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Permeability of benzoic acid derivatives in excised guinea pig dorsal skin and effects of L-menthol

Shuji Kitagawa *, Akio Hosokai, Yoshiyuki Kaseda, Nobuya Yamamoto, Yoh Kaneko, Emiko Matsuoka

Niigata College of Pharmacy, Kamishin'ei-cho 5-13-2, Niigata 950-21, Japan

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Abstract

Penetration through excised guinea pig dorsal skin was examined for nine mono-substituents of benzoic acid derivatives. Permeability coefficients of the derivatives correlated well with their *n*-octanol/water partition coefficients. Since the regression coefficient was similar to the value obtained in human skin, it is suggested that the lipid lamellae of guinea pig skin resembled that of human skin in lipophilicity. Addition of penetration enhancer, 1% L-menthol in 15% ethanol markedly increased the flux and permeability coefficients of relatively hydrophilic derivatives and decreased the dependency of the permeability coefficients on the partition coefficients. Electron spin resonance analysis using 5-doxylstearic acid revealed the presence of a strongly immobilized component of the spin label in the skin and its disappearance in the presence of 1% L-menthol in 15% ethanol. These results suggest that the rigid lamellar structure of the stratum corneum was disrupted by L-menthol with ethanol, and caused the enhancement of penetration of relatively hydrophilic benzoic acid derivatives. © 1998 Elsevier Science B.V.

Keywords: Benzoic acid derivatives; Skin penetration; Partition coefficient; Penetration enhancer; ESR

1. Introduction

Skin is a promising route for drug administration because the hepatic first pass effect can be avoided, good compliance can be anticipated and side effects can be reduced. However, since prompt penetration is essential to transdermal drug application, only a few drugs are administered in this way. For prompt skin permeation lipophilicity is essential. In the study of skin drug penetration, guinea pigs as well as hairless mice and rats are often used as experimental animals. With such animals it is important to reveal the dependency of drug permeabilities on lipophilicity to establish their suitability as a model of human skin. To determine whether guinea pig skin is a

* Corresponding author.

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good model, we first examined the dependency of drug permeability in excised guinea pig dorsal skin on *n*-octanol/water partition coefficients by using nine mono-substituents of benzoic acid derivatives, with *n*-octanol/water partition coefficients ranging from 0.68 to 2.29, as model drugs. We compared the results with the recent findings in human skin (Potts and Guy, 1992b; Barratt, 1995).

To improve the skin permeabilities of drugs, penetration enhancers have been applied. L-Menthol is a candidate for such an enhancer (Obata et al., 1991; Sugibayashi et al., 1995). However, its efficacy seems to be changed by the lipophilicity of the drugs (Kitagawa et al., 1997). Since quantitative relationships between the drug skin permeability coefficients in the presence of enhancers and *n*-octanol/water partition coefficients of the drugs are unknown, we examined the change in the correlation of the partition coefficients when 1% L-menthol in 15% ethanol was used as a penetration enhancer.

As a mechanism of the enhancement by L-menthol, perturbation of the lipid lamellae in the stratum corneum have been suggested (Walker and Smith, 1996). However, the exact mechanism is still not clear. Therefore, in this work we also studied the mechanism of the enhancement of this system by measuring electron spin resonance (ESR) spectra of 5-doxylstearic acid labeled skin. Spin labeling is a good method for obtaining information on the structure and physical state of stratum corneum lipid lamellae. By observing ESR spectra the presence of lipid structures with different physical states can be found and the effects of skin penetration enhancers on the structure and physical properties of the lipid lamellae can be made clear (Quan and Maibach, 1994, 1995).

2. Materials and methods

2.1. Materials

Benzoic acid derivatives were purchased from Wako (Osaka, Japan) and Tokyo Chemical (Tokyo, Japan). 5-Doxylstearic acid was obtained from Aldrich (Milwaukee, WI).

