Effect of Ethanol Pretreatment on Skin Permeation of Drugs

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It has been demonstrated that ethanol (EtOH) can enhance skin permeation of drugs when simultaneously applied with drugs. However, only a few studies have reported on the pretreatment effect of EtOH on skin permeations. In this study, the pretreatment effects of EtOH on skin permeation of drugs were investigated by measuring changes in skin permeation and electrical skin resistance. Permeabilities of deuterium oxide (D_2O), isosorbide mononitrate (ISMN), isosorbide dinitrate (ISDN), calcein sodium (CA-Na), and fluorescein isothiocyanate-dextran 4 kDa (FD-4, 3.3–4.4 kDa) were evaluated through Yucatan micropig skin pretreated with different concentrations of EtOH solution. From the results, almost constant skin permeabilities of D_2O and ISDN were observed independent of EtOH concentration. Skin permeabilities of ISMN, CA, and FD-4 increased with low concentrations of EtOH, but decreased with high concentrations of EtOH. At 99.5% EtOH pretreatment, skin permeabilities of hydrophilic compounds (ISMN, CA, and FD-4) decreased to non-detectable levels. In addition, low molecular ion transports were almost constant at any EtOH concentration. Since molecular (ion) sizes of ISMN, CA, and FD-4 are larger than Na^+, Cl^-, and D_2O, permeation pathway sizes for hydrophilic compounds in the skin barrier may be remarkably decreased by pretreatment with high concentrations of EtOH. However, the permeability coefficient of ISDN was not influenced by any EtOH concentration, since ISDN is a lipophilic, low-molecular compound that permeated through the lipophilic stratum corneum pathway. The present results show useful information for repeatedly and topically applied formulations containing EtOH, and also contribute to the effective use of alcohol formulations.

Key words skin permeation; ethanol; pretreatment; electrical skin resistance; enhancer; permeation route

Ethanol (EtOH) is well known as an ingredient of alcoholic beverages and has been utilized as a medicine for humans with different efficacies since ancient times. Currently, several skin disinfectants containing EtOH are frequently utilized in hospitals and public facilities for preventive purposes against viral infections. EtOH is a commonly used solvent just like water in pharmaceutical formulations, and is applied as a skin permeation enhancer, a skin disinfectant, and a solubilizer for poorly-soluble drugs. Tinctures, lotions, gels and liniments are dosage forms containing EtOH. Transdermal drug delivery systems and topical formulations often have the problem of low skin permeation of active ingredients. The skin barrier is mainly constituted by the outermost layer of skin, the stratum corneum, which dramatically restricts skin permeability of drugs. EtOH in topical formulations greatly enhances skin permeation of drugs. Estradiol and fentanyl dermal patches are typical examples developed using EtOH to enhance their transdermal deliveries.[1–3] It has been proposed that the mechanisms of EtOH effects on the stratum corneum are extraction of lipids, increases in lipid fluidity, enhancement of drug solubility in stratum corneum lipids, changes in skin hydration, effects on the putative pore pathway, alterations in keratinized proteins, and effects on solvent drugs.[4,5] Regarding the pretreatment effects of EtOH, however, little was found on skin permeation of drugs compared with simultaneous application of EtOH and drugs. Furthermore, few reports were found on the relation between the skin permeation of drugs and their lipophilicity and/or molecular weight after treatment with EtOH.

In the present study, deuterium oxide (D_2O), isosorbide mononitrate (ISMN), isosorbide dinitrate (ISDN), calcein sodium (CA-Na), and fluorescein isothiocyanate-dextran 4 kDa (FD-4, 3.3–4.4 kDa) were selected as model drugs and the pretreated effect of EtOH was determined on their *in vitro* skin permeations. These five model drugs were selected because of their different molecular weights and log K_{ow} (logarithm of octanol–water partition coefficient at 37°C). Table 1 shows the physicochemical properties of these model drugs. Yucatan micropig (YMP) skin was selected as a permeation membrane as it has been frequently used for skin permeation studies.[10–12] Drug permeability and histological characteristics such as hair density in porcine skin closely resemble human skin.[13–16] Furthermore, electrical skin resistance was determined to investigate the effects of EtOH pretreatment on skin permeabilities of small ions such as Na^+ and Cl^-.

