

Effects of Removal of Stratum Corneum, Delipidization and Addition of Enhancers, Ethanol and *l*-Menthol, on Skin Permeation of Benzoic Acid and Its 4-*n*-Alkyl Substituents in Excised Guinea Pig Dorsal Skin

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Skin penetration of benzoic acid and its 4-alkyl substituents (methyl, ethyl, *n*-propyl and *n*-butyl) through excised guinea pig dorsal skin was examined, and effects of removal of stratum corneum, delipidization and addition of the penetration enhancers, ethanol and *l*-menthol plus ethanol, were observed. Permeability coefficients, which increased with the increase in their alkyl chain lengths, depended on the ratio of undissociated form of the derivatives. Removal of stratum corneum by tape stripping and delipidization by a chloroform–methanol mixture, whose effects on the permeation were similar, increased the permeability coefficients of the derivatives, especially those of relatively hydrophilic derivatives. Addition of 1% *l*-menthol plus 15% ethanol increased the permeability coefficient of benzoic acid, but decreased those of ethyl-, *n*-propyl- and *n*-butyl-substituents, and differences in the permeability coefficients among these acids almost disappeared. A similar though weaker tendency was observed for the effects of 15% ethanol itself. Analysis of transfer free energy of the methylene group from vehicle to skin revealed that tape stripping and delipidization induced the reduction of lipophilic barrier property, although it still remained after these treatments. The analysis also showed that the addition of the enhancers made the skin relatively more hydrophilic compared to the vehicle, which induced an increase in permeability coefficient of benzoic acid and decreases in those of its lipophilic substituents.

Key words alkyl benzoic acid; ethanol; *l*-menthol; stratum corneum; transfer free energy

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. Due to the presence of the rigid lipid lamella in the stratum corneum,¹⁾ lipophilicity is essential for prompt skin permeation. This makes the addition of chemical or physical penetration enhancers or conversion to hydrophobic prodrugs necessary to use hydrophilic drugs in practical transdermal preparations.^{2,3)} In this work we examined the skin permeation of benzoic acid and its 4-alkyl substituents, and observed the increase in their permeability coefficients by introduction of longer alkyl chains which makes the substituents more lipophilic and is expected to increase the partition to the skin. We used guinea pig skin as a model of human skin, since the lipid lamella of guinea pig dorsal skin has been suggested to resemble that of human skin in lipophilicity; there is a similar dependency on *n*-octanol/water partition coefficients of the skin permeability coefficients of the 2- or 4-substituents of benzoic acid as we previously reported.⁴⁾

To determine the barrier property of stripped skin and delipidized skin, we observed the change in the relation between permeability coefficients of benzoic acid and its 4-alkyl substituents and their alkyl chain lengths after removal of stratum corneum by tape stripping and lipid extraction by chloroform–methanol mixture. We examined the changes in relative lipophilicity of the skin after these treatments by analyzing free energy of the transfer of methylene group of the 4-alkyl substituents of benzoic acid from vehicle to skin. We also examined the effects of penetration enhancers, *l*-menthol and ethanol, which have been reported to improve the skin permeabilities of various drugs.^{5–7)} We attempted to determine the changes in the barrier property of the skin by removal of the stratum corneum, lipid extraction and addition

of the enhancers.

Experimental

Materials Benzoic acid, its 4-methyl, 4-ethyl and 4-*n*-propyl substituents, and *l*-menthol were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-*n*-Butyl substituent was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries.

Skin Preparations Full thickness dorsal skin was excised from male guinea pigs and subcutaneous fat and other extraneous tissues were trimmed. The stratum corneum was removed by twenty successive strippings with cellophane tape until the surface glistened. Stratum corneum lipids were extracted by incubating the excised skin with a chloroform–methanol mixture (2:1 vol) for 12 h and washing it extensively with phosphate buffered saline (PBS, pH 7.4).

