Analysis of the Pretreatment Effect of Ethanol on the Stratum Corneum- and Hair Follicular-Penetration of Drugs Using the Hair Follicle-Plugging Method

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The hair follicle-plugging method was used to analyze the effects of EtOH on skin permeation pathways. Methods: *In vitro* permeation experiments were performed on 4 model drugs [isosorbide mononitrate (ISMN), ionized lidocaine (ionized LC), fluorescein (FL), and fluorescein isothiocyanate (FITC)-dextran 4 kDa (FD-4)] using excised pig ear skin. The skin permeations of ionized LC, FL, and FD-4 were decreased by hair follicle-plugging. Hair follicle-plugging prevented the skin permeation of FL and FD-4 in EtOH-pretreated skin, but did not prevent that of ISMN. On the other hand, the effect of hair follicle-plugging on the permeation of ionized LC was different among the pretreatment conditions. These results indicate that the EtOH pretreatment greatly affected the aqueous pathway in the stratum corneum and hair follicles.

Key words   skin permeation; aqueous pathway; hair follicle-plugging; EtOH pretreatment; permeation route; enhancer

Drug delivery systems (DDSs) have been investigated extensively to provide drug formulations with sustained release and/or targeting properties. Transdermal therapeutic systems (TTSs) are a typical example of DDSs. Therapeutic medicines in TTSs that have already been marketed include tulobuterol for the treatment of bronchial asthma,[1] clonidine for hypertension,[2] nitroglycerin and isosorbide dinitrate for angina pectoris,[3,4] and rivastigmine for Alzheimer's disease.[5] However, the enhancement of skin permeation is sometimes required for drugs. Skin penetration enhancers are one of the methods used to increase the skin permeation of drugs.

Skin penetration enhancers include alcohols,[6,7] fatty acids,[8,9] fatty acid esters,[10,11] terpenes,[12,13] pyrrolidones,[14,15] surfactants,[16,17] and Azone.[18,19] Among these enhancers, ethanol (EtOH) is the most commonly used additive in topical formulations. It is applied not only as a skin penetration enhancer, but also a solvent, such as water, for skin disinfection, as well as a solubilizer for poorly-soluble drugs. Estradiol and fentanyl dermal patches are typical examples that contain EtOH to enhance their absorption into the skin.[20–22] The mechanisms underlying the penetration-enhancement effect of EtOH on the stratum corneum barrier were previously reported to be lipid extraction, an increase in lipid fluidity, the 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.[23,24] Although many studies have already examined skin permeability with the simultaneous application of EtOH and drugs, few have investigated the effects of an EtOH pretreatment on the skin permeation of drugs. EtOH-containing formulations are frequently applied to the skin surface for the purpose of preventing disinfection and other therapeutic treatments. Other topical formulations may also be applied following treatments with EtOH-containing formulations.

We previously investigated the effects of an EtOH pretreatment on the pig skin permeability of drugs.[25] Our findings demonstrated that the skin permeation of hydrophilic drugs was increased and decreased by pretreatments with low and high concentrations of EtOH, respectively. We then speculated that EtOH may have affected the permeation pathway of these drugs. The permeation pathway of hydrophilic drugs may be skin appendages as well as the aqueous region of the stratum corneum.[26–28] Therefore, we developed a method for hair follicle infundibulum-plugging with a mixture of silicone grease, cyanoacrylate adhesive and nile red, referred to as the hair follicle-plugging method, to analyze drug permeability through skin appendages.[29] This method revealed that the hair follicle could be the primary permeation pathway for water-soluble compounds such as ionized lidocaine (ionized LC) and fluorescein isothiocyanate-dextran 4 kDa (FD-4).

As described above, we previously reported the effects of EtOH on the skin permeability of drugs and developed an evaluation method for drug permeation through specific pathways in the skin. The next step was to investigate the effects of EtOH on different aqueous permeation pathways. Few studies have examined hair follicle penetration in association with the application of EtOH, which is commonly used as a skin penetration enhancer. The purpose of the present study was to analyze the effects of an EtOH pretreatment on stratum corneum- and hair follicle-permeation. Isosorbide mononitrate (ISMN), ionized LC, fluorescein sodium (FL), FD-4 were selected as model drugs and the individual contribution of the stratum corneum and hair follicles was determined from total skin permeation using *in vitro* skin permeation experiments. Furthermore, we considered the effects of the EtOH pretreatment on electrical skin resistance and lipid composition in skin, as determined by TLC analysis.

Experimental

Materials   LC and FD-4 were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, U.S.A.). Nile red was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). ISMN was ob-

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Table 1. Physicochemical Properties of Model Drugs

Model compounds	Molecular weights	Log K_{ow}[a]	pK_a[e]
Isosoibide-5-mononitrate (ISMN)	191	-0.15[b]	$-$[f]
Lidocaine hydrochloride (ionized LC)	271	-0.93[c]	7.9[31]
Fluorescein sodium (FL)	376	-0.62[d]	2.3, 4.3, 6.5[32]
Fluorescein isothiocyanate-dextran 4 kDa (FD-4)	3300–4400	-0.77[d]	6.4[33]

a) Logarithm of the octanol/phosphate buffer partition coefficient at 37°C. *b*) Determined with pH 7.4 PBS.[30] *c*) Determined with pH 5.0 PB. *d*) Determined with pH 7.4 PBS.[28] *e*) Acid dissociation constant. *f*) Electrically neutral compound.

tained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). FL, EtOH (99.5%, HPLC grade), sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents were of reagent grade or HPLC grade, and used without further purification. Table 1 shows the physicochemical properties of the model drugs.

Animals Pig ear skin sets were obtained from Saitama Experimental Animals Supply Co., Ltd. (Sugito, Saitama, Japan), and stored at -30°C until the permeation experiments. Pig ear skin was thawed at 37°C on the day of the experiment. Pig ear skin slices of 1.0 mm in 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. All animal studies were performed following the recommendations of the Institutional Board for Animal Studies, Josai University (Sakado, Saitama, Japan).