MATERIALS AND METHODS

Materials EtOH (99.5%, HPLC grade), D_2O (99.9%, NMR grade), sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ISMN and CA-Na were obtained from Tokyo Kasei Kogyo Co., Ltd.

Table 1. Physicochemical Parameters of Model Drugs

Model drugs	Molecular weights	Log K_{ow} [a)]
Deuterium oxide (D_2O)	20	—
Isosorbide-5-mononitrate (ISMN)	191	0.442[b)]
Isosorbide dinitrate (ISDN)	236	1.22[c)]
Calcein sodium (CA-Na)	668	−3.5[d)]
Fluorescein isothiocyanate-dextran 4 kDa (FD-4)	3300–4400	−0.773[e)]

a) Logarithm of octanol/water partition coefficient at 37°C. *b*) Hatanaka *et al.*[6)] *c*) Hatanaka *et al.*[7)] *d*) Yamada *et al.*[8)] *e*) Todo *et al.*[9)]

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(Tokyo, Japan). ISDN was supplied by Chugoku Kayaku Co., Ltd. (Hiroshima, Japan). FD-4 was obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, U.S.A.). All other reagents and solvents were of reagent grade or HPLC grade, and were used without further purification.

Animals Frozen YMP skin sets (female: 5 months old) were obtained from Charles River Japan Inc. (Yokohama, Japan). Frozen YMP skin sets were stored at −80°C until permeation experiments. Frozen YMP skin was thawed at room temperature. YMP skin slices of 1.2 mm-thickness were prepared using a dermatome (Acculan® 3Ti Dermatome; B. Braun, Tuttlingen, Germany) to separate the stratum corneum and upper epidermis from the dermis and subcutaneous tissue. Stripped YMP skin was prepared by 50 consecutive tape-strippings of the stratum corneum from YMP skin. All animal studies were done with the recommendations of the Institutional Board for Animal Studies, Josai University (Sakado, Saitama, Japan).

***In Vitro* Skin Permeation Experiments** Dermatomed YMP skin membranes were mounted in vertical-typed diffusion cells (effective diffusion area: 3.14 cm^2). Then, 1.5 mL of 0, 20, 40, 60, 80, or 99.5% (v/v) EtOH aqueous solution was applied to the stratum corneum side and twice-diluted pH 7.4 phosphate-buffered saline (PBS) (for D_2O) or PBS (for ISMN, ISDN, CA-Na and FD-4) was applied to the dermal side (receiver cell volume: 18 mL). Each concentration of EtOH aqueous solution was removed after EtOH treatment for 12 h, and skin surfaces were washed out three times with distilled water. In addition, the receiver solution was replaced by twice-diluted PBS or PBS. Then, 1.0 mL of 55.2 mM D_2O, 700 mM ISMN, 4.0 mM ISDN, 1.0 mM CA-Na, or 1.0 mM FD-4 in PBS was applied to the stratum corneum side. Since D_2O was twice diluted with PBS to measure its concentration in receiver solution, the twice-diluted PBS was applied to the dermal side. Permeation experiments were performed at 32°C over 8 h (ISDN), 12 h (D_2O and ISMN), or 30 h (CA-Na and FD-4) through YMP skin, while the receiver solution was continuously stirred with a star-head-type magnetic stirrer. At predetermined times, an aliquot (0.5 mL) was withdrawn from the receiver solution and the same volume of twice-diluted fresh PBS or PBS was added to keep the volume constant.

Determination of D_2O Concentrations of D_2O in the sample were determined by an FT-IR (IRAffinity-1; Shimadzu, Kyoto, Japan). Determination of D_2O was performed without further preparation of the samples. D_2O was quantified by measuring the intensity of the O-D stretching vibrational band at 2512 cm^{-1}.

