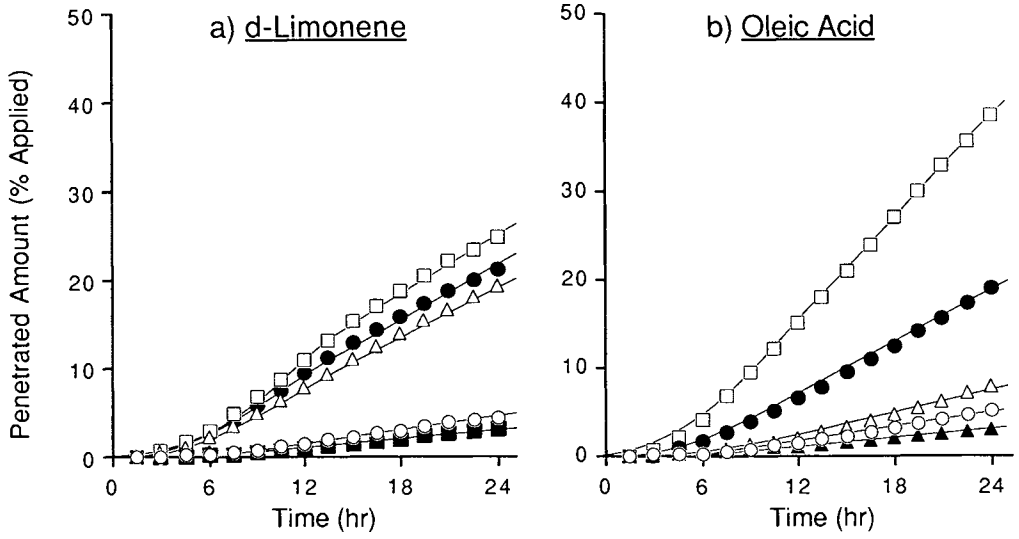

Fig. 1. Penetration profile of 6-MP through intact guinea pig skin pretreated with ethanolic solutions of 0 (▲), 96 (○), 144 (■), 192 (△), 288 (●), and 384 (□) μmol of *d*-limonene and 0 (▲), 7.8 (○), 15.7 (△), 31.4 (●), and 62.7 (□) μmol of oleic acid. Each point represents the mean value of at least three experiments. The connected lines were calculated from the parameters listed in Table II.

Calculation of Diffusion and Partition Parameters

We analyzed all permeation profiles of drugs based on the two-layer skin diffusion model with parallel routes. Estimated parameters for each drug are summarized in Table II. The previous results for GACH are also shown in this table to compare the enhancing mechanisms of the three enhancers. All three enhancers affected mainly the parameters representing drug transport through the nonpolar route. Concerning the effects on the nonpolar route in the stratum corneum (Fig. 3), *d*-limonene increased predominantly the diffusion parameter, approximately 15-fold, while GACH enhanced mainly partitioning, up to 60-fold. Oleic acid increased both diffusion and partition parameters by six- to sevenfold.

DISCUSSION

The mechanism of percutaneous penetration enhancers has been studied by spectroscopic observations (22–26), and using structure–activity relationships (6,11,23,27). However, it remains difficult to understand fully drug penetration under coadministration with an enhancer. We focused our attention on the combined study of penetration routes of drugs and the mechanism and site of action of enhancers, because these factors are dominant determinants of drug penetration enhancement. The present skin model enables us to dissect drug penetration into distinct processes. Since all penetration processes occur by passive diffusion, we can discuss the effect of enhancers in terms of drugs diffusivity and partitioning for each route of drug transport.

Guinea pig skin was used as a model skin under standardized experimental conditions, such as age and anatomical site. Although permeabilities of most drugs through guinea pig skin are higher than those through human skin (28), the basic mechanism of drug permeation is expected to be similar between the two species.

In this study, *d*-limonene and oleic acid as well as GACH (5) showed large enhancements for the amphiphilic 6-MP but had little effect on the hydrophilic MT. Enhancer doses required to achieve 20% penetration of an applied 6-MP dose at 24 hr were about 30 μmol for oleic acid, 15 μmol for GACH (5), and 200 μmol for *d*-limonene. Analysis based on the diffusion model indicated that they influenced mainly the nonpolar route of the stratum corneum. However, the three enhancers caused distinct effects on drug transport through the nonpolar route.

The nonpolar route of the stratum corneum is implicated as the action site of enhancers, since penetration profiles through stripped skin were not affected by enhancers (data not shown), and skin penetration of MT, a marker of the polar route of penetration, was also hardly changed by any enhancers. The latter result contradicts the finding of Barry *et al.* (29) that penetration of MT was enhanced 130-fold in oleic acid/propylene glycol-treated skin. However, the present result was obtained by pretreatment only with oleic acid, and propylene glycol could play an important role in penetration enhancement (30). In addition, 24-hr pretreatment with ethanol (control condition) might already accelerate MT penetration to some extent (1) and mask the effect of oleic acid in this study.