Hair Follicle-Plugging Process Hair follicles were plugged with a silicone grease–cyanoacrylate adhesive mixture (PA-mixture) to block drug penetration through them.[29] The PA-mixture consisted of equal parts of silicone grease (Super Lube® Silicone Dielectric Grease; Synco Chemical Corp., Bohemia, NY, U.S.A.) and α-cyanoacrylate adhesives (Aron Alpha Jelly; Konishi Co., Ltd., Osaka, Japan) with small amounts of nile red being applied to the hair follicles. Nile red was used to visualize the skin area treated by the PA-mixture. Thus, hair follicles were plugged to prevent drug penetration through the follicular pathway. There were approximately 60 hair follicles on the effective skin penetration area (1.77 cm²), 30 of which were plugged with the PA-mixture.

In Vitro **Skin Permeation Experiments** A dermatomed pig ear skin membrane was mounted on vertical-typed diffusion cells (effective diffusion area: 1.77 cm²), followed by the application of 0, 20, or 99.5% (v/v) EtOH aqueous solution to the stratum corneum side and pH 7.4 phosphate-buffered saline (PBS) to the dermal side (receiver cell volume: 6 mL). Each concentration of the EtOH aqueous solution was removed after the EtOH treatment for 12 h and the skin permeation experiment was then performed. When the skin permeation experiment through hair follicle-plugged skin was being conducted, EtOH-pretreated skin was removed from the vertical-typed diffusion cell and the skin was treated with the hair follicle-plugging method. The receiver solution was also replaced by fresh PBS. A total of 0.8 mL of 700 mM ISMN, 100 mM LC, 1 mM FL, or 5 mM FD-4 was then applied to the stratum corneum side. The donor solution of LC was adjusted to pH 5.0 by 1/30 mM phosphate buffer to become the LC cation, while that of ISMN, FL and FD-4 was dissolved in PBS, such that LC predominantly existed in an ionized state in the pH 5.0 solution, and FL and FD-4 predominantly existed in an

ionized state in the pH 7.4 solution. Permeation experiments were performed at 32°C over 8 h (ISMN and LC) or 12 h (FL and FD-4) through pig ear skin, while the receiver solution was continuously stirred with a star-head-type magnetic stirrer. An aliquot (0.5 mL) was withdrawn from the receiver solution at predetermined times and the same volume of fresh PBS was added to keep the volume constant. Each experiment was repeated five times.

Determination of ISMN and LC ISMN and LC concentrations were determined in the samples using an HPLC system (Prominence; Shimadzu, Kyoto, Japan) equipped with a UV detector (SPD-M20A; Shimadzu). Briefly, 0.2 mL of ISMN and LC samples were added to the same volume of neat methanol for ISMN or acetonitrile containing an internal standard for LC (4-hydroxybenzoic acid *n*-amyl ester; 20 μg/mL), respectively, and vortex-mixed. After centrifugation at $3600 \times \boldsymbol{g}$ and 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.; Imtakt, Kyoto, Japan) at 40°C. The mobile phases for ISMN and LC were distilled in water–acetonitrile (9:1, v/v) and 10 mM phosphate buffer (pH 6.5)–acetonitrile (1:1, v/v), respectively, and the flow rate was 1.0 mL/min. Detection was performed at UV 220 nm (ISMN) and 230 nm (LC).

Determination of FD-4 and FL FL and FD-4 concentrations in the samples were analyzed using a spectrofluorophotometer (RF 5300PC; Shimadzu) at excitation wavelengths of 480 nm and 490 nm, respectively, and at fluorescent emission wavelengths of 535 nm and 520 nm, respectively.

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

Identification of Lipid Extracted from the Skin with the EtOH Treatment Thin-layer chromatography (TLC) was performed to identify the lipids extracted from EtOH-pretreated skin. A total of 0, 20, and 99.5% EtOH solution (1.0 mL) and chloroform–methanol (2:1, v/v) solution (1.0 mL) were applied to the stratum corneum side (1.77 cm²) over 12 h, and the pretreatment solution was then withdrawn. The withdrawn solution was evaporated to dryness under a N₂ purge. The residue was reconstituted with chloroform/methanol solution (1.0 mL). The sample (50 μL) was applied to a HPTLC plate (HPTLC Silica gel 60 F254; Merck, Darmstadt, Germany) developed using chloroform/methanol/acetic acid (190/9/1) for approximately 40 min. Detection was performed by placing 10% copper sulfate in 8% phosphoric acid and heating at 180°C for 10 min. A total of 0.05% triolein, 0.05% cholesterol, 0.05% palmitic acid, and 0.05% *N*-stearoyl-phytosphingosine

(ceramide III) in chloroform/methanol solution was used as a control sample in the TLC analysis.

Analysis of Permeation Parameters The cumulative amount of the drug permeating through the skin into the receptor compartment was plotted against time to obtain the skin permeation profile. The PA-mixture-treated area was measured with a digital camera attached to a microscope (DP21; Olympus Corp., Tokyo, Japan). The corrected effective permeation area was calculated by subtract the PA-mixture-treated area from the effective permeation area. Then, the cumulative amount of drugs permeated through a unit area of the skin was calculated using the corrected effective permeation area.[29] The steady state flux (J) was estimated from the slope of the linear portion of the profile. The permeability coefficient (P) was calculated from J and the donor concentration (C_v), by the following equation,

$$P = \frac{J}{C_v} \tag{1}$$

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 effects of the EtOH pretreatment on the P-value.

Statistical Analysis

Statistical analysis was performed using an unpaired Student's t-test and ANOVA. A p value less than 0.05 was considered significant.