Determination of CA-Na and FD-4 Concentrations of CA-Na and FD-4 in samples were analyzed using a spectrofluorophotometer (RF 5300PC; Shimadzu) at excitation wavelengths of 488 and 490 nm, respectively, and at fluorescent emission wavelengths of 515 and 520 nm, respectively.

Determination of ISMN and ISDN Concentrations of ISMN and ISDN in samples were determined using an HPLC system (Prominence; Shimadzu) equipped with a UV detector (SPD-M20A; Shimadzu). Briefly, 0.2 mL of ISMN and ISDN samples were added to the same volume of neat methanol for ISMN or methanol containing an internal standard of ISDN (butyl 4-aminobenzoate; 1.0 μg/mL), respectively, and were vortex-mixed. After centrifugation at 3600×***g*** at 4°C for 5 min, the resulting supernatant (20 μL) was directly injected into the HPLC system. Chromatographic separation was performed using a Unison UK-C18 (3 μm, 75×4.6 mm i.d.; Imtact, Kyoto, Japan) at 40°C. Mobile phases were distilled water–acetonitrile (ISMN; 9 : 1, v/v, ISDN; 3/2, v/v) and the flow rate was 1.0 mL/min. Detection was performed at UV 220 nm.

Measurement of Skin Resistance Skin resistance was determined by an impedance meter (AS-TZ-1; Asahi Techno Lab. Ltd., Yokohama, Japan) after YMP skin permeation experiments. Skin resistance indicates reversal of ion transport through the skin.

Analysis of Permeation Parameters Cumulative amounts of drug permeating through skin into the receptor compartment were plotted against time to obtain skin permeation profiles. The steady state flux (J) was estimated from the slope of the linear portion of the profile. From J and donor concentrations (C_v), the permeability coefficient (P) was calculated using the following Eq. 1:

$$P = J/C_v \quad (1)$$

In addition, the enhancement ratio was calculated by dividing the P-value of model drugs through skin pretreated with various EtOH concentrations by that through pretreated skin with water to determine the effect of EtOH pretreatment on the P-value. Furthermore, the other permeation parameters were analyzed by Scheuplein equation.[17] The Diffusion coefficient (D) was estimated by time-lag method. The partition coefficient (K) from the donor solution into the skin was estimated from J, C_v, D and the thickness of the skin (L) in the following Eq. 2:

$$J = K \cdot C_v \cdot D / L \quad (2)$$

RESULTS

Effect of EtOH Pretreatment on D_2O Permeation through YMP Skin The effect of EtOH pretreatment was investigated on YMP full-thickness skin permeation of D_2O. The skin permeation test was performed using EtOH-pretreated skin. Figure 1 shows that the cumulative amount of D_2O that permeated over 12 h through skin pretreated with different concentrations of EtOH. Little effect was observed on skin permeability of D_2O after pretreatment with different concentrations of EtOH. In addition, skin permeability of D_2O through pretreated skin with different concentrations of EtOH was almost the same as that through skin with water alone over 12 h.

Effect of EtOH Pretreatment on FD-4 Permeation through YMP Skin The effect of EtOH pretreatment was investigated on skin (full-thickness skin or stripped skin) permeability of FD-4. Figures 2a and b show the time course of the cumulative amount of FD-4 that permeated through full-thickness skin or stripped skin, respectively. Permeabilities of FD-4 through full-thickness skin were increased by pretreatment with low concentrations of EtOH, whereas they were decreased by pretreatment with high concentrations of EtOH. The cumulative amount of FD-4 that permeated through full-thickness skin pretreated with 99.5% EtOH was reduced to 10% over that with water pretreatment, whereas that through stripped skin was reduced to 50%. In other words, pretreatment with 99.5% EtOH affected barrier function in the stratum corneum more than that in the viable epidermis and