2.2. Measurement of in vitro skin penetration

In vitro skin penetration of drugs was examined as described previously (Kitagawa et al., 1995). Full thickness dorsal skin was excised from male guinea pigs and subcutaneous fat and other extraneous tissues were trimmed. The skin was then mounted in a two-chamber diffusion cell with a water jacket (37°C). The available diffusion area was about 0.65 cm^2, and each half-cell volume was about 5.4 ml. The donor cells were filled with saline either in the presence or absence of 1% menthol in 15% ethanol and the receiver cells with phosphate buffered saline (pH 7.4). Pretreatment was carried out for 12 h with stirring by a magnetic stirrer. After washing both cells, the suspension of benzoic acid derivatives in saline either in the presence or absence of the penetration enhancer was added to the donor cells and the penetration experiment was started. 150 μl of sample was taken from the receiver cells periodically and analyzed by HPLC (2-Cl and 4-NO_2 derivatives) or UV absorbance (other derivatives). The derivatives used in this study were stable during the experiment. The permeability coefficient, K_p, was calculated from the initial straight portion of the penetration curve, dC_R/dt according to Eq. (1).

$$K_p = \frac{dC_R}{dt} \cdot \frac{V_R}{A} \cdot \frac{1}{C_d} \quad (1)$$

where C_R and V_R are the concentration of benzoic acid derivatives in the receiver compartment and the volume there, respectively, C_d is the concentration of the derivatives in the donor compartment which is equal to their solubility, and A is the diffusion area. The solubility of each benzoic acid derivative was determined from the concentration of the filtrate of a nitrocellulose type membrane filter (pore size 0.45 μm), which was calculated by measuring UV absorbance or by HPLC analysis. The latter was performed with HPLC (L-6000, Hitachi, Tokyo, Japan) equipped with an L-4000 UV detector

Table 1
Logarithm values of *n*-octanol/water partition coefficients (log P_{oct}), MW, solubility (C_d), flux (J) and permeability coefficients (K_p) of benzoic acid derivatives

Substituent	log P^a_{oct}	MW	C_d (μmol ml^{-1})	J^b (μmol cm^{-1} h^{-1})	K^b_p ($\times 10^{-2}$cm h^{-1})
4-NH_2	0.68	137.14	52.0	0.54 ± 0.16	1.04 ± 0.31
2-NH_2	1.21	137.14	32.7	0.33 ± 0.05	1.01 ± 0.15
2-NO_2	1.46	167.12	49.5	0.90 ± 0.04	1.82 ± 0.07
H	1.87	122.12	31.2	2.79 ± 0.54	8.95 ± 1.73
4-NO_2	1.89	167.12	2.29	0.16 ± 0.05	6.82 ± 2.15
2-Cl	2.05	156.57	17.1	1.44 ± 0.31	8.43 ± 1.82
2-CH_3	2.18	136.15	7.20	0.85 ± 0.30	11.8 ± 4.2
2-OH	2.26	138.12	24.0	2.27 ± 0.14	9.46 ± 0.59
4-CH_3	2.29	136.15	3.80	0.65 ± 0.19	17.2 ± 5.0

[a] Data are cited from Leo et al., 1971 and Sotomatsu et al., 1993.
[b] Data are means $\pm$ S.D. for three experiments.

(Hitachi). Separation was achieved on a reversed phase column (ODS, Shodex C18-5A, 4.6 mm i.d., 250 mm long) using a mobile phase consisting of either acetonitrile with 10 mM phosphate buffer (pH 2.5) (30:70, v/v) for the 2-Cl derivative or methanol with 10 mM phosphate buffer (pH 2.5) (55:45, v/v) for the 4-NO_2 derivative.

2.3. Measurement of electron spin resonance spectra

The epidermis of the dorsal skin was incubated at 37°C in the presence or absence of 1% L-menthol in 15% ethanol for 2 h. After removing the medium, 200 μl 50 μM 5-doxylstearic acid solubilized in phosphate buffered saline was added on to the surface of the epidermis (area 0.65 cm^2) and incubated at 37°C for 2 h. The dermis side of the skin was incubated with phosphate buffered saline at 37°C. Then the spin label solution left on the epidermis was removed and the skin was dried under a nitrogen gas stream. Slices of the spin-labeled skin samples were inserted into a quartz cell (Labotech, Tokyo) and ESR spectra were measured with a TE-200 (X-band) spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation frequency and 0.2 mT modulation amplitude at an out-put power of 8 mW. Order parameter and apparent rotational correlation time was obtained from the spectra as described (Kitagawa et al., 1990).