Results

Effect of the EtOH Pretreatment on the Pig Ear Skin Permeation of Several Hydrophilic Drugs The effect of the pretreatment with EtOH was investigated on the dermatomed pig ear skin permeation of four hydrophilic drugs: ISMN, ionized LC, FL, and FD-4. Based on the findings of a previous study, EtOH concentrations for the pretreatment aqueous EtOH solution were set at 0%, 20%, and 99.5% EtOH, in which skin permeation was predicted to markedly increase and decrease by 20% and 99.5% EtOH, respectively. *In vitro* skin permeation experiments were performed on the four compounds using aqueous drug solutions after the treatment with any concentration of the EtOH solution without the drug. Figure 1 illustrates the relationship observed between the concentration of EtOH used to pretreat the skin surface and logarithm of the skin permeability coefficient of the drug tested. The skin permeability coefficient was calculated from the steady-state flux and applied drug concentrations. In the case of the 0% EtOH (100% water) pretreatment, which was used as the control group, the highest permeability was observed for ISMN, followed by ionized FL, LC, and FD-4. The permeability coefficients of all model drugs through skin pretreated with 20% EtOH were higher than those through purified water-pretreated skin. In addition, in the case of 99.5% EtOH, the permeability coefficients were lower than those with 20% EtOH, although a marked difference was observed in the decreasing ratios. The permeability coefficients of FL and FD-4 for the pretreatment with 99.5% EtOH were markedly lower than those of the control group. The permeability coefficient of FL for 99.5% EtOH was lower than that of ionized LC, unlike that for 0% EtOH.
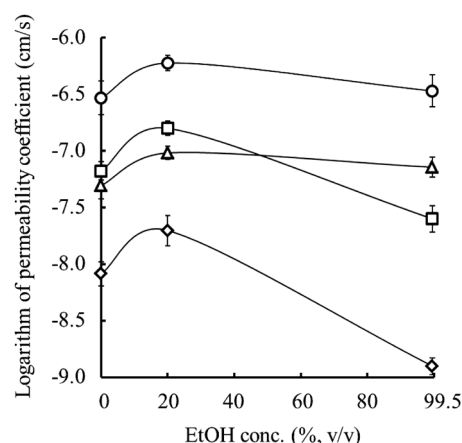

Fig. 1. EtOH Concentration Profile of the Logarithm of Permeability Coefficients of ISMN (○), Ionized LC (△), FL (□), and FD-4 (◇)

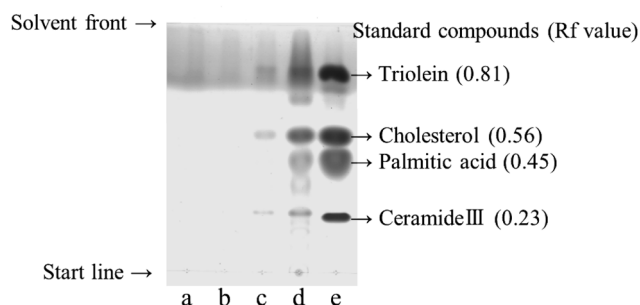Each point represents the mean±S.E. (n=5).

Fig. 2. Thin-Layer Chromatogram of Skin Lipids Extracted with Pretreated Water (a), 20% EtOH (b), 99.5% EtOH (c), Chloroform/Methanol (d), and Standard Solution (e)

Analysis of Lipids Extracted from Skin Pretreated with Various Concentrations of EtOH Figure 1 shows that the permeability coefficients of hydrophilic drugs could be changed by the different EtOH concentrations. These changes may be related to the composition and amount of lipids extracted from the skin surface. The pretreatment solution was withdrawn after skin was pretreated with different concentrations of EtOH, and the composition and amount of lipids were qualitatively analyzed using TLC. Chloroform–methanol (2:1), which has a strong extracting effect on skin lipids, was used as a positive control. Figure 2 shows the results obtained by the TLC analysis. No lipid spots were detected for 0% and 20% EtOH. Although weak spots for triglycerides, cholesterols, and ceramides were detected for 99.5% EtOH, these levels were markedly smaller than those of the positive control, the chloroform/methanol group. Moreover, fatty acids were not detected for every EtOH solution.

Pretreatment Effect of Different Concentrations of EtOH on the Skin Permeability of Hydrophilic Drugs and Electrical Skin Resistance Relationships were investigated between the skin permeability coefficients of drugs and the reciprocal of electrical skin resistance and pretreatment concentrations of EtOH. The reciprocal of electrical skin resistance was calculated from the electrical impedance of the skin following skin permeation experiments. Figure 3 shows the effects of the EtOH pretreatment on the enhancement ratio of the skin permeability coefficient of drugs or the reciprocal of electrical skin resistance. As shown in Fig. 1, the permeability
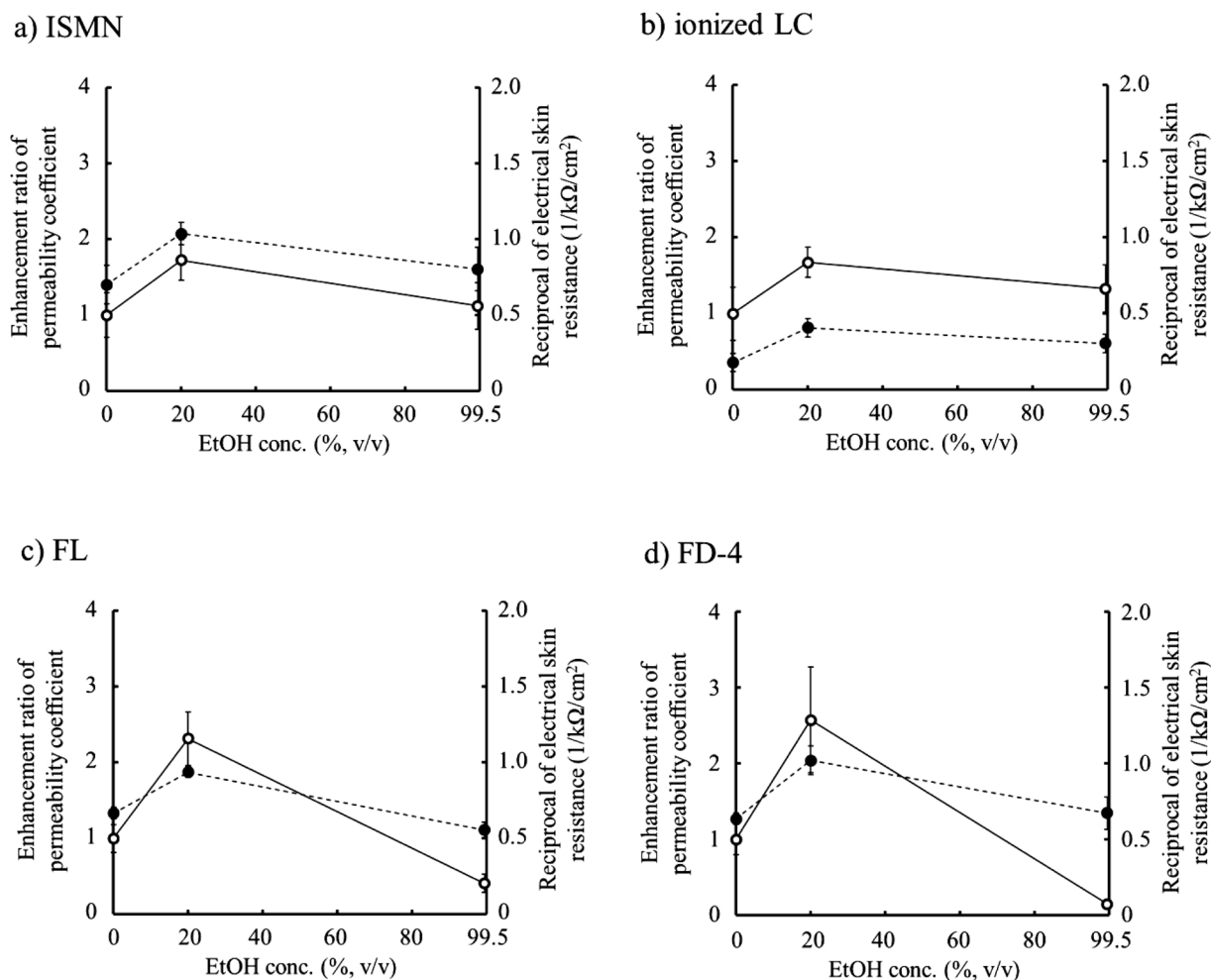
a) ISMN