Although the lipophilic BP is expected to permeate the nonpolar route better than the amphiphilic 6-MP, the measured enhancement effect on BP was lower than that on 6-MP. This is explained by a difference in the rate-limiting step for BP and 6-MP; the stratum corneum is not rate-limiting for BP penetration but the viable epidermis plus dermis layer is. Thus, enhanced BP penetration through the stratum corneum has little effect on the total penetrated drug amount. However, *d*-limonene showed some enhancement effect for BP, suggesting that *d*-limonene remarkably increased drug permeation through the stratum corneum at high doses.

In our analysis, oleic acid as well as GACH increased the partition parameter in the nonpolar route of the stratum

Table I. Amount of Drugs Recovered at the End of the Skin Diffusion Experiment[a]

Drug enhancer (μmol)	Drug recovery (%)				N
	Receptor	Skin	Donor	Total	
MT					
None (control)	0.53 ± 0.04	1.1 ± 0.2	96 ± 0	97 ± 0	3
d-Limonene					
96	0.27 ± 0.14	2.2 ± 0.3	96 ± 0	98 ± 1	4
144	2.6 ± 0.8	3.9 ± 1.3	87 ± 4	94 ± 4	3
192	2.4 ± 1.4	3.2 ± 0.9	86 ± 5	91 ± 3	5
288	2.2 ± 0.9	5.0 ± 0.4	86 ± 5	93 ± 4	3
384	2.5 ± 0.3	3.7 ± 1.9	77 ± 8	83 ± 6	3
Oleic acid					
7.8	0.25 ± 0.12	2.3 ± 0.6	92 ± 5	95 ± 6	4
15.7	0.25 ± 0.04	2.8 ± 0.2	95 ± 2	98 ± 2	3
31.4	0.68 ± 0.41	3.8 ± 1.1	94 ± 2	98 ± 3	3
62.7	0.32 ± 0.15	3.1 ± 0.9	89 ± 7	92 ± 7	5
Stripping	52 ± 10	4.5 ± 2.5	28 ± 14	84 ± 9	3
6-MP					
None (control)	3.0 ± 1.6	6.7 ± 2.1	91 ± 4	101 ± 4	3
d-Limonene					
48	2.8 ± 0.3	5.3 ± 2.0	85 ± 3	93 ± 3	3
96	4.5 ± 0.2	8.5 ± 1.5	84 ± 3	97 ± 4	3
144	3.3 ± 0.8	6.6 ± 1.0	79 ± 9	89 ± 8	3
192	19 ± 8	7.0 ± 2.0	64 ± 11	91 ± 5	4
288	21 ± 2	7.0 ± 1.0	65 ± 5	93 ± 5	3
384	25 ± 2	7.3 ± 1.2	58 ± 4	90 ± 7	3
Oleic acid					
7.8	5.2 ± 1.9	6.3 ± 1.0	80 ± 2	92 ± 2	3
15.7	7.8 ± 2.3	8.2 ± 1.5	76 ± 5	92 ± 4	4
31.4	19 ± 5	6.7 ± 3.5	66 ± 5	91 ± 5	3
62.7	38 ± 5	7.1 ± 1.5	42 ± 8	87 ± 2	3
Stripping	57 ± 8	8.3 ± 2.3	24 ± 6	89 ± 3	4
BP					
None (control)	21 ± 3	49 ± 3	18 ± 5	88 ± 5	3
d-Limonene					
96	25 ± 3	58 ± 5	12 ± 2	95 ± 3	3
144	27 ± 6	53 ± 6	8.8 ± 0.7	88 ± 3	4
192	29 ± 3	52 ± 4	10 ± 1	91 ± 5	3
288	34 ± 3	46 ± 4	10 ± 4	90 ± 4	3
Oleic acid					
7.8	21 ± 4	56 ± 10	14 ± 2	91 ± 8	5
15.7	22 ± 1	58 ± 7	14 ± 2	94 ± 6	3
31.4	22 ± 2	52 ± 4	14 ± 1	88 ± 4	3
62.7	20 ± 4	52 ± 9	14 ± 2	86 ± 6	3
Stripping	38 ± 3	26 ± 6	9.2 ± 1.6	74 ± 3	4

[a] Means ± standard deviations.