Measurement of *in Vitro* Skin Penetration *In vitro* skin penetration of drugs was examined as described previously.⁸⁾ The skin preparations were mounted in a two-chamber diffusion cell with water jackets (37 °C). The available diffusion area was about 0.65 cm², and each half-cell volume was about 5.4 ml. The donor cells were filled with saline either in the presence or absence of the enhancer unless otherwise mentioned, and the receiver cells with PBS (pH 7.4). Cells were pretreated for 12 h with stirring at 450 rpm by a magnetic stirrer. After washing of both compartments, the suspension of excess amount of benzoic acid or its 4-alkyl substituents in saline either in the presence or absence of the penetration enhancer was added to the donor compartments unless otherwise mentioned, and the penetration experiment was begun. One hundred fifty μ l of sample was taken from the receiver cells periodically over a maximum period of 29 h, diluted with PBS twenty times or more and analyzed by UV absorbance at 224 nm for benzoic acid, at 235 nm for 4-methyl and 4-ethyl substituents and at 236 nm for 4-*n*-propyl and 4-*n*-butyl substituents. Solubilized components from skin and penetrated enhancers did not interfere with the UV absorbance.

Dependency on pH in the donor compartment of the permeability coefficients of the derivatives was examined with suspension of their excess amount (pH 3.0, 4.0) or a 50 mM solution of the derivatives (pH 5.0–7.0), using either phosphate buffer (pH 3.0, 6.0, 7.0) or citrate buffer (pH 4.0, 5.0), and was observed by the procedure described above.

Calculation of Permeability Coefficients The permeability coefficient was calculated according to Eq. 1 from the initial straight portion of the penetration curve, dC_R/dt , shown in Fig. 1 for benzoic acid as an example,⁸⁾

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

where C_R and V_R are the concentration of the 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.

Solubility Measurement The solubility of benzoic acid and its 4-alkyl substituents was measured after their incubation in an excess amount in saline either in the presence or absence of the enhancer at 37°C for 24 h. After quick centrifugation at 1000×*g* for 2 min at 37°C, concentration of the supernatant was obtained by measuring UV absorbance as described above.

Results

Permeability of Benzoic Acid and 4-Alkyl Substituents and Effects of Removal of Stratum Corneum and Delipidization

We first examined the permeation of benzoic acid and its 4-*n*-alkyl (methyl, ethyl, *n*-propyl, *n*-butyl) substituents through excised guinea pig dorsal skin. Permeation proceeded with short lag time in control skin as shown in Fig. 1 for benzoic acid. Solubility and permeability coefficients of benzoic acid and its 4-alkyl substituents are listed in Table 1. Among these compounds flux was largest with benzoic acid in control skin, however, the permeability coefficients increased with increase in the alkyl chain lengths of the compounds as shown in Fig. 2 and Eq. 2,

$$\log K_p = 0.236n - 0.976 \quad (r = 0.983) \quad (2)$$

where n is the number of carbon atoms of the 4-alkyl groups and r is the correlation coefficient.

We also observed the changes in permeability in the stripped skin and delipidized skin. For delipidization we used a chloroform-methanol mixture (2:1 vol) as a solvent, because this mixed solvent system has been shown to work as an effective delipidizing agent in skin.⁹⁾ Both removal of the stratum corneum by tape stripping and delipidization of skin decreased the dependency of the permeability coefficients on the alkyl chain lengths. As shown in Fig. 2, relatively hydrophilic benzoic acid permeated in these skin preparations and its permeability coefficient markedly increased, while permeability coefficients of the 4-alkyl substituents with larger lipophilicity such as 4-butyl substituent only slightly increased. Therefore, the correlation between logarithm value of the permeability coefficient and the alkyl chain length in stripped skin was changed as shown in Fig. 2 and Eq. 3,

$$\log K_p = 0.174n - 0.668 \quad (r = 0.937) \quad (3)$$

and that in delipidized skin was changed as shown in Fig. 2 and Eq. 4.

$$\log K_p = 0.168n - 0.620 \quad (r = 0.957) \quad (4)$$

These findings indicated that removal of the stratum corneum by tape stripping and delipidization by the organic solvent mixture induced similar enhancement effects on the permeability of benzoic acid and its 4-alkyl substituents.

