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Differences in enhancing effect of l-menthol, ethanol and their combination between hairless rat and human skin

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Abstract

The differences in skin permeation enhancing effect by l-menthol, ethanol and their combination between hairless rat and human skin were investigated by measuring the permeation of isosorbide dinitrate (ISDN) as a model drug and all components in the donor vehicle through excised skin. Deuterium oxide (D_2O) and ethanol-d_6 (EtOD) were used in place of water and ethanol, respectively. Vehicle compositions containing enhancers were 5% l-menthol/D_2O (MW), 40% EtOD/D_2O (EW) and 5% l-menthol/40% EtOD/D_2O (MEW). D_2O (W) was also used as a control vehicle. The permeability of each component (including ISDN) through human skin was lower than that through hairless rat skin in all the vehicles. A strong enhancement effect was achieved only by the combination of l-menthol and ethanol (MEW) in human skin. The most marked difference in enhancing effect between hairless rat and human skin was observed with the application of MW. Comparison of the cumulative amount of each component between human and rat (human/rat) using MW showed l-menthol to be 1/2, ISDN 1/8 and D_2O 1/30. With MEW, on the other hand, human/rat ratios were similar (l-menthol, 1/3; ISDN, 1/3; EtOD, 1/3; D_2O, 1/2) independent of the component species. In addition, there was a lag time of about 2 h for all components when MEW was applied to human skin, whereas no lag time was observed in hairless rat skin. Lipid leached from skin to the donor compartment was followed to evaluate the species difference. The results suggested that differences in lipid leaching between human and hairless rat skin might be one reason for the variation in enhancing effect or lag time.

Keywords: Skin permeation enhancer; Species difference; l-Menthol; Ethanol; Delipidization

1. Introduction

A good understanding of the enhancing characteristics of a skin permeation enhancer is im-

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portant for its effective application in a transdermal drug delivery system (TDS). Several studies have been reported using different enhancers (Barry, 1987; Hori et al., 1990, 1991; Williams and Barry, 1991). We have also reported on the combination of l-menthol and ethanol (Morimoto et al., 1993), and found that the enhancing effect in human was different from that in hairless rat (Kobayashi et al., 1993). That is, the enhancing ratio for the permeation of water soluble or polar drugs through human skin was higher than that through hairless rat skin. Since hairless rat skin has been used broadly to evaluate skin permeation enhancers, it is important to elucidate the difference in the enhancing effects between hairless rat and human skins for the fabrication of TDS.

Elias (1981) reported that species differences in drug permeability between animal and human skin were causally related to its morphology. Sato et al. (1991) reported that the enhancement effect of Azone or isopropyl myristate on the permeation of nicorandil through human skin was weaker than through the surface-lipid rich animal skins (hairless mice, hairless rats and guinea pigs). More reports, however, are needed to clarify the species differences of the effect by enhancers.

In the present study, we carried out in vitro permeation experiments through hairless rat and human skin using isosorbide dinitrate (ISDN) as a model drug and either one or a combination system of l-menthol and ethanol as enhancers. Because of the restricted availability of human skin, the amount of all the components (ISDN, enhancers and water) permeating from the donor side of the diffusion cell was measured. Since the l-menthol concentration in skin was considered to be one of the factors for its enhancing effect in our previous study (Kobayashi et al., 1994), the concentration dependence on the enhancing effect was determined. Furthermore, the enhancing effect of a high concentration of ethanol is probably caused by delipidization of skin (Hatanaka et al., 1992), hence the cumulative amount of lipid leaching from the skin into the donor side of the diffusion cell was evaluated.

Deuterium oxide (D_2O) and ethanol-d_6 (EtOD) were used instead of water and ethanol (EtOH), respectively, for ease of assay. The donor vehicles including 5% ISDN were D_2O (W), 5% l-menthol in D_2O (MW), 40% EtOD in D_2O (EW) and 5% l-menthol and 40% EtOD in D_2O (MEW).