corneum. Contrary to this result, several groups reported that the action mechanism of these types of enhancers correlated with a change in drug diffusivity in the lipid layer. Oleic acid (22–25) and Azone (26) treatment cause an increase in fluidity of the lipid phase of the stratum corneum, i.e., in acyl-chain disorder. On the other hand, incorporation of Azone into the lamellar bilayer phase was shown to lead to a marked increase in the ability of the lipid phase to take up water (31). In addition, skin treated with oleic acid might be able to retain much more water because of the formation of a permeable interfacial defect within the stratum corneum lipid bilayers, i.e., phase separation (15). These phenomena should be closely related to a change in fluidity of the lipid layer. We reported previously that even ethanol treatment (control) accelerated drug penetration by increasing its diffusivity (1) and that GACH treatment further increased drug partitioning into the nonpolar route of the stratum corneum in a way similar to that of Azone (6). The analysis based on linear-energy relationships suggested that the increase in drug partitioning was explained by increasing polarity of the nonpolar route of the stratum corneum with GACH treatment (5). These findings indicate that the enhancing mechanism of oleic acid and GACH is attributable at least in part to a decrease in lipophilicity of the nonpolar route due to increased water uptake. The analysis based on the skin diffusion model supports this hypothesis.

Although drug diffusivity was accelerated severalfold by pretreatment with ethanol alone (1), *d*-limonene was

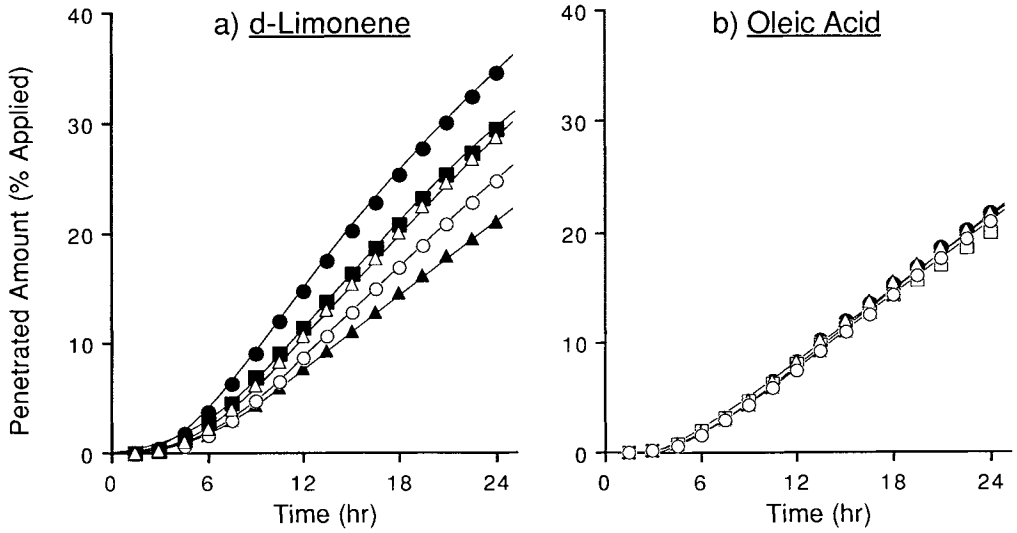

Fig. 2. Penetration profile of BP through intact guinea pig skin pretreated with ethanolic solutions of 0 (▲), 96 (○), 144 (■), 192 (△), and 288 (●) μmol of *d*-limonene and 0 (▲), 7.8 (○), 15.7 (△), 31.4 (●), and 62.7 (□) μmol of oleic acid. Each point represents the mean value of at least three experiments. The connected lines were calculated from the parameters listed in Table II.

shown here to increase drug diffusivity further. The penetration-enhancing effect of *d*-limonene was not linear with dose, and large quantities of *d*-limonene were necessary for enhancement. Further, the lag time of the penetration profiles was shortened by *d*-limonene. *d*-Limonene possesses a single-ring structure which might cause a disruption of normal lipid packing, but its effect on acyl-chain disorder is unclear. In addition to an increase in diffusivity, the effect of *d*-limonene on BP penetration was also attributed to a gradual decrease in partitioning into the nonpolar route in our analysis. In a finite dosing system, decreased partitioning of highly lipophilic drug to the nonpolar route of the stratum corneum leads to an acceleration of penetration (5). Consequently, *d*-limonene is proposed to accelerate drug diffusivity and decrease partitioning (increase in lipophilicity of the nonpolar route). In the former case, reorganization in systems of skin lipids and enhancer should occur at doses higher than the threshold. The latter means that *d*-limonene does not increase water uptake and acts just as an inert hydrophobic solvent.