b) ionized LC

c) FL

d) FD-4

Fig. 3.   Effect of the EtOH Pretreatment on the Enhancement Ratio of Permeability Coefficients (○) or Reciprocal of Electrical Skin Resistances (●) of ISMN (a), Ionized LC (b), CA (c), and FD-4 (d)

Each point represents the mean±S.E. (n=5).

coefficients of all model drugs were increased by the pretreatment with 20% EtOH. In addition, the permeation of FL was decreased by 99.5% EtOH, while that of FD-4 was decreased even further. The reciprocal of electrical skin resistance was increased and decreased after the pretreatment with 20% and 99.5% EtOH, respectively. Similar profiles were found for ISMN and ionized LC due to the effects of EtOH concentrations between the enhancement ratio of permeability coefficient and reciprocal of electrical resistance.

Effect of the EtOH Pretreatment on the Skin Permeation Pathway of Hydrophilic Drugs   Hair follicles, which are considered to be one of the permeation pathways of drugs through skin, were plugged using a newly established method. Approximately 60 hairs could be visually recognized in the effective penetration area (1.77 cm^2) on pig ear skin. Therefore, the number of hair follicles plugged was set at 30 (approximately half of the hair follicles) in the present study. *In vitro* skin permeation experiments were performed after hair follicle-plugging, and the contribution of hair follicles to skin permeation was estimated from the decreasing ratio in the cumulative amount of the drug that permeated through the skin with hair follicle-plugging against that without the treatment. Figure 4 shows the time course of the cumulative amount of model drugs that permeated through pig ear skin,

and Table 2a summarizes the cumulative amount of the drugs permeated over 8 h (ISMN and ionized LC) or 12 h (FL and FD-4) through the skin. With or without hair follicle-plugging, approximately the same amount of permeation was observed in the case of ISMN permeation. On the other hand, in the case of ionized LC, the cumulative permeation amount was decreased by hair follicle-plugging through 0% EtOH-treated skin, while negligible changes were observed through 20% and 99.5% EtOH-treated skin. The cumulative permeation amounts of FL and FD-4 were decreased by hair follicle-plugging, and the decreasing ratio was independent of the EtOH concentration. Table 2b summarizes the decreasing amount and ratio of the skin permeation of drugs by hair follicle-plugging after the pretreatment with different concentrations of EtOH. The decreasing amount and ratio of the ISMN permeation were low at any EtOH concentration, while those of ionized LC were high with the 0% EtOH pretreatment only. A high decreasing amount and ratio were observed under all conditions for the permeation of FL and FD-4, and that after 99.5% EtOH pretreatment was lower than those of the 0% and 20% EtOH pretreatments.