2. Experimental

2.1. Materials

ISDN was a gift from Toko Pharmaceutical Industries Co., Ltd (Tokyo, Japan). l-Menthol (JP grade) and EtOH (Wako Pure Chemical Industries, Osaka, Japan) were used as skin permeation enhancers. l-Carvone, internal standard for assaying l-menthol, and triolein (practical grade), cholesterol and vanillin for measuring total lipid leached from skin were purchased from Wako Pure Chemical Industries, Ltd. D_2O and EtOD were obtained from Merck Co. (Darmstadt, Germany) and Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), respectively. Other reagents were of reagent grade or HPLC grade products.

2.2. Skin membrane preparations

Abdominal skin was excised from male WBN/ILA-Ht rats (average weight 160 g, Life Science Research Center, Josai University, Saitama, Japan) immediately before the permeation experiment. Human skin was obtained following unrelated surgical operations at the Department of Surgery, Saitama Medical Center, Saitama Medical School; sources were the chests of 32–41-year-old female patients. The human skin samples were stored at −20°C until use. The thickness was adjusted to about 750 μm by whittling the dermis side. Prior to use in the permeation experiment, the dermis side was washed with distilled water. It was confirmed that these treatments did not affect the skin permeability of drugs (Morimoto et al., 1992).

2.3. Skin permeation procedure

Skin permeation experiments were performed according to the method of Okumura et al. (1989).

The diffusion cell used consisted of two half-cells with a water jacket connected to a water bath at 37°C. Each half-cell had a volume of 2.5 ml and an effective diffusion area of 0.95 cm^2. The dermis side of the skin was in contact with the receiver compartment and the stratum corneum with the donor compartment. The donor compartment was filled with 5% ISDN suspension of W, MW, EW or MEW, and the receiver compartment with water. Both compartments were stirred with a starhead bar driven by a constant-speed synchronous motor (MC-301, Scinics, Tokyo) at 1200 rpm. The whole volume was withdrawn from the receiver compartment at predetermined times for assay and filled again with water. Each experiment was triplicated.

2.4. Measurement of l-menthol concentration in full thickness skin

l-Menthol concentration in full thickness skin was determined immediately after the 8 h permeation experiments. After rinsing of the skin surface with water, excess water was blotted off, and the effective diffusion area (0.95 cm^2) of the skin was punched. The skin was splitted into pieces and immersed in a test tube containing 0.1% l-carvone in dioxane, and the sample was sonicated (Branson, BM-42, Yamato, Tokyo) for 10 min (recovery ratio, $99.6 \pm 1.26\%$). After centrifugation, the concentration of l-menthol in the supernatant was measured using a gas chromatograph (GC). A calibration curve was constructed using the supernatant of the skin homogenate with dioxane containing 0.1% l-carvone and a known amount of l-menthol.

2.5. Lipid leaching from skin into donor compartment

The time course of lipid leaching from the skin to the donor compartment containing EW, MW or MEW without ISDN was measured. The diffusion cell, temperature and stirring conditions employed were the same as in the permeation procedure. The entire volume was withdrawn from the donor and receiver compartment at 5, 10 and 30 min, 1, 2, 4, 6 and 8 h after beginning the experiment. The donor and the receiver were filled again with EW, MW or MEW and water, respectively, immediately after sampling.

Fig. 1. Electron microgram of cross-section of human stratum corneum with scanning electron microanalyzer (3000× magnification).

2.6. Stratum corneum (SC) preparation

The SC of human and hairless rat skin was separated from the viable epidermis to measure the total lipid content in SC. Human skin with a thickness of less than 750 μm and hairless rat skin were mounted on a large size half-cell (effective diffusion area, 4.52 cm^2) facing the dermis side to the inside. The half-cell was filled with water and stirred for 4 days. After finding water depot between the SC and viable epidermis, the SC sheet was separated. Unlike the usual separation methods using trypsin (Knutson et al., 1985), microwave (Kumar et al., 1989) and heating (Kligman and Christopher, 1963), this method is considered to be less damaging (e.g., little lipid leaching) to the SC but does take longer for

preparation. Fig. 1 shows an electron micrograph of a cross-section of human SC which was intentionally torn by hand for easy observation of the multiple layer of SC. A Hitachi X-6500 electron microscope (Tokyo) was used.

2.7. Extraction of lipids

Lipids were extracted from the SC according to the method of Knutson et al. (1985).