Effect of Ionization on Skin Permeability Effect of ionization of the derivatives on their skin permeability was determined based on the changes in the permeability coefficients with change of pH in the vehicle. As shown in Fig. 3 for benzoic acid, permeability coefficients of the derivatives decreased with the increase of pH. The change of pH in the

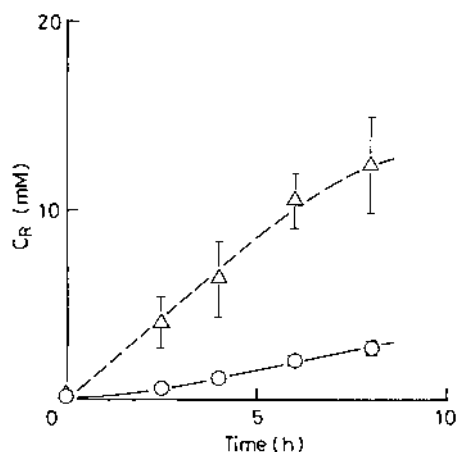


Fig. 1. Increase in Concentration of Benzoic Acid in Receiver Compartment, C_R , due to Transfer through Guinea Pig Dorsal Skin

○, control skin; △, delipidized skin. Data are means ± S.D. of three experiments.

Table 1. Solubility (C_d) and Permeability Coefficients (K_p) of Benzoic Acid and Its 4-Alkyl Substituents

Substituent	$C_d^{a)}$ (mM)	$K_p^{a)}$ ($\times 10^{-2} \text{ cm} \cdot \text{h}^{-1}$)
None	32.7 ± 1.6	9.01 ± 1.51
Methyl	3.80 ± 0.03	20.3 ± 5.8
Ethyl	3.34 ± 0.10	34.8 ± 1.2
<i>n</i> -Propyl	0.70 ± 0.03	62.7 ± 13.0
<i>n</i> -Butyl	0.37 ± 0.02	79.9 ± 10.1

a) Data are means ± S.D. of three experiments.

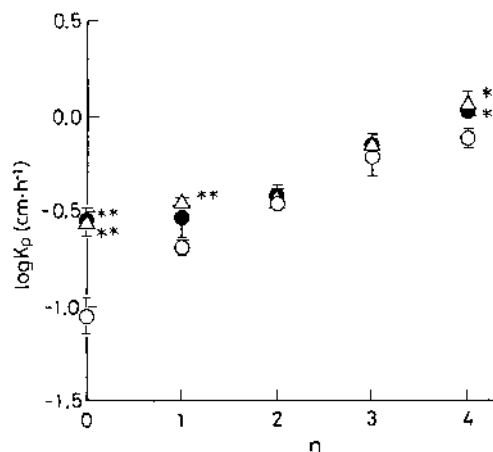


Fig. 2. Permeability Coefficients of Benzoic Acid Derivatives (K_p) and Its 4-Alkyl Substituents through Guinea Pig Dorsal Skin, Its Stratum Corneum Removed Skin and Delipidized Skin and Their Relationships with Alkyl Chain Lengths

○, control skin; ●, stratum corneum removed skin; △, delipidized skin. Data are means ± S.D. of three experiments. The significance of difference between permeability coefficient of each substituent in control skin and that in either stratum corneum removed skin or delipidized skin was determined by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$.

vehicle during the permeation experiment was less than 0.1. As shown in Fig. 3, below pH 6.0 in which more than 2% of the acid is present in the undissociated form, observed permeability coefficients were consistent with the estimated values, which were obtained assuming that only an undissociated form of the acid can permeate through the skin. How-

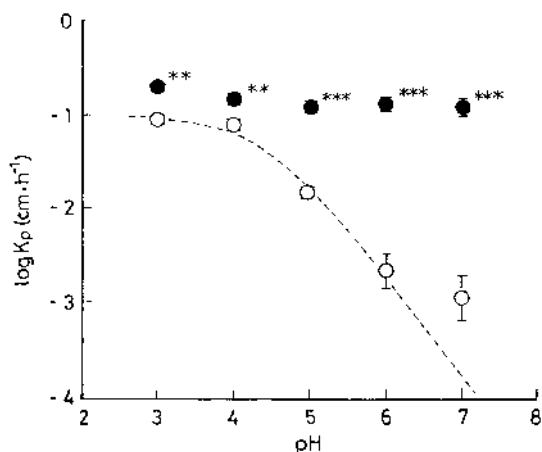


Fig. 3. Effect of Ionization on Permeability Coefficient (K_p) of Benzoic Acid through Guinea Pig Dorsal Skin and Stratum Corneum Removed Skin