Discussion

In the present study, the effects of the EtOH pretreatment

a) ISMN

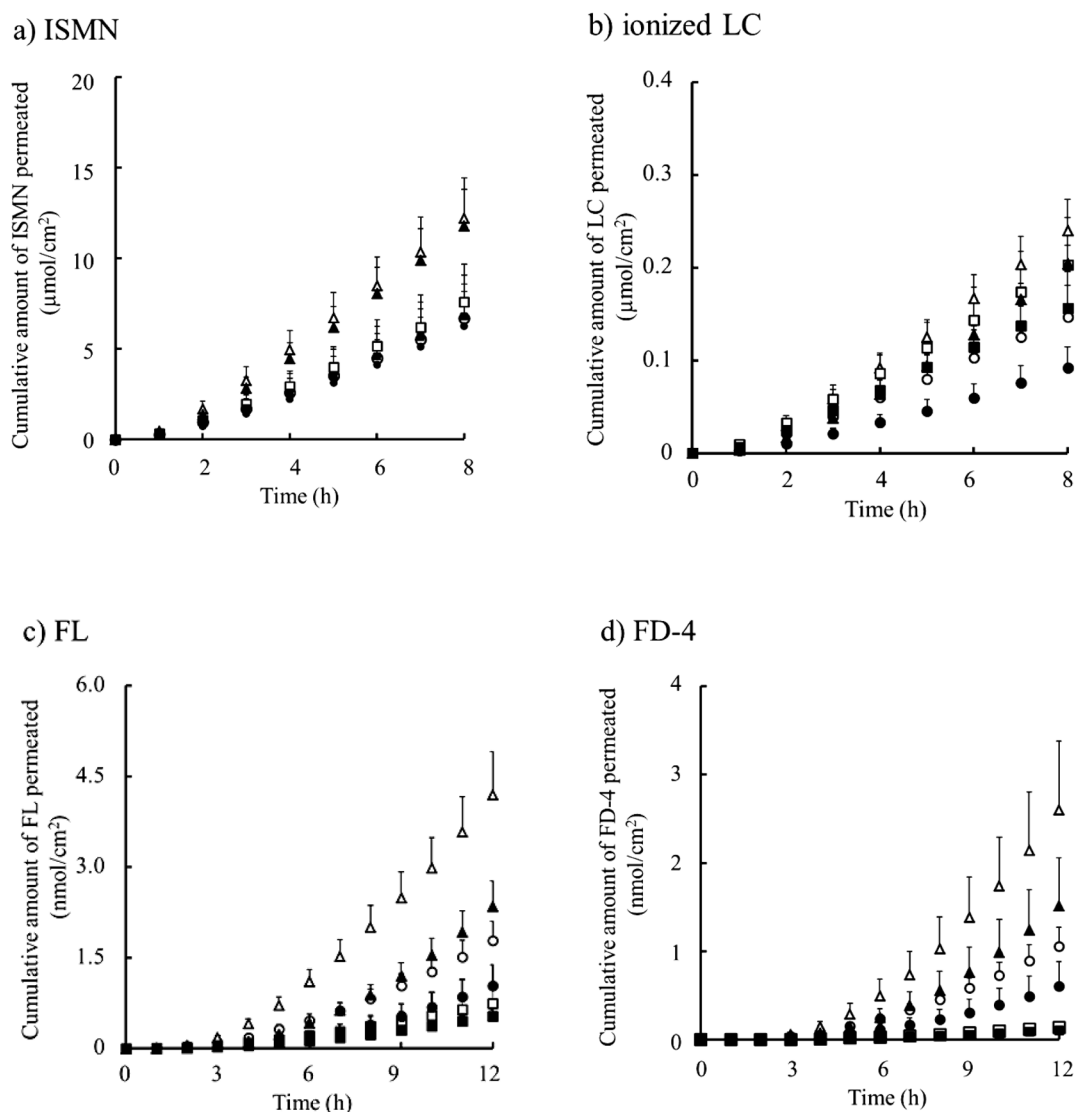## b) ionized LC

c) FL

d) FD-4

Fig. 4. Time Course of the Cumulative Amount of FD-4 That Permeated through Different Concentrations of EtOH Pretreated-Skin with or without 30-Hair Follicles-Plugging

Symbols; water pretreated-skin without hair follicle-plugging (○), 20% EtOH pretreated-skin without hair follicle-plugging (△), 99.5% EtOH pretreated-skin without hair follicle-plugging (□), water pretreated-skin with 30-hair follicles-plugging (●), 20% EtOH pretreated-skin with 30-hair follicles-plugging (▲), and 99.5% EtOH pretreated-skin with 30-hair follicles-plugging (■). Each point represents the mean±S.E. (n=5).

were investigated on the skin permeations of four hydrophilic drugs: ISMN, ionized LC, FL, and FD-4. The permeability coefficients of ISMN with a higher $\text{Log}K_{ow}$ and ionized LC with a smaller molecular weight among these four drugs were not significantly affected by the treatment with a high concentration of EtOH. When the skin permeations of FL and FD-4, which have similar $\text{Log}K_{ow}$, were compared, the decreasing ratio in FD-4 permeation was lower than that in FL after the pretreatment with a high concentration of EtOH. The permeation of FD-4 with a high molecular weight was strongly prevented by the high concentration of EtOH, which suggested that a high concentration of EtOH may decrease the size of the aqueous pathway in skin by the high concentration of EtOH. The decreasing ratio in the skin permeation of FD-4 obtained in the present study was higher than our previous findings for the permeation of calcein (molecular weight (MW); 645) after a pretreatment with a high concentration of EtOH.[25] On the other hand, the reciprocal of electrical skin resistance[34] obtained in the present study suggests that ion transport through

skin was detected under all conditions independently of the EtOH concentration. The low reciprocal of skin resistance of ionized LC has been attributed to low ion concentrations in the buffered solution (we used 1/30 mM PB instead of PBS) in the donor compartment, and the relationship between skin resistance and the EtOH concentration was similar to that of the other drugs. These results indicate that the permeability coefficients of Na^+ and Cl^- remained unchanged after the pretreatment with a high concentration of EtOH. Nevertheless, the permeability coefficients of FL and FD-4 were decreased, which indicated that the size of the aqueous pathway may have been decreased into a narrow pathway by the high concentration of EtOH or that the permeation routes of FL and FD-4 may be markedly different from those of Na^+ and Cl^-.

Although the present TLC analysis revealed that no lipids were extracted from the skin by 20% EtOH, the skin permeation of drugs was increased. Extracted skin lipids were detected following 99.5% EtOH; however, the amount was markedly smaller than that with the positive control, chloro-

Table 2. Difference in the Cumulative Amount of Model Drugs That Permeated through EtOH-Pretreated Skin at Each Concentration with or without 30-Hair Follicles-Plugging

a)

EtOH conc.	Cumulative amount of model drugs permeated (hair follicle-plugged skin)		
	0	20	99.5
ISMN (over 8 h, μmol/cm^2)	6.68±2.39 (6.26±1.91)	12.21±2.22 (11.79±2.01)	7.58±2.10 (6.80±1.77)
Ionized LC (over 8 h, μmol/cm^2)	0.147±0.049 (0.092±0.023)	0.240±0.034 (0.205±0.020)	0.203±0.051 (0.156±0.025)
FL (over 12 h, nmol/cm^2)	1.78±0.32 (1.04±0.34)	4.20±0.71* (2.35±0.42)	0.75±0.24* (0.53±0.11)
FD-4 (over 12 h, nmol/cm^2)	1.06±0.22 (0.60±0.28)	2.60±0.78 (1.51±0.55)	0.14±0.02* (0.10±0.03)

b)

EtOH conc.	Decreasing amount of model drugs permeated (reduction ratio)		
	0	20	99.5
ISMN	0.42 μmol −6.30%	0.42 μmol −3.40%	0.78 μmol −10.30%
Ionized LC	0.055 μmol −37.40%	0.035 μmol −14.60%	0.047 μmol −23.20%
FL	0.74 nmol −41.60%	1.85 nmol −44.00%	0.22 nmol −29.30%
FD-4	0.44 nmol −43.20%	1.09 nmol −41.90%	0.04 nmol −28.60%