2.8. Cholesterol solubility in vehicle

Excess cholesterol was added to 1 ml of MW, EW or MEW and equilibrated at 37°C for 24 h. The saturated concentration of cholesterol was determined using the method of Allain et al. (1974).

2.9. Assay

All the correlation coefficients of calibration curves used were above 0.999.

ISDN was determined by HPLC. Each sample was added to the same volume of methanol containing ethyl *p*-hydroxybenzoate as an internal standard. The HPLC system consisted of a pump (LC-6A, Shimadzu Seisakusho, Kyoto, Japan), a UV detector (SPD-6A, Shimadzu Seisakusho), Chromatopac (CR-3A, Shimadzu Seisakusho) and a stainless-steel column (4.0 × 15 mm) packed with Nucleosil $5C_{18}$ (Macherey-Nargel, Germany). Conditions were: elution phase, acetonitrile/water (55:45); flow rate, 1.0 ml/min; room temperature; detection, UV 220 nm.

EtOD was measured by GC. To 100 μl of sample, the same volume of distilled water containing *n*-propanol as an internal standard was added and 1 μl of the supernatant after centrifugation was injected in a GC-FID system (GC-6A Shimadzu Seisakusho). Conditions were: column, Gaskuropak 54 (GL Sciences, Tokyo, Japan); column, injection and detection temperature, 140, 180 and 180°C, respectively; carrier gas, N_2; flow rate, 50 ml/min.

l-Menthol was also assayed by GC. Sample

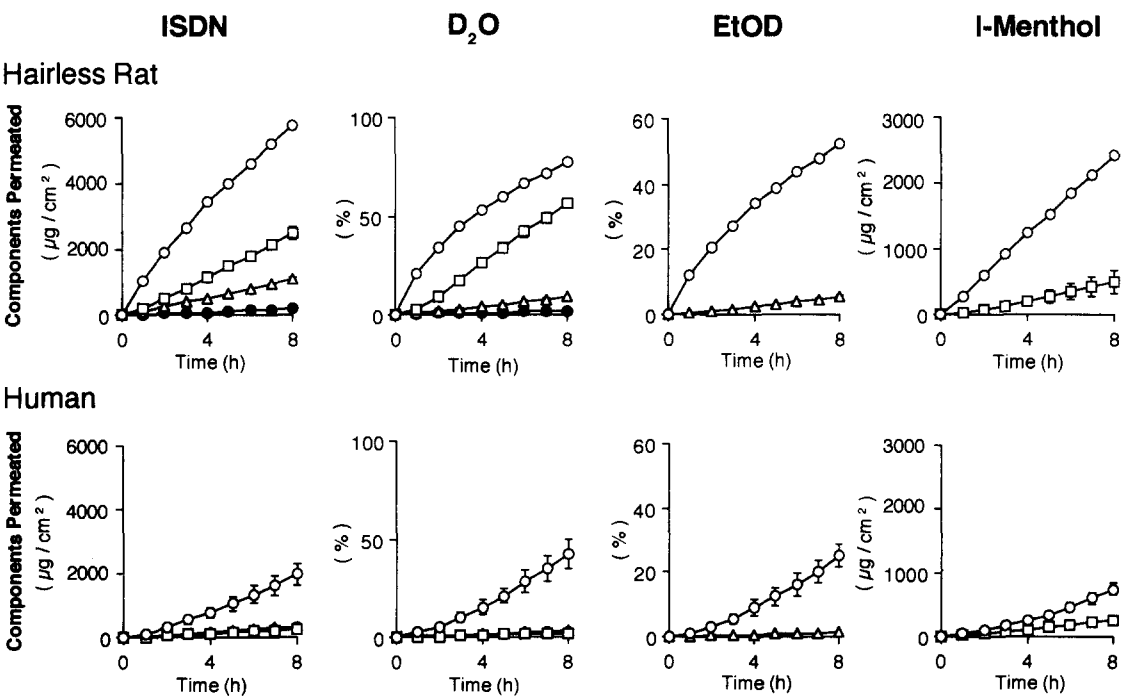

Fig. 2. Effect of enhancers on the permeation of several components through hairless rat and human skin. Each point represents the mean and SD of three experiments. W (●), EW (△), MW (□), MEW (○).

containing l-menthol was added to an equal volume of dioxane containing l-carvone as an internal standard. The supernatant (1 μl) after centrifugation was injected in a GC-FID system (GC-14A, Shimadzu Seisakusho). Conditions were: column OV-17 (GL Sciences, Tokyo); column, injection and detection temperature, 130, 160 and 160°C, respectively; carrier gas, N_2; flow rate, 50 ml/min.