3. Results

3.1. Skin absorption of benzoic acid derivatives

At first we examined the absorption of nine mono-substituents of benzoic acid derivatives from suspension in saline. As shown in Table 1, the flux of benzoic acid was the largest. The low flux value for 4-NO_2 derivative seems to be due to its low solubility in saline. We calculated the permeability coefficients of the derivatives and examined relationships with the *n*-octanol/water partition coefficients. The relations between logarithm values of the permeability coefficients, K_p, and logarithm values of *n*-octanol/water partition coefficients, P_{oct}, and molecular weights, MW, of the derivatives were analyzed as shown in Eqs. (2) and (3), because *n*-octanol/water partition coefficients and molecular weights are reported to be determinants of drug skin permeability (Potts and Guy, 1992a).

$$\log K_p = 0.819 \log P_{oct} - 2.760 \quad (n = 9, r = 0.944, s = 0.16, F = 57.1) \qquad (2)$$

$$\log K_p = 0.812 \log P_{oct} - 0.00393\ \mathrm{MW} - 2.178 \quad (n = 9, r = 0.955, s = 0.14, F = 31.4) \qquad (3)$$

Here, *n* is the number of the derivatives tested, *r* is the correlation coefficient, *s* is the S.D., and *F* is the ratio between regression and residual variances. As shown in Fig. 1 and Eq. (2), a linear

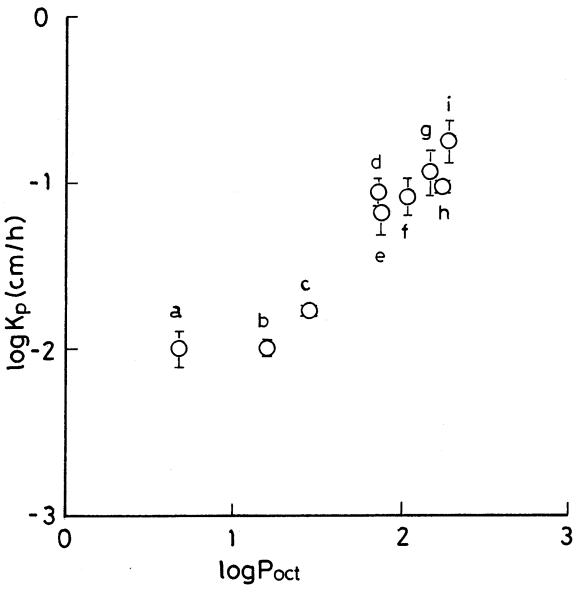

Fig. 1. Relationship between *n*-octanol/water partition coefficients, P_{oct}, of benzoic acid derivatives and their permeability coefficients, K_p, through guinea pig dorsal skin. Substituents of benzoic acid: a, 4-NH_2; b, 2-NH_2; c, 2-NO_2; d, H; e, 4-NO_2; f, 2-Cl; g, 2-CH_3; h, 2-OH; i, 4-CH_3.

correlation between the logarithm values of the permeability coefficients and the *n*-octanol/water partition coefficients was observed. Since differences of molecular weight among the derivatives examined here were small, a good correlation was observed even without consideration of the term for molecular weights shown in Eq. (3). The chemical structure of the derivatives did not have any significant effect on the permeability coefficients.

3.2. Effects of L*-menthol on skin absorption of benzoic acid derivatives*

It has been revealed that cyclic mono-terpenes such as L-menthol enhance transdermal drug penetration (Obata et al., 1991; Sugibayashi et al., 1995). The enhancing effect of L-menthol is known to depend on ethanol concentration (Obata et al., 1991). To avoid the delipidization of the stratum corneum at a high percentage of ethanol (Hatanaka et al., 1992), we examined the effect of 1% L-menthol in 15% ethanol on skin absorption of the benzoic acid derivatives.