Each value represents the mean±S.E. ($n=5$). *$p<0.05$: significantly different from 0% EtOH-pretreated skin.

form/methanol. Hatta *et al.* previously reported that EtOH had a moderate effect on lipid extraction from skin.[35] In addition, low concentrations of EtOH were reported to increase lipid fluidity in the intercellular lipids in the stratum corneum, and high concentration of EtOH might decrease the permeation pathway by protein denature in the stratum corneum, viable epidermis and dermis.[36]

At least two pathways have been proposed for the permeation of hydrophilic drugs and ions through skin: the aqueous region of the stratum corneum and skin appendages such as hair follicles.[26,37] It is difficult to evaluate the permeability of drugs through each specific penetration pathway using permeation experiments because topically-applied drugs are simultaneously permeated through these different pathways. One millimeter thick split porcine skin may have tunnel-like hair follicles. Several studies have already reported that these tunnel-like hair follicles may become a permeation route for nanoparticles, although nanoparticles could not permeate through full-thickness skin. Therefore, dermatomed skin was selected for the present study. However, the effects of EtOH on the skin permeation of chemical compounds differed between hydrophilic and lipophilic compounds in this experiment. Furthermore, the permeability coefficient of lipophilic compounds has been shown to decrease with an increase in the thickness of the dermis,[38] which also affected estimations of the blood concentrations of topically applied drugs.[39] Thus, unlike the skin permeation experiments using nanoparticles, skin permeation can be examined with the 1-mm thick skin used in the present study. We also selected 1-mm porcine split skin for one more reason. The highest skin permeation of hydrophilic chemical compounds was observed after the pretreatment with 20% EtOH in the present study. Manabe *et al.* reported that the skin permeation of hydrophilic compounds

was increased by the simultaneous application of 10 to 20% EtOH on full-thickness skin.[36] These findings were consistent with the results obtained in the present study. Therefore, 1-mm thick split porcine skin can also be used to evaluate enhancements caused by aqueous EtOH as well as full-thickness skin.

Several studies recently assessed hair follicle pathways.[40–47] The findings of our previous study, which used a newly developed hair follicle-plugging method, demonstrated that hair follicles played an importance role in the skin permeation of ionized LC and FD-4.[29] The present study was based on the assumption that hydrophilic drugs permeated through the aqueous pathway in the stratum corneum and hair follicles, and we evaluated the effects of the EtOH pretreatment on each specific skin permeation pathway using the hair follicle-plugging method. The number of hair follicles plugged was set as 30 because the amount of permeation through hair follicles should be markedly decreased by plugging approximately half of the recognizable hair follicles in the effective penetration area, and a good relationship was observed between the decreasing ratio of FD-4 permeation and the number of hair follicles plugged. The total number of hair follicles was estimated to be approximately 60 based on the interception of the linear extrapolation of the relationship. It was also difficult to plug all hair follicles because many unrecognized hair follicles existed under a stereoscopic microscope. Therefore, drug permeation through the hair follicle pathway could be estimated from the decreasing ratio or amount achieved by the hair follicle-plugging of 30 follicles.

Drug distribution and diffusion in and through skin barriers are very important factors for the skin permeation or penetration of chemical compounds. Patzelt *et al.* showed that follicular penetration with nanoparticles was reduced by 90% in excised skin.[48] This may have been due to a reduction in

permeation through the hair follicle pathway. The skin permeation of chemical compounds can generally be expressed by dissolution-diffusion phenomena. Thus, skin-insoluble nanoparticles could not permeate into the stratum corneum and remained in tube-like tissue, the hair follicles. Thus, a reduction in hair follicle size may directly affect the distribution of nanoparticles. On the other hand, high molecular weight hydrophilic compounds such as calcein and FD-4 could permeate into the stratum corneum and viable epidermis/dermis, although the permeation rates through full-thickness skin were extremely low.

Many studies on the transdermal deliveries of hydrophilic compounds have been performed using excised skin.[28,40,42,43,45,49] Evaluations of skin permeation parameters using excised skin are very useful for developing transdermal formulations. The skin permeation ratios of drugs through hair follicles against those through the whole permeation area should be considered to clarify the contribution of the hair follicle route. Thus, in vitro skin permeation experiments utilizing hair follicle-plugging may be of importance in evaluating skin permeation parameters both for the stratum corneum and hair follicle pathways. However, further studies, such as those using in-situ experiments, may be needed to consider the contribution of hair follicles against the overall skin permeation of chemical compounds. Since no significant difference was found in the skin permeation of ISMN with or without hair follicle-plugging, the aqueous pathway in the stratum corneum must be the primary permeation route for this drug. Ionized LC could permeate through both the aqueous pathway of the stratum corneum and hair follicles; however, the latter may be a more important route. In addition, the EtOH pretreat-

ment affected the aqueous pathway in the stratum corneum, and the contribution of the stratum corneum to the total skin permeation of ionized LC may have been high. Hair follicles may be the primary route for the permeation of FL and FD-4 because hair follicle-plugging decreased the amount of FL and FD-4 that permeated through. Furthermore, the permeability coefficients of FL and FD-4 were increased after the 20% EtOH pretreatment, and the decreasing ratio due to hair follicle-plugging for 20% EtOH was similar to that for 0% EtOH, which indicated that EtOH affected the aqueous pathway in the hair follicles. The reduction ratio of permeability coefficients by hair follicle-plugging for 99.5% EtOH was slightly lower than that for 0% or 20% EtOH because of the intrinsically very low permeabilities of FL and FD-4 for 99.5% EtOH. We previously reported that FL and FD-4 permeabilities through three-dimensional cultured skin were markedly lower than those through hairless rat skin,[28] and concluded that this may have been due to the absence of appendages in the cultured skin. The results of the present study, in which FL and FD-4 preferentially permeated through skin appendages, support our previous conclusion.