D_2O was determined by IR spectrometry (IR-450, Shimadzu Seisakusho) with $CaCF_2$ cell (light distance, 0.025 mm; window, 30 mm diameter × 4 mm). Detection wave number was 2515 cm^{-1}. The amount of D_2O was calculated by subtracting the total (D_2O + EtOD) from the EtOD value (Sugibayashi et al., 1992).

Total lipid was measured according to the method of Knight et al. (1972). In the case of the sample withdrawn from the donor compartment, the same volume of EtOH was added to dissolve suspended l-menthol and to make one clear layer. 1 ml of the resulting solution was evaporated in boiling water until the odor of l-menthol disappeared. In the case of lipid extracted from SC, the extraction solvent was evaporated under nitrogen gas at 50°C, and each sample was reconstituted with 200 μl of EtOH. The sample was added to 300 μl of conc. H_2SO_4 and heated in boiling water for 5 min. After cooling to room temperature, 2 ml of phosphovanillin test solution (0.6% w/v aqueous vanillin solution/phosphoric acid = 1:4) was added and the mixture was incubated for 15 min at 37°C. Then, the absorbance was measured at 535 nm using a spectrophotometer (U 3200, Hitachi, Tokyo, Japan). A mixture of cholesterol and triolein (3:1) was used as a reference standard (Knight et al., 1972).

3. Results and discussion

Fig. 2 shows the time course of the cumulative amount of each component (ISDN, D_2O, EtOD and l-menthol) contained in the donor compartment permeating through hairless rat and human skin. Fig. 3 shows the time course of the ratio of the cumulative amount through human skin to

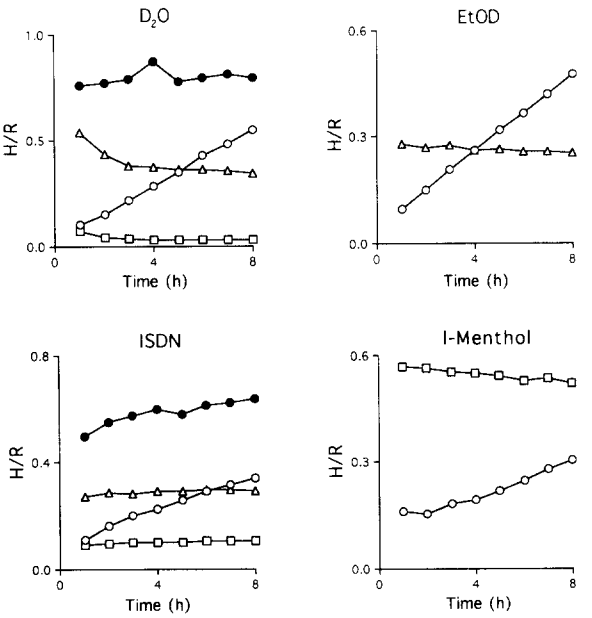

Fig. 3. Time course of H/R in each permeant. W (●), EW (△), MW (□), MEW: (○).

that through hairless rat skin (H/R) to compare the permeability of each component between the two skins. Table 1 summarizes in H/R values at 8 h. These data show that the permeability of each component through human skin was lower than that in hairless rat skin in all solvent systems (W, EW, MW and MEW). The H/R ratio was almost constant, independent of the time elapsed for each permeant in all solvent systems except MEW. In the case of MEW the H/R ratio increased with time. However, the ratio was evenly increased with time and the slope of the ratio/time was almost the same for each permeant. The H/R ratio at 8 h (Table 1) was used to

Table 1
Approximate ratio of cumulative amount of each component permeated over 8 h through human skin to that through hairless rat skin

Vehicle	Component			
	D_2O	EtOD	ISDN	l-Menthol
W	1		1/2	
EW	1/4	1/3	1/4	
MW	1/30		1/8	1/2
MEW	1/2	1/2	1/3	1/3

Table 2
l-Menthol concentration in hairless rat and human skin after the 8 h permeation experiment

Vehicle	l-Menthol concentration (mg/g)	
	Hairless rat	Human
MW	33.8	8.5
MEW	72.9	17.4

simply evaluate the enhancers. The obtained species differences in the effect of enhancers will be explained by dividing three points of view as follows.