As shown in Table 2, in the presence of the enhancer, the flux of all the derivatives tested increased, especially those with low *n*-octanol/water partition coefficients. That is, the flux of 4-NH_2, 2-NH_2 and 2-NO_2 derivatives increased more than 10-fold, whereas that of 2-CH_3, 2-OH and 4-CH_3 derivatives increased only about 2-fold. The solubilities of the latter derivatives were increased by about 2-fold, whereas those of the former were increased only slightly. Permeability coefficients of the relatively hydrophilic derivatives also increased more than 10-fold. On the other hand, no significant increase of permeability

Table 2
Solubility (C_d), flux (J) and permeability coefficients (K_p) of benzoic acid derivatives in the presence of 1% L-menthol and 15% ethanol

Substituent	C_d (mM) (μmol ml^{-1})	J[a] (μmol cm^{-2} h^{-1})	K_p^a ($\times 10^{-2}$cm h^{-1})
4-NH_2	62.8	7.22 ± 0.57***	11.5 ± 0.9***
2-NH_2	35.4	6.12 ± 0.50***	17.3 ± 1.4***
2-NO_2	56.6	12.9 ± 0.7***	22.8 ± 1.2***
H	43.6	8.81 ± 1.74**	20.2 ± 4.0*
4-NO_2	4.79	1.04 ± 0.07***	21.8 ± 1.4**
2-Cl	28.1	5.51 ± 1.35*	19.6 ± 4.8*
2-CH_3	13.9	2.18 ± 0.21**	15.7 ± 1.5
2-OH	43.8	6.70 ± 1.18*	15.3 ± 2.7
4-CH_3	6.88	1.41 ± 0.16*	20.5 ± 2.3

[a] Data are means ± S.D. for three experiments.
Statistical significances of differences of values from control values shown in Table 1 were determined by Student's *t*-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

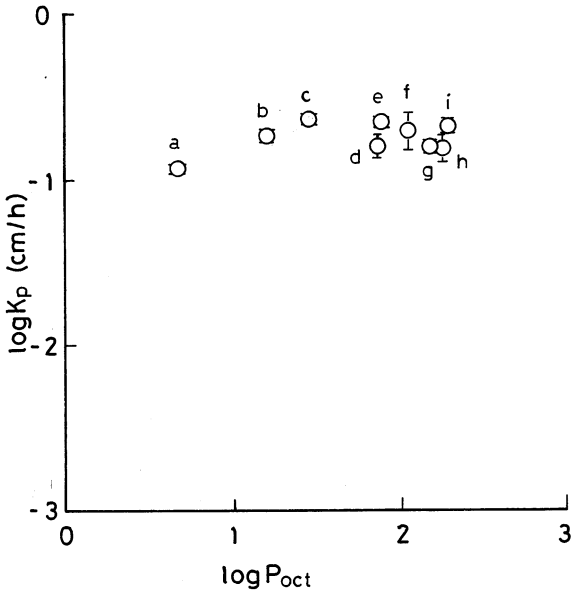

Fig. 2. Effects of 1% L-menthol in 15% ethanol on the relationship between *n*-octanol/water partition coefficients, P_{oct}, of benzoic acid derivatives and their permeability coefficients, K_p, through guinea pig dorsal skin. Substituents of benzoic acid: a, 4-NH_2; b, 2-NH_2; c, 2-NO_2; d, H; e, 4-NO_2; f, 2-Cl; g, 2-CH_3; h, 2-OH; i, 4-CH_3.

coefficients was observed for the lipophilic derivatives. Therefore, the dependency of the permeability coefficients on lipophilicity markedly decreased because of the marked enhancement of absorption of the hydrophilic derivatives by L-menthol. As shown in Fig. 2 and Eqs. (4) and (5), no significant correlation was observed between permeability and *n*-octanol/water partition coefficients. Addition of the square term of $\log P_{oct}$, often observed as a parabolic relationship between the permeability and $\log P_{oct}$ (Tayar et al., 1991), significantly improved the correlation as shown in Eq. (6).