In summary, the permeability coefficients of ISMN, ionized LC, FL, and FD-4 were increased by the pretreatment with 20% EtOH and decreased by that with 99.5% (especially for the permeation of FL and FD-4); however, the approximately constant transport of ions was observed by the pretreatment with any concentration of EtOH. In addition, the decrease in drug permeation by hair follicle-plugging was observed for LC after the pretreatment with 0% EtOH and for FL and FD-4 with any concentration of EtOH. We finally decided on the skin permeation pathway of drugs following the EtOH
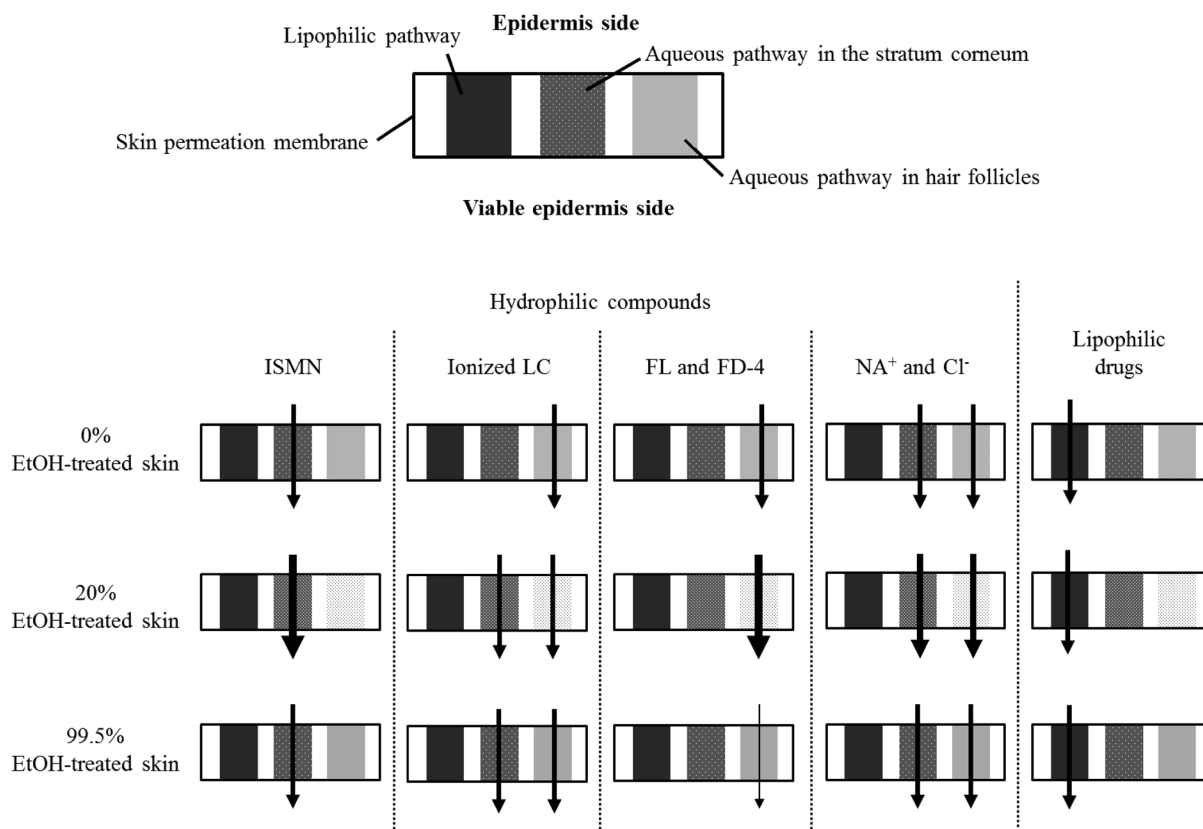

Fig. 5. Schematic Pathway Model of the Effects of EtOH on the Skin Permeation of Drugs

pretreatment from the results obtained as above. Figure 5 shows a schematic model of the skin permeation pathway. The penetrants were categorized into 5 groups for their primary permeation route (ISMN; aqueous pathway in the stratum corneum, ionized LC or Na$^+$ and Cl$^-$; both aqueous pathways in the stratum corneum and hair follicles, FL and FD-4; aqueous pathway in hair follicles and lipophilic compounds; lipophilic pathway). Moreover, the permeability coefficients of lipophilic compounds were estimated from our previously reported results in which EtOH did not have a significant effect on the skin permeation of isosorbide dinitrate (MW; 236, Log K_{ow}; 1.22).[25] The reason why different behaviors were observed between ionized LC and FL, which have similar molecular weights and Log K_{ow}, was attributed to the existence of different permeation pathways dependent on the different physicochemical properties of penetrants such as the electrical charge or functional group. Thus, EtOH could affect both aqueous pathways to change the permeability coefficients of hydrophilic drugs.

In conclusion, multiple pathways were found for the skin permeation of hydrophilic compounds, and the skin permeation route may be determined depending on the physicochemical properties of the drugs. In addition, EtOH, when used as a skin penetration enhancer, affects the hair follicle pathway, which plays an importance role in penetration especially that of hydrophilic drugs.