Firstly, species difference in the enhancing effect by several solvent systems will be discussed. The order of enhancement effect on the permeation of all components was $W < EW < MW < MEW$ in hairless rat skin, while a strong enhancement effect was achieved only with MEW in human skin ($W \leq EW \approx MW \ll MEW$). This may be due to a weak effect of MW on human skin. The l-menthol concentration in human skin when using the MW or MEW system was 1/4 of that in hairless rat skin for each case (Table 2), which shows that the penetration of l-menthol into human skin was more difficult than into the rat skin. This is not direct evidence, however, of the weak effect of MW on human skin.

Lipid leaching from the skin was then measured (Fig. 4). About 30% of the total lipid in SC of the rat skin was leached when the MW system was used, whereas only 5% was leached from human skin. On the other hand, marked delipidization was observed in both the skins when using MEW. Since more than the total lipid in SC was leached after 1 h of the experiment in hairless rat, lipids even in the under layer of SC would be leached due to a decrease in barrier function of SC. We (Hatanaka et al., 1992) reported that the degree of delipidization by ethanol solution (20, 40, 60 and 100% solution was used) was paralleled by its enhancement effect, and that about 20% of the sterol was leached from SC in rat skin using neat ethanol. The amount of lipid leached by the MW system in the present study, 30% of the total lipid in SC, was considered to be enough to exert its enhancing effect, whereas the amount delipidized from human skin

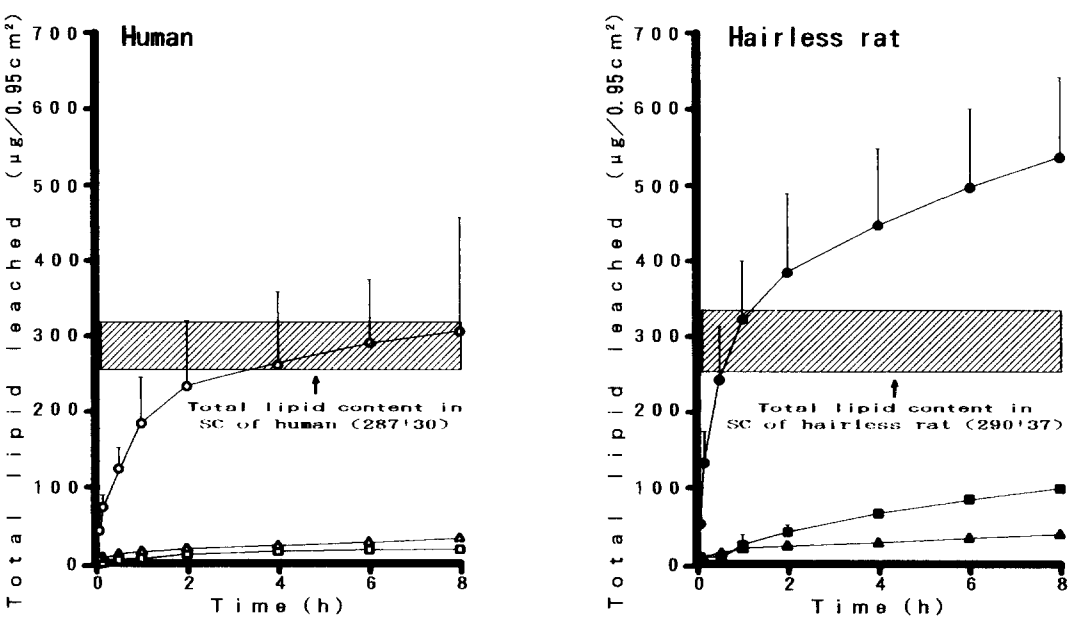

Fig. 4. Effect of enhancers on the lipid leaching from skin into the donor compartment. Each point represents the mean and SE of three experiments. Hairless rat skin with EW (▲), hairless rat skin with MW (■), hairless rat skin with MEW (●), human skin with EW (△), human skin with MW (□), human skin with MEW (○).