$$\log K_p = 0.073 \log P_{oct} - 0.875$$
$$(n = 9, r = 0.419, s = 0.09, F = 1.49) \quad (4)$$

$$\log K_p = 0.075 \log P_{oct} + 0.00288\ \mathrm{MW} - 1.295$$
$$(n = 9, r = 0.634, s = 0.07, F = 2.02) \quad (5)$$

$$\log K_p = 0.955 \log P_{oct} - 0.288(\log P_{oct})^2 - 1.459$$
$$(n = 9, r = 0.875, s = 0.05, F = 9.82) \quad (6)$$

3.3. Effect of L-menthol on the ESR spectra

It has been suggested that absorption enhancement of drugs by cyclic monoterpenes is due to the perturbation of the stratum corneum lipid lamellae (Hashida and Yamashita, 1995). To clarify the enhancement mechanism by L-menthol, we investigated the effect of 1% L-menthol in 15% ethanol on the electron spin resonance spectra of 5-doxylstearic acid labeled skin. The result in the absence of the enhancer shown in Fig. 3a indicates the presence of a weakly and a strongly immobilized component of the spin labels. The order parameter calculated from the strongly immobilized component was 0.656 ± 0.009 ($n = 3$). This value reflects the rigid environment of the spin label. Therefore, it is possible that this immobilized component of the spectrum represent the spin labels in the stratum corneum lipid lamella which has been suggested to be in a rigid state (Potts et al., 1991). On the other hand, the result shown in Fig. 3b indicates the disappearance of the strongly immobilized component by incubation with this enhancer. Only the signal of the weakly immobilized component, whose rotational correlation time was $2.07 \pm 0.14 \times 10^{-9}$ s, was observed.

4. Discussion

The results shown here indicate that the permeability of benzoic acid derivatives is well controlled by *n*-octanol/water partition coefficients. The results suggest that transfer of these compounds through the stratum corneum lipid lamella is a limiting step in the skin permeation of these compounds as has been suggested for other drugs. The slope of $\log P_{oct}$ represents the lipophilic nature of the skin. The value in guinea pig skin given by Eq. (3) (about 0.81) was similar to the values in human skin (0.74 by Potts and Guy (1992b) for 89 chemicals and 0.769 by Barratt (1995) for 91 chemicals). The value was smaller than that in shed snake skin (1.11 by Itoh et al. (1990b) for 13 chemicals) which has been suggested to be a good model for human stratum corneum (Itoh et al., 1990a). Therefore, it is sug-