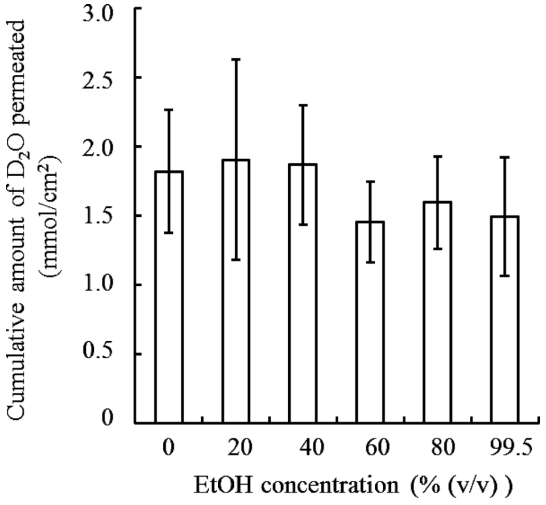

Fig. 1. Effect of EtOH Pretreatment on Cumulative Amounts of D_2O Permeated through YMP Skin over 12 h

Each value represents the mean±S.D. (n=3–4).

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Pretreatment Effect of Different EtOH Concentrations on the Skin Permeability Coefficient and Reciprocal of Electrical Skin Resistance Relationships were evaluated between the reciprocal of electrical skin resistance or skin permeability of drugs and pretreatment concentrations of EtOH. The skin permeability coefficient was calculated from the steady-state flux and applied drug concentrations. The reciprocal of electrical skin resistance was calculated from electrical impedance of the skin after the skin permeation test.

Table 2 shows skin permeability coefficients of model drugs obtained from skin permeation profiles after treatment with different concentrations of EtOH. Figures 3a and b show the effect of EtOH pretreatment on the enhancement ratio of skin permeability coefficient of drugs. Constant skin permeabilities of D_2O and ISDN were observed independently of EtOH concentrations. Although skin permeabilities of ISMN, CA, and FD-4 were increased by EtOH pretreatment until 40%, a gradual decrease was observed with an increase in EtOH concentrations of more than 60%. Interestingly, skin permeabilities of ISMN, CA, and FD-4 were dramatically lower with 99.5% EtOH than that with water pretreatment. In spite of the similar molecular weights of ISMN and ISDN, the effects of EtOH pretreatment were much different on skin permeabilities of these drugs. Skin permeation coefficients of FD-4 were greatly changed by EtOH concentration. Thus, the effect of EtOH concentration on the diffusion coefficient and partition coefficient of FD-4 was investigated. Figure 4 shows the effect of EtOH pretreatment on the enhancement ratio of diffusion coefficient and partition coefficient. Constant diffusion coefficients of FD-4 were observed independently of EtOH concentration, whereas partition coefficients of FD-4 was increased by EtOH pretreatment until 40% and a gradual decrease was observed with an increase in EtOH concentration of more than 60%. The profile of partition coefficient was similar to that of permeation coefficient.

Figure 5 shows the effect of EtOH pretreatment on the reciprocal of electrical skin resistance. In the case of all model drugs, the reciprocal of electrical skin resistance was higher after pretreatment with 20% and 40% EtOH, whereas it was