by MW, 5%, might be insufficient. Moreover, the solubility of cholesterol, a major lipid in SC (Elias, 1981), in the EW, MW and MEW systems was 8.1, 20.8 and 683 μg/ml, respectively; the MEW system thus markedly increased cholesterol solubility. The amount of lipid leached from skin and solubility of cholesterol when using the EW system was much lower than we had expected. Human skin was also structurally affected by the MEW system in spite of its strong barrier function to l-menthol.

Secondly, we paid attention to species difference in lag time. All the permeants showed a similar lag time (2 h) when using the MEW system in human skin, whereas almost no lag time was observed in rat skin (Fig. 2). This difference reflected the time course of H/R using MEW (Fig. 3). These phenomena may not be explained by the simple partition and diffusion theory with a constant skin barrier function.

Based on the partition and diffusion theory, lag time (τ) is expressed as $L^2/6D$ (Scheuplein, 1967), where L and D are the thickness of membrane and the effective diffusion coefficient of a drug in skin barrier, respectively. D can be described by the equation, $\log D = -A \times \log \mathrm{mw} + B$ (Flynn et al., 1974), where A and B are constants and mw denotes the molecular weight of drug. If the skin barrier function is constant during a permeation experiment, the lag time should be dependent on the molecular weight. Williams and Barry (1991) calculated the D value of 5-fluorouracil to evaluate effect of skin permeation enhancers by the lag time method using in vitro permeation data through human skin. The molecular weights of the permeants in this study are different; 20, 54, 156 and 236 for D_2O, EtOD, l-menthol and ISDN, respectively. Therefore, the fact that all the permeants showed a similar lag time when using MEW in human skin cannot be explained by the theory. This may be due to the time-dependent skin change due to the MEW system.

Under the assumption that the skin consists of two layers (Kobayashi et al., 1993),

$$R_T = R_{SC} + R_{ED} \quad (1)$$

where R_T is the total permeation resistance of skin, and R_{SC} and R_{ED} represent the resistance of the SC and viable epidermis and dermis (ED), respectively. Interestingly, the time course of lipid leaching by the MEW system showed a convex curvature (Fig. 4) in contrast to the concave curvature of the time profile of each component permeated through human skin (including lag time) (Fig. 2). If the extent of the decrease in R_{SC} with time corresponds to the amount of lipid leached from the SC and the R_{SC} value decreases in the first-order process (Fig. 4), the following equation can be derived.

$$R_{SC_t} = R_{SC0} e^{-kt} \quad (2)$$

where R_{SC_t} and R_{SC0} are the resistance of the SC at time t after MEW treatment and at time zero, respectively, and k denotes a rate constant. Substituting Eq. 2 into Eq. 1, and using the relation that the permeability coefficient (P) is the reciprocal of the resistance (Martin, 1993),

$$P_T = \left(P_{SC0} e^{kt} \cdot P_{ED}\right) / \left(P_{SC0} e^{kt} + P_{ED}\right) \quad (3)$$

where all the subscripts have the same meaning as that of the resistances. If P_{ED} is A times higher than P_{SC0} ($P_{ED} = A \cdot P_{SC0}$)

$$P_T = \left(A \cdot P_{SC0} e^{kt}\right) / \left(A + e^{kt}\right)$$

$$= A \cdot P_{SC0} / \left(A \cdot e^{-kt} + 1\right) \quad (4)$$

Using the cumulative amount (Q), the flux (J) of permeant is described as:

$$J = dQ/dt = P_T C_V$$

$$= C_V \cdot P_{SC0} \cdot A / \left(A \cdot e^{-kt} + 1\right) \quad (5)$$

where C_V is the permeant concentration in the vehicle. Then Q is (Yano, 1981):

$$Q = C_V \cdot P_{SC0} \cdot A \int_0^t 1 / \left(A \cdot e^{-kt} + 1\right) dt$$

$$= C_V P_{SC0} (A/k) \ln\left(\left(A + e^{kt}\right) / (A + 1)\right) \quad (6)$$