In this study, diffusivity and partitioning were evaluated using hybrid parameters [Eqs. (14) and (15)]. Therefore, the

Table II. Parameters for Percutaneous Absorption of Drugs[a]

Enhancer (μmol)	MT		6-MP		BP	
	D_p' (hr^{-1})	K_p' ($\times 10^6$ cm^3)	D_{np}' (hr^{-1})	K_{np}' ($\times 10^4$ cm^3)	D_{np}' (hr^{-1})	K_{np}' (cm^3)
None (control)	40	7.3	3.5	4.8	0.19	2.0
d-Limonene						
96	39	3.7	6.4	4.8	0.17	1.4
144	42	33	3.6	2.8	0.26	1.1
192	40	25	41	4.5	0.26	0.86
288	42	32	46	4.7	0.81	0.59
384	47	38	54	5.5		
Oleic acid						
7.8	36	3.9	3.5	10	0.18	2.2
15.7	38	3.5	4.1	13	0.24	2.3
31.4	43	8.4	11	16	0.25	2.0
62.7	46	3.8	25	27	0.41	2.4
GACH						
3.2	49	7.0	3.5	7.6	0.068	13
6.4	42	20	3.4	14	0.066	27
12.7	41	13	2.9	42	0.034	51
25.5	50	22	3.5	370	0.094	79
51.0	48	90	4.2	430		

[a] Parameters of penetration through viable dermis were $D_d' = 0.081\ hr^{-1}$ and $K_d' = 0.69\ cm^3$ for MT, $D_d' = 0.060\ hr^{-1}$ and $K_d' = 1.0\ cm^3$ for 6-MP, and $D_d' = 0.037\ hr^{-1}$ and $K_d' = 1.5\ cm^3$ for BP. The data for GACH are cited from Ref. 5.

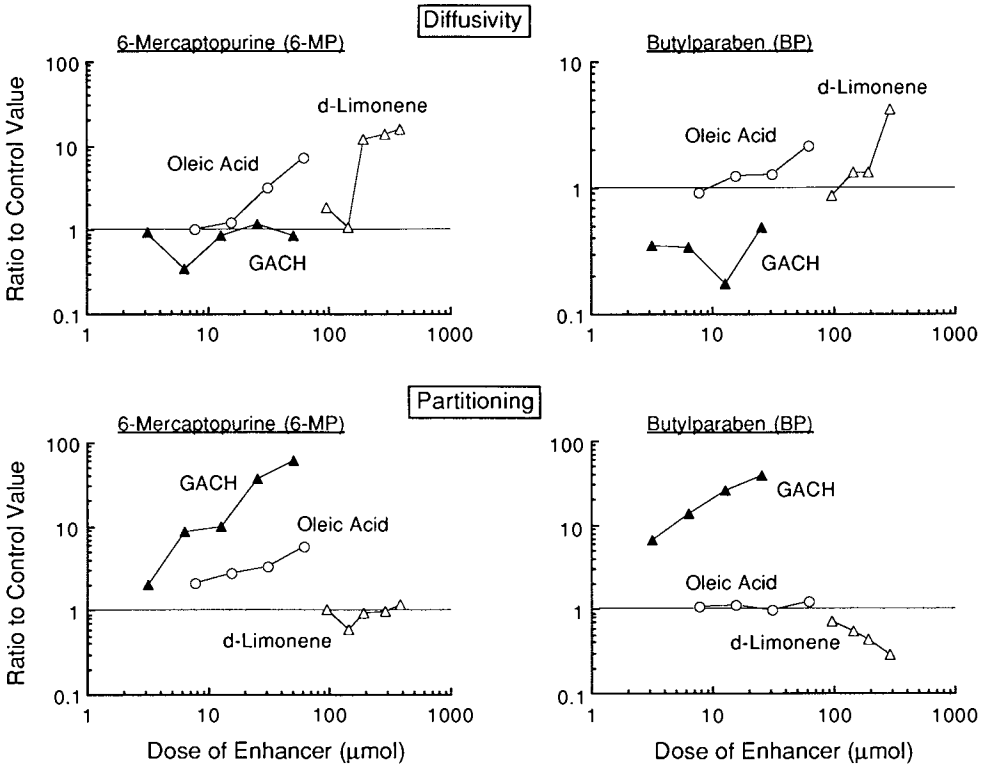

Fig. 3. Comparison of diffusion and partition parameters of 6-MP and BP for nonpolar-route penetration in the stratum corneum. Each value represents the ratio to the control condition. (△) *d*-Limonene; (○) oleic acid; (▲) GACH.

changes in diffusion length and volume of each absorption route may also be included in these parameters. However, it is difficult to evaluate these elemental parameters, since the real diffusion length is not necessarily consistent with the anatomical thickness. For GACH, the increased K_{np}' value was attributed to an increase in the partition coefficient between the nonpolar route and the aqueous vehicle because it is hardly possible that the volume of the nonpolar route increases by two orders of magnitude. On the other hand, the small increase in the K_p' value by enhancers may be due to enlargement of the polar route (the increase in its volume) concomitant with perturbation of the lipid structure, since the polar route is considered to be a water-filled region (17,18).

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