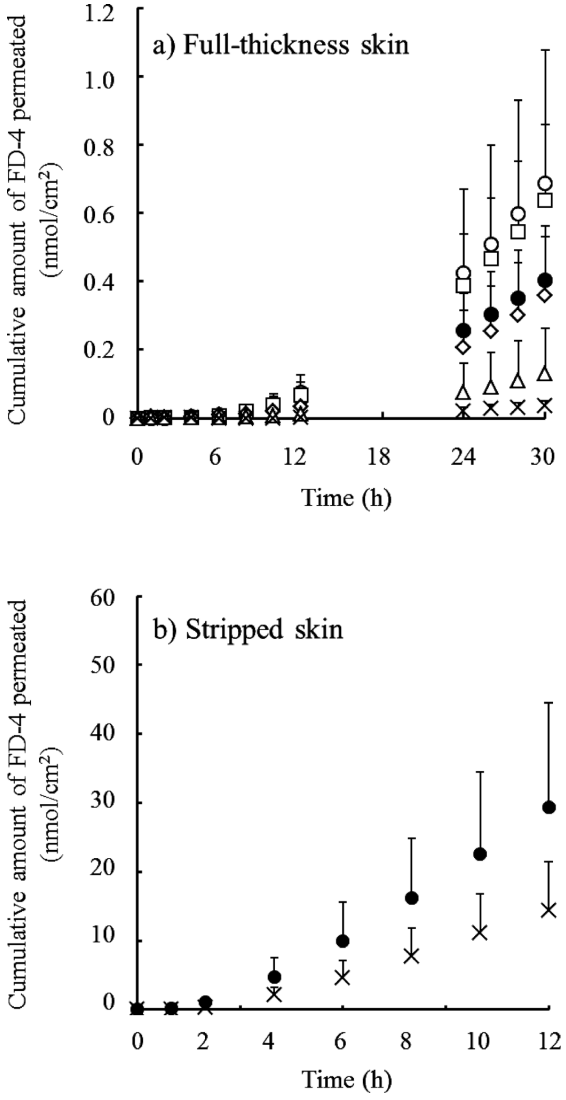

Fig. 2. Time Course of Cumulative Amounts of FD-4 Permeated through Full-Thickness Skin (a) and Stripped Skin (b) after Pretreatment with Different Concentrations of EtOH

Water (●), 20% EtOH (○), 40% EtOH (□), 60% EtOH (◇), 80% EtOH (△), 99.5% EtOH (×). Each point represents the mean±S.D. (n=4–7).

decreased with an increased EtOH concentration of more than 60%.

Figure 6 shows relationships between the reciprocal of electrical skin resistance and skin permeability coefficient of drugs. Good relationships were observed between the skin permeability coefficient and reciprocal of electrical skin resistance ($p<0.05$) in cases of ISMN, CA, and FD-4. Intercept of the regression line was positive in D_2O and ISDN permeations, whereas it was negative in ISMN, CA, and FD-4 permeations.

DISCUSSION

In the present study, the effect of EtOH pretreatment on skin permeation of drugs was investigated using dermatomed YMP skin. YMP skin thickness was set at 1.2 mm, and this was thicker than that used in ordinary skin permeation tests. YMP skin permeabilities of drugs were reported to be increased with an increase in age.[18] Increases in flux were

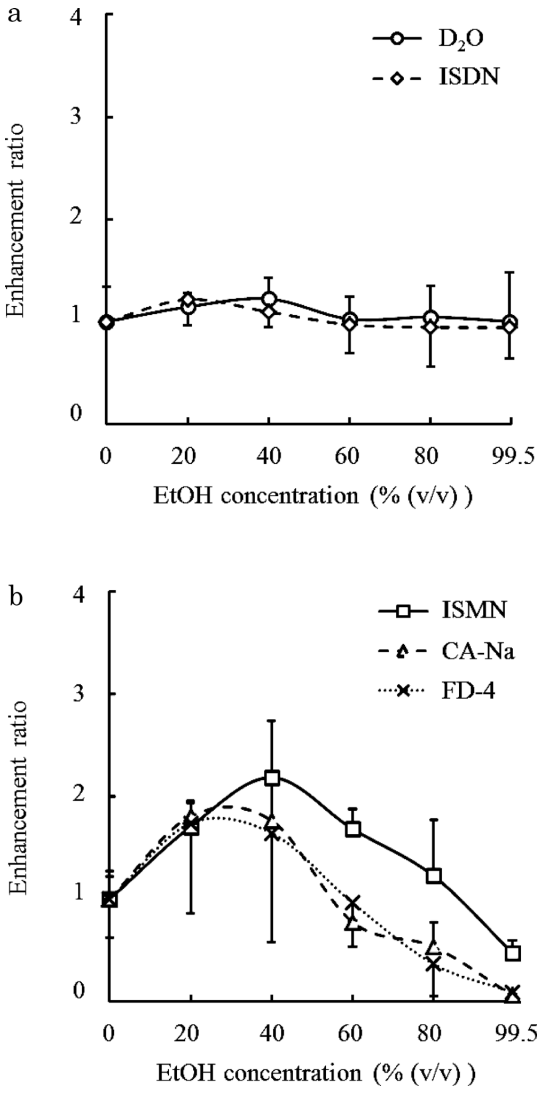

Fig. 3. Effect of EtOH Pretreatment on the Enhancement Ratio of Permeability Coefficient of Several Model Drugs

Each point represents the mean±S.D. (n=3–7).