○, control skin; ●, stratum corneum removed skin. Data are means \pm S.D. of three experiments. The significance of difference between permeability coefficient of benzoic acid in control skin and that in stratum corneum removed skin was determined by Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$. Dotted line represents the estimated permeability coefficients which were obtained by assuming that only an undissociated form of benzoic acid can permeate. The permeability coefficients were estimated using the permeability coefficient of the undissociated form and the ratio of undissociated form of the acid which was calculated using pK_a value. The pK_a value (4.3) was obtained by measuring pH-dependent change in UV absorbance at 230 nm at 37°C. The permeability coefficient of the undissociated form ($0.0965 \text{ cm} \cdot \text{h}^{-1}$) was estimated using the observed permeability coefficient ($0.0919 \text{ cm} \cdot \text{h}^{-1}$) and the ratio of undissociated form (0.952) at pH 3.0.

ever, the observed permeability coefficient was higher than the estimated value at pH 7.0 at which about 99.8 % of the acid was present as the dissociated form. Similar results were obtained for the 4-alkyl substituents.

Dependence of permeability coefficients on pH markedly decreased in stratum corneum removed skin, however. These findings are consistent with the well-known characteristics of the barrier function of stratum corneum to an ionic drug.

Effects of *l*-Menthol and Ethanol on Skin Permeation

Ethanol has been shown to enhance transdermal drug penetration dose-dependently.^{10,11} *l*-Menthol has also been shown to work as an enhancer dependent on ethanol concentration.⁵ To avoid the delipidization of stratum corneum by a high percentage of ethanol,¹² in this work we examined the effect of 1% *l*-menthol in 15% ethanol on skin absorption of benzoic acid and its 4-alkyl substituents. As shown in Fig. 4, with the addition of 1% *l*-menthol plus 15% ethanol the permeability coefficient of benzoic acid, whose logarithm value of *n*-octanol/water partition coefficient, P_{oct} , is 1.87,¹³ increased about two-fold. In contrast, those of 4-ethyl, 4-*n*-propyl and 4-*n*-butyl substituents, which have larger lipophilicity, decreased. A similar though weaker tendency was observed in the presence of 15% ethanol itself without *l*-menthol as also shown in the figure.

Free Energy of Transfer of Methylene Group from Vehicle to Skin and Effects of Removal of Stratum Corneum, Delipidization and Enhancer Addition The free energy of transfer of the methylene group from the aqueous phase to skin was calculated from the permeability data according to the following equation,¹⁴

$$\Delta(\Delta G) = -RT\Delta \ln(K_p) \quad (5)$$

where $\Delta(\Delta G)$ represents the free energy of transfer of methylene group from the aqueous phase to the skin, R is the

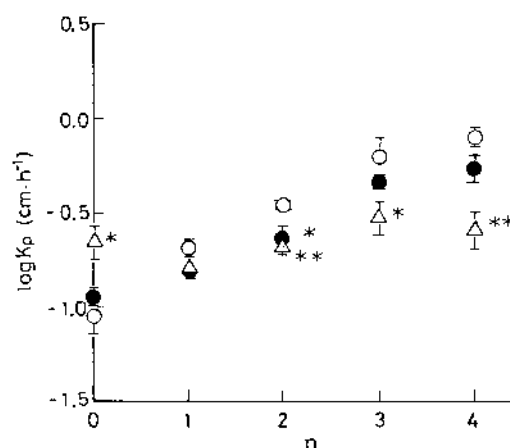


Fig. 4. Effects of 15% Ethanol and 1% *l*-Menthol in 15% Ethanol on Permeability Coefficients (K_p) of Benzoic Acid Derivatives and Its 4-Alkyl Substituents through Guinea Pig Dorsal Skin

○, control skin; ●, treated with 15% ethanol; △, treated with 1% *l*-menthol plus 15% ethanol. Data are means \pm S.D. of three experiments. The significance of difference between permeability coefficient of each substituent without enhancer and that with either 15% ethanol or 1% *l*-menthol plus 15% ethanol was determined by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$.