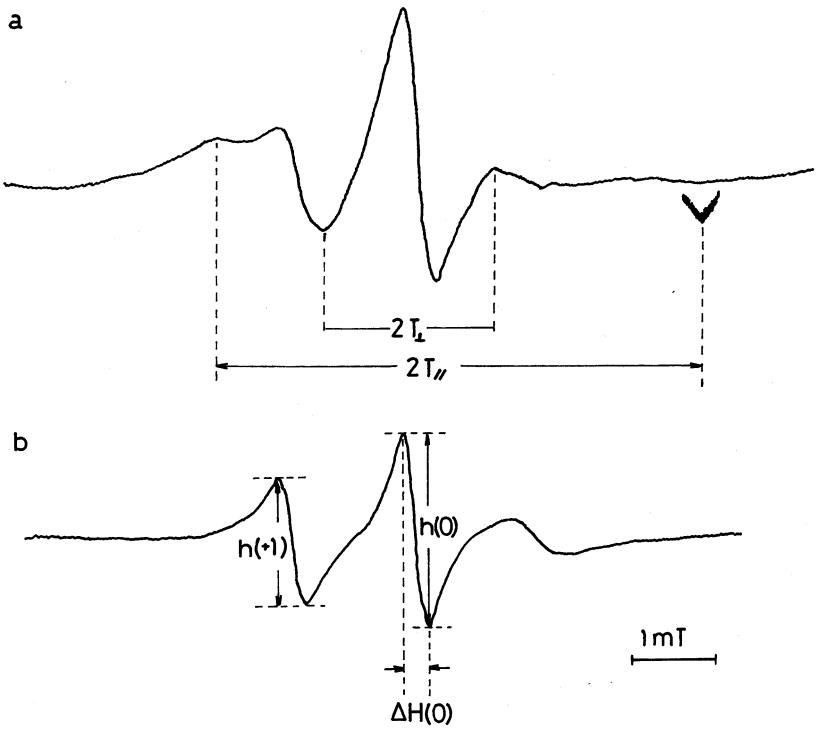

Fig. 3. ESR spectrum of 5-doxylstearic acid in excised guinea pig dorsal skin and effects of 1% L-menthol in 15% ethanol. a, control; b, treated with 1% L-menthol in 15% ethanol. From spectrum a, the outer and inner hyperfine splittings, $2T_{//}$ and $2T_{\perp}$, were measured, and the order parameter was calculated as described (Kitagawa et al., 1990). From spectrum b, the central peak width, H_0, central peak height, $h(0)$, and peak height of lower magnetic fields, $h(+1)$, were measured, and the apparent rotational correlation time was similarly calculated.

gested that the lipid lamellae of guinea pig dorsal skin resembles that of human skin in lipophilicity. This agrees with our recent findings of the free energy of the transfer of the methylene group of alkyl parabens from the aqueous phase in guinea pig dorsal skin which was also similar to that in human skin (Kitagawa et al., 1997). These results suggest that guinea pig skin is a good model for human skin.

The combination of L-menthol with ethanol has been revealed to enhance the skin permeation of various drugs (Obata et al., 1991; Sugibayashi et al., 1995). As revealed in this study for the system of 1% L-menthol plus 15% ethanol, it also significantly enhanced the penetration of relatively hydrophilic benzoic acid derivatives through guinea pig dorsal skin with $\log P_{oct}$ values less than 2. The degree of enhancement was much greater than that by ethanol itself. Therefore, this system is useful for improving the skin penetration of hydrophilic drugs. Addition of a square term of $\log P_{oct}$ improved the correlation between permeability coefficients and n-octanol/water partition coefficients. This means that the optimal lipophilicity for the skin penetration by benzoic acid derivatives is in the presence of L-menthol with ethanol. This corresponded to the previous finding on the effects of the same enhancer on the penetration of alkyl parabens (Kitagawa et al., 1997). The decrease in permeability of derivatives with more than optimal lipophilicity may be due to the decrease of partitioning of the lipophilic derivatives between skin and vehicle as suggested by the marked increase in their solubilities in the presence of 1% L-menthol plus 15% ethanol. This is consistent with the effects of ethanol (Ghanem et al., 1987; Obata et al., 1993) and D-limonene (Koyama et al., 1994). Perturbation of lipid lamel-

lae by enhancers may also modify partitioning of drugs into the stratum corneum by changing the hydration of the lipid lamellae (Ward and Tallon, 1988).

ESR spectra showed the presence of strongly and weakly immobilized components. As suggested by Quan and Maibach in their study on 5-doxylstearic acid-labeled human stratum corneum, the spin label partitions between a nonpolar (immobilized) environment and a fluid environment. It is likely that the strongly immobilized component in the spectra corresponds to the rigid lipid lamellae. On the other hand, the weakly immobilized component seems to show the presence of a fluid bilayer-like component in the stratum corneum in addition to the rigid lipid lamellae (Quan and Maibach, 1994). The disappearance of the immobilized component in the presence of L-menthol with ethanol suggested that the rigid lipid lamellar structure was significantly disordered by the enhancer, resulting in a fluid bilayer-like structure. This agrees with our recent findings on the effects of L-menthol with ethanol on the fluidity of the lipid bilayer of stratum corneum lipid liposomes (Kitagawa et al., 1997). These results are also consistent with a previous report on the effects of Azone® (1-dodecylazacycloheptan-2-one) on 5-doxylstearic acid-labeled stratum corneum (Quan and Maibach, 1994). Therefore, disruption of the rigid lipid structure by the enhancer seems to permit much easier drug diffusion through the skin, which will promote the penetration of hydrophilic drugs.

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