A and k must be determined to simulate the relation of Q and t using Eq. 6. From the previous study (Morimoto et al., 1992; Kobayashi et al., 1994) A was considered to be dependent on the permeant polarity, and the value for hydrophilic permeants was greater than that for lipophilic permeants. Furthermore, the range of the value was about 5–100 for most permeants in

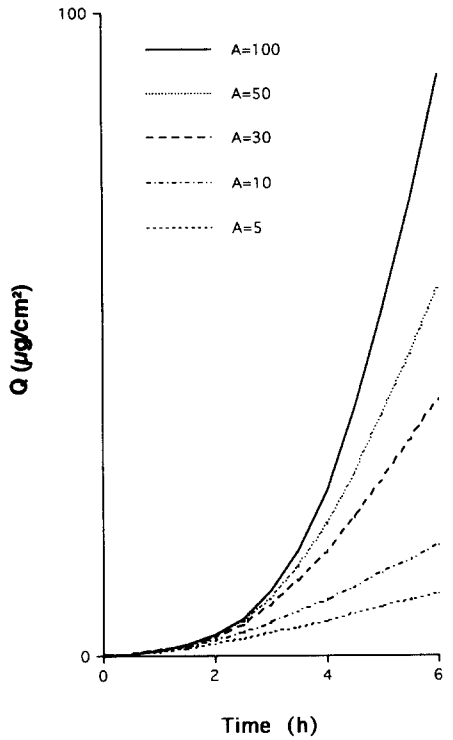

Fig. 5. Simulation curves based on Eq. 6. Product of C_v and P_{SC0}, proportionality constant in Eq. 6, is assumed to be unity (1 μg/h per cm^2). A and k are assumed to be 5, 10, 30, 50 and 100, and 1.15, respectively.

hairless rat skin. Since similar considerations could be applied to human skin, A was selected from the range. Assuming that the barrier function of SC after 2 h treatment of MEW is 1/10 of P_{SC0} (Fig. 4), k is calculated as 1.15 h^{-1} using Eq. 2. Fig. 5 shows simulation curves using these values and Eq. 6. In each line, the extrapolation to the time axis (apparent lag time) exists around 2 h. The lag time obtained from the experiment using MEW and human skin in this study would be affected by the time-dependent decrease in barrier function of SC caused by delipidization.

Thirdly, we focus on the H/R ratio between penetrants. All the components showed lower permeability in human skin than in hairless rat skin, but the H/R ratios at 8 h using MW was different among the penetrants (D_2O, 1/30; ISDN, 1/8; l-menthol, 1/2) compared to the ratios using EW of MEW (similar ratio from 1/2 to 1/4) (Table 1). Our previous study (Morimoto et al., 1992) to derive a predictable equation of drug permeation through hairless rat or human skin, where the dependent and independent variables were the permeability coefficient and octanol/water partitioning coefficient (K_{OW}), respectively, showed that the P of hydrophilic drugs (log $K_{OW} < 0$) through human skin was smaller than that of hairless rat skin and that the larger the K_{OW}, the smaller was the difference in P through the rat and human skin. A high contribution of follicles (transappendageal route) to the overall permeation of hydrophilic drug through hairless rat skin (Illel et al., 1990) supports the above results. Because the penetration enhancing effect of MW on the permeation of drug through rat skin was greater than through the human skin and the effect was more marked on the permeation of hydrophilic drug due to its delipidization in rat than human, the H/R of hydrophilic permeant was more strongly emphasized than that of lipophilic permeant. The difference in polarity of permeant: D_2O, polar: ISDN, log $K_{OW} = 1.34$ (Hatanaka et al., 1990) and l-menthol, nonpolar: might demonstrate the difference in the H/R when using the MW system.

4. Conclusion

The extent of drug permeability through human skin from two solvent systems containing ethanol, EW and MEW, was considered to be about 1/2–1/4 lower than that through hairless rat skin. In contrast, the enhancement effect of MW might be different in skin species, depending on the permeant polarity. The lag time of permeation through human skin using MEW might be caused by the decrease in barrier function of SC due to delipidization with time.

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