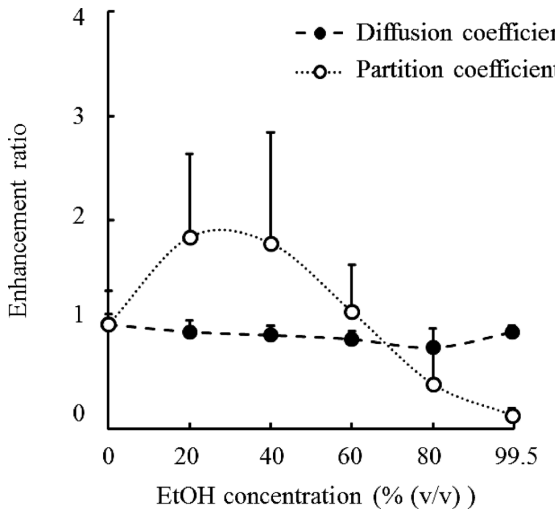

Fig. 4. Effect of EtOH Pretreatment on the Enhancement Ratio of Diffusion Coefficient or Partition Coefficient of FD-4

Each point represents the mean±S.D. (n=4–7).

observed with growth of hair follicle tissue, probably because the long hairs of dermatomed skin would be cut by dermatomation.[19] Therefore, we used thicker skin membranes to avoid variations in skin permeation data.

Skin permeabilities of hydrophilic compounds, ISMN, CA, and FD-4, altered by EtOH pretreatment. The present skin permeation profiles of FD-4 through full-thickness skin or stripped skin suggest that EtOH pretreatment may mainly affect the hydrophilic pathway of drugs in the stratum corneum and slightly affect the viable epidermis and dermis. EtOH is known to affect skin permeabilities of various compounds. Low concentrations of EtOH were reported to increase liquid fluidity especially near the polar region in intercellular lipids of the stratum corneum, and high concentrations of EtOH was found to extract lipids from the stratum corneum.[20] Additionally, 100% EtOH has been suggested to markedly alter skin permeability, because 100% EtOH stabilizes the gel phase of the lipid bilayer in the stratum corneum or removes water from the skin.[21] Hatta *et al.* proposed that EtOH applied to the stratum corneum disrupted the orthorhombic hydrocarbon-chain packing structure, and partially disrupted the structure of soft keratin in the corneocytes to form EtOH pools in the intercellular lipid matrix and was a transcellular pathway for hydrophilic molecules.[22] Furthermore, EtOH might cause a protein denaturation in the stratum corneum, viable epidermis and dermis. Consequently, changes in skin permeabilities of hydrophilic drugs in this study may result from changes of partition coefficients by EtOH pretreatment. On the other hand, constant skin permeabilities of D_2O and ISDN were observed independently of EtOH concentration, suggesting that EtOH pretreatment could not affect the diffusivity and partition coefficients of these compounds.

In the case of all permeation studies using different model drugs, the effect of EtOH pretreatment showed the same tendency on electrical skin resistance. The reciprocal of electrical skin resistance was slightly increased by pretreatment with low concentrations of EtOH and was slightly decreased by pretreatment with high concentrations of EtOH. Because

Table 2. Permeability Coefficient of Model Drugs Obtained from Their Skin Permeation Profiles