References

1) Iikura Y., Uchiyama H., Akimoto K., Ebisawa M., Sakaguchi N., Tsubaki T., Ishizu H., Kabayama H., Yagi K., Miura K., *Ann. Allergy Asthma Immunol.*, **74**, 147–151 (1995).
2) Shah V. P., Tymes N. W., Skelly J. P., *Pharm. Res.*, **6**, 346–351 (1989).
3) Chien Y. W., Keshary P. R., Huang Y. C., Sarpotdar P. P., *J. Pharm. Sci.*, **72**, 968–970 (1983).
4) Arra G. S., Arutla S., Krishna D. R., *Drug Dev. Ind. Pharm.*, **24**, 489–492 (1998).
5) Oertel W., Ross J. S., Eggert K., Adler G., *Neurology*, **69** (Suppl. 1), S4–S9 (2007).
6) Liu P., Bergstrom T. K., *J. Pharm. Sci.*, **85**, 320–325 (1996).
7) Duracher L., Blasco L., Hubaud J.-C., Vian L., Marti-Mestres G., *Int. J. Pharm.*, **374**, 39–45 (2009).
8) Naik A., Pechtold L. A. R. M., Potts R. O., Guy R. H., *J. Control. Release*, **37**, 299–306 (1995).
9) Kim M.-J., Doh H.-J., Choi M.-K., Chung S.-J., Shim C.-K., Kim D.-D., Kim J. S., Yong C.-S., Choi H.-G., *Drug Deliv.*, **15**, 373–379 (2008).
10) Okumura M., Nakamori Y., Yoshida Y., Niwa H., Sugibayashi K., Morimoto Y., *Drug Des. Deliv.*, **6**, 137–148 (1990).
11) Bhandari K. H., Lee D. X., Newa M., Yoon S. I., Kim J. S., Kim D.-D., Kim J.-A., Yoo B. K., Woo J. S., Lyoo W. S., Lee J. H., Choi H.-G., Yong C.-S., *Arch. Pharm. Res.*, **31**, 242–249 (2008).
12) Kararli T. T., Kirchhoff C. F., Penzotti S. C. Jr., *J. Control. Release*, **34**, 43–51 (1995).
13) Song Y. H., Gwak H. S., Chun I. K., *Drug Deliv.*, **16**, 75–81 (2009).
14) Akhter S. A., Barry B. W., *J. Pharm. Pharmacol.*, **37**, 27–37 (1985).
15) Babu R. J., Pandit J. K., *Int. J. Pharm.*, **288**, 325–334 (2005).
16) Ashton P., Walters K. A., Brain K. R., Hadgraft J., *Int. J. Pharm.*, **87**, 261–264 (1992).
17) Som I., Bhatia K., Yasir M., *J. Pharm. Bioallied Sci.*, **4**, 2–9 (2012).
18) Sugibayashi K., Hosoya K., Morimoto Y., Higuchi W. I., *J. Pharm.*
19) Singh B. N., Singh R. B., Singh J., *Int. J. Pharm.*, **298**, 98–107 (2005).
20) Pershing L. K., Lambert L. D., Knutson K., *Pharm. Res.*, **7**, 170–175 (1990).
21) Good W. R., Powers M. S., Campbell P., Schenkel L., *J. Control. Release*, **2**, 89–97 (1985).
22) Berner B., Mazzenga G. C., Otte J. H., Steffens R. J., Juang R. H., Ebert C. D., *J. Pharm. Sci.*, **78**, 402–407 (1989).
23) Williams A. C., Barry B. W., *Adv. Drug Deliv. Rev.*, **64**, 128–137 (2012).
24) Van der Merwe D., Riviere J. E., *Toxicol. In Vitro*, **19**, 69–77 (2005).
25) Horita D., Todo H., Sugibayashi K., *Biol. Pharm. Bull.*, **35**, 1343–1348 (2012).
26) Scheuplein R. J., *J. Invest. Dermatol.*, **48**, 79–88 (1967).
27) Hayashi T., Sugibayashi K., Morimoto Y., *Chem. Pharm. Bull.*, **40**, 3090–3093 (1992).
28) Todo H., Kimura E., Yasuno H., Tokudome Y., Hashimoto F., Ikarashi Y., Sugibayashi K., *Biol. Pharm. Bull.*, **33**, 1394–1399 (2010).
29) Horita D., Yoshimoto M., Todo H., Sugibayashi K., *Drug Dev. Ind. Pharm.*, **40**, 345–351 (2014).
30) Hatanaka T., Suzuki R., Katayama K., Koizumi T., *Int. J. Pharm.*, **168**, 199–208 (1998).
31) Okamoto H., Sakai T., Danjo K., *Biol. Pharm. Bull.*, **28**, 1689–1694 (2005).
32) Mota M. C., Carvalho P., Ramalho J., Leite E., *Int. Ophthalmol.*, **15**, 321–326 (1991).
33) Gerasimenko J. V., Tepikin A. V., Petersen O. H., Gerasimenko O. V., *Curr. Biol.*, **8**, 1335–1338 (1998).
34) Kalia Y. N., Pirot F., Potts R. O., Guy R. H., *J. Pharm. Sci.*, **87**, 1508–1511 (1998).
35) Hatta I., Nakazawa H., Obata Y., Ohta N., Inoue K., Yagi N., *Chem. Phys. Lipids*, **163**, 381–389 (2010).
36) Manabe E., Sugibayashi K., Morimoto Y., *Int. J. Pharm.*, **129**, 211–221 (1996).
37) Naik A., Kalia Y., Guy R., *Pharm. Sci. Technol. Today*, **3**, 318–326 (2000).
38) Yamaguchi K., Mitsui T., Aso Y., Sugibayashi K., *J. Pharm. Sci.*, **97**, 4391–4403 (2008).
39) Takeuchi H., Ishida M., Furuya A., Todo H., Urano H., Sugibayashi K., *Biol. Pharm. Bull.*, **35**, 192–202 (2012).
40) Stahl J., Niedorf F., Wohlert M., Kietzmann M., *Altern. Lab. Anim.*, **40**, 51–57 (2012).
41) Blume-Peytavi U., Massoudy L., Patzelt A., Lademann J., Dietz E., Rasulev U., Garcia Bartels N., *Eur. J. Pharm. Biopharm.*, **76**, 450–453 (2010).
42) Trauer S., Lademann J., Knorr F., Richter H., Liebsch M., Rozycki C., Balizs G., Büttemeyer R., Linscheid M., Patzelt A., *Skin Pharmacol. Physiol.*, **23**, 320–327 (2010).
43) Otberg N., Teichmann A., Rasuljev U., Sinkgraven R., Sterry W., Lademann J., *Skin Pharmacol. Physiol.*, **20**, 195–198 (2007).
44) Teichmann A., Otberg N., Jacobi U., Sterry W., Lademann J., *Skin Pharmacol. Physiol.*, **19**, 216–223 (2006).
45) Ogiso T., Shiraki T., Okajima K., Tanino T., Iwaki M., Wada T., *J. Drug Target.*, **10**, 369–378 (2002).
46) Barry B. W., *Adv. Drug Deliv. Rev.*, **54**, 31–40 (2002).
47) Lauer A. C., Lieb L. M., Ramachandran C., Flynn G. L., Weiner N. D., *Pharm. Res.*, **12**, 179–186 (1995).
48) Patzelt A., Richter H., Buettemeyer R., Huber H. J., Blume-Peytavi U., Sterry W., Lademann J., *Eur. J. Pharm. Biopharm.*, **70**, 234–238 (2008).
49) Kimura E., Kawano Y., Todo H., Ikarashi Y., Sugibayashi K., *Biol. Pharm. Bull.*, **35**, 1476–1486 (2012).