Table 2. Free Energy of Transfer of Methylene Group From Vehicle to Skin

Condition	Free Energy (kJ/mol)
Control skin	-1.40
Stratum corneum removed skin	-1.03
Delipidized skin	-0.98
Treated with 15% ethanol	-1.08
Treated with 1% <i>l</i> -menthol plus 15% ethanol	-0.28

gas constant, T is the absolute temperature and $\Delta \ln(K_p)$ means the slope of $\ln(K_p)$ vs. carbon number plot shown in Figs. 2 and 4. The free energy of transfer thus calculated for the methylene group in the excised guinea pig dorsal skin in the presence or absence of enhancers, and those in the stratum corneum removed skin and delipidized skin are listed in Table 2. From these values we can estimate the lipophilic barrier property of the skin and its changes by removal of stratum corneum, delipidization and addition of an enhancer. The negative free energy value in the control skin indicates that the skin is a lipophilic barrier and addition of lipophilic functional groups increases permeability through it.¹⁴ The free energy value of methylene group found for benzoic acid and its 4-*n*-alkyl substituents in the absence of enhancers was smaller than that previously reported for alkylparabens in the same types of skin.¹⁵ The absolute values of the free energy decreased in stripped or delipidized skin and in the skin treated with enhancers. The value in the presence of *l*-menthol plus ethanol was especially small.

Discussion

The present findings revealed that permeability through guinea pig dorsal skin of benzoic acid and its 4-alkyl substituents increased with the increase in alkyl chain length, indicating that the permeability increases depended on the lipophilicity, as reported for various other compounds. For these derivatives skin permeation proceeds by undissociated forms and permeation of ionic forms is neglected, except in

the pH range in which almost all forms present are dissociated. Therefore, as has been shown,¹⁾ stratum corneum lipid lamella seems to work as a barrier for ionic compounds. However, in the pH range in which almost all the derivatives are present as ionic forms, a porous or polar pathway may also contribute to the skin permeation of these forms,¹⁶⁾ because the observed permeability was higher than the estimated values calculated by the ratio of the undissociated forms in the pH range.

About thirty percent decrease in the absolute value of the free energy of transfer of the methylene group in both stripped skin and delipidized skin suggested that removal of stratum corneum and delipidization induced the reduction of lipophilic barrier property and changed the skin to be more hydrophilic, although the lipophilic barrier property still remained after these treatments. This change seems to be the cause of the marked increases in the permeability of relatively hydrophilic benzoic acid shown here and other hydrophilic compounds reported previously.¹⁵⁾ Delipidization by chloroform-methanol mixture decreased the barrier property of skin, and as a result the permeability coefficients of benzoic acid and its 4-alkyl substituents increased to a similar extent with the removal of stratum corneum by tape stripping.

We previously reported that the treatment with *l*-menthol plus ethanol significantly increased the skin permeability of methyl paraben whose logarithm value of *n*-octanol/water partition coefficient ($\log P_{\text{oct}}$) was 1.66, but markedly decreased that of *n*-butyl paraben which has much larger lipophilicity.¹⁵⁾ Ethanol itself had similar though weaker effects. The present findings are consistent with these because the enhancer systems only stimulated the skin permeation of benzoic acid whose $\log P_{\text{oct}}$ value is less than 2, but decreased the 4-alkyl substituents which have much larger lipophilicity.

In the case of skin treated with 15% ethanol, the absolute value of the free energy of transfer of the methylene group also decreased. This is probably due to the increase of the energetic stability of the group in the vehicle by the addition of ethanol, which seems to have induced the decreased partition of the 4-alkyl substituents to the skin and thus lowered the skin permeability coefficients; this is often observed when organic solvents are added as penetration enhancers.^{10,15,17-19)} For example, solubility of 4-*n*-butyl substituent, which was the most lipophilic substituent tested, increased 2.8 times with the addition of 15% ethanol. The treatment using 1% *l*-menthol plus 15% ethanol induced a much more marked decrease in the absolute value of the free energy of transfer than that of ethanol itself. This is possibly due to the increase in the hydrophilic property of the stratum corneum in addi-

tion to an increase in the energetic stability of the group in the medium. We have reported the marked increase in lipid fluidity of the stratum corneum with the addition of *l*-menthol plus ethanol.^{4,15)} The perturbation of the rigid packing of stratum corneum lipid lamella seems to produce the hydrophilic property in the lipid lamella as well as an increase in diffusion coefficients of drugs in the permeation process through the lamella. Both these effects induced by *l*-menthol plus ethanol might be related to the significant increases in the permeability coefficients of hydrophilic drugs as shown for benzoic acid in this study, as well as the significant reduction in those of hydrophobic drugs as shown for 4-ethyl, 4-*n*-propyl and 4-*n*-butyl substituents.

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