EtOH concentration (% (v/v))	0	20	40	60	80	99.5
P_{D_2O} (cm/s)×10^{-6}	1.46±0.50	1.67±0.19	1.78±0.30	1.49±0.32	1.52±0.45	1.46±0.70
P_{ISMN} (cm/s)×10^{-7}	1.57±0.43	2.68±0.39	3.43±0.88	2.64±0.31	1.92±0.86	0.74±0.19
P_{ISDN} (cm/s)×10^{-6}	2.73±0.07	3.32±0.69	2.99±0.42	2.65±0.77	2.58±1.06	2.57±0.82
$P_{CA\text{-}Na}$ (cm/s)×10^{-8}	4.99±1.10	9.04±0.65	8.82±1.74	3.87±0.06	2.67±1.20	0.29±0.07
$P_{FD\text{-}4}$ (cm/s)×10^{-9}	7.97±2.99	13.9±7.03	13.1±8.51	7.68±3.40	2.92±2.50	0.73±0.66

Mean±S.D. (n=3–7).

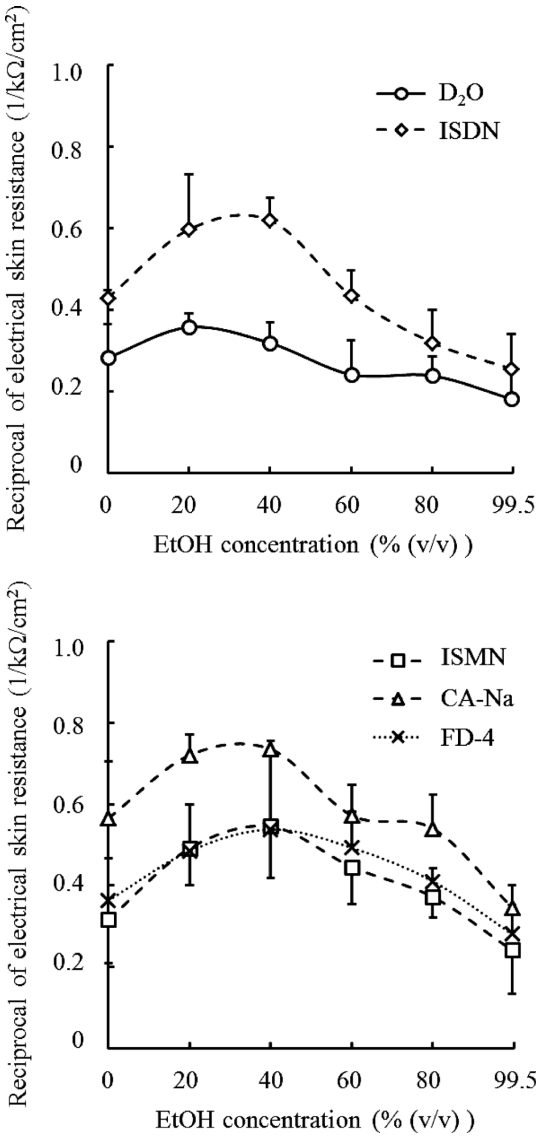

Fig. 5. Effect of EtOH Pretreatment on the Reciprocal of Electrical Skin Resistance

Each point represents the mean±S.D. (n=3–7).

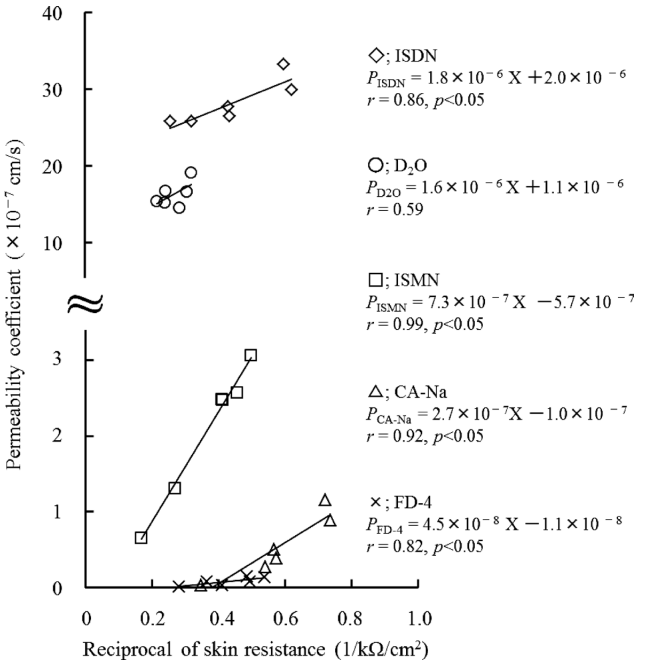

Fig. 6. Relationships between the Reciprocal of Electrical Skin Resistance and Permeability Coefficient of Several Model Drugs

Solid straight lines were calculated by the least-square method. D_2O (○), ISMN (□), ISDN (◇), CA-Na (△), FD-4 (×).

electrical skin resistance indicates the difficulty for skin permeation of total (low molecular) ions through the skin, the present results suggest that EtOH pretreatment alters the flux of low molecular ions through the skin. Additionally, the reciprocal of electrical skin resistance was well correlated with skin permeability of hydrophilic compounds. Higher skin permeation of compounds was observed with lower electrical skin resistance and *vice versa*. Thus, EtOH pretreatment on the skin could affect permeation pathways of ionically formed compounds. Although certain levels of ion permeabilities were confirmed under all pretreatment conditions, skin permeabilities of the hydrophilic compounds, CA and FD-4, were dramatically decreased after pretreatment with 99.5% EtOH. Because the molecular (ion) size of ISMN (191 Da), CA (668 Da), and FD-4 (3.3–4.4 kDa) are larger than Na^+, Cl^- and D_2O, pretreatment with high concentrations of EtOH could change pathway sizes through aqueous (pore) domains of the skin by reducing partition of drugs into the skin. These results suggest that skin permeation may depend on the molecular (ion) size of penetrants through the skin. Hence, EtOH may affect permeation pathway sizes of hydrophilic compounds. Electrical skin resistance was decreased more by 25 to 75% EtOH treatment than that by 0 or 100% EtOH treatment.[23] On the other hand, skin permeability of lipophilic and low-molecular ISDN was not influenced by EtOH pretreatment. By EtOH pretreatment, the reciprocal of electrical skin resistance was changed after the skin permeation test. Those after the permeation test of D_2O and CA-Na were in a range from 0.3 to 0.4 (1/kΩ/cm^2) and from 0.3 to 0.7 (1/kΩ/cm^2), respectively. That each of ISMN, ISDN, and FD-4 were in a range from 0.2 to 0.6 (1/kΩ/cm^2). The low reciprocal of skin resistance after the permeation test of D_2O would be due to low ion concentrations of twice-diluted PBS in receiver and donor solutions. The high reciprocal of skin resistance after the permeation test of CA would be due to the existence of drug derived Na^+ in CA-Na.

It was revealed from the relationships between the reciprocal of electrical skin resistance and permeability coefficient of drugs that high skin permeabilities of drugs were observed with low electrical skin resistance, and *vice versa*. A good relationship was observed between electrical skin impedance and skin permeability of several drugs in the presence of chemical enhancers.[24] In addition, the intercept in the regression line (Fig. 6) revealed that permeation pathways of ISMN, CA and FD-4 would partly corresponded to those of the ion transport pathway, and that these drug permeation pathways with negative intercepts would be larger than that for Na^+ or Cl^-. On the other hand, the intercept of D_2O and ISMN permeations were positive, as shown in Fig. 6, suggesting that the permeation routes of D_2O and ISMN were much different from those of hydrophilic substances.

Many kinds of skin disinfectants containing EtOH are used in hospitals or public facilities for prevention against viral

infections. The present results revealed that skin disinfectants excessively applied could alter skin permeability of substances, and thus attention should be paid to application of topical drugs after such EtOH treatments.

In conclusion, EtOH pretreatment greatly affected skin permeation of drugs and the effects were highly dependent on the physicochemical properties of the drugs. Furthermore, decreased skin permeabilities of ISMN, CA, and FD-4 after pretreatment with high concentrations of EtOH suggest that EtOH may affect the permeation pathway of hydrophilic compounds in the stratum corneum, especially at high concentrations.

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