Skin has various types of transporters and is a biochemically active organ. These aspects of skin influence the distribution of chemicals in skin and their elimination from skin. The biochemical and histological variations of the skin must be taken into account when conducting transdermal penetration research. Here we used hairless mouse skin to investigate the percutaneous absorption of chemicals in vitro from the stratum corneum (SC) side to the viable skin (VS) side (forward direction) and from the VS side to the SC side (backward direction). We examined the effects of molecular weight, lipophilicity (Log $K_{ow}$), electric charge, and the molecular structure of penetrants. The penetration flux of verapamil hydrochloride (VRP) for the backward direction was 3.2 times larger than that for the forward direction. The flux values of benzoic acid (BA) and para-hydroxybenzoic acid (pHBA) for the forward direction were 2.1 and 4.6 times larger than those for the backward direction, respectively. This directional difference was caused by the active transporter for VRP, the histological distribution of BA solubility, and the intermolecular hydrogen bonding between pHBA and skin tissue in the stripped skin. Across intact skin, in contrast, there was no difference in the skin penetration profile between the forward direction and backward directions.

**Key words** skin penetration; diffusive direction; pH gradient; active transporter; skin distribution; molecular structure

Skin consists of two different layers, the stratum corneum (SC, the lipophilic layer) and viable skin (VS, the hydrophilic layer). The diffusion-partitioning model based on Fick’s law of diffusion is widely applied to the mathematical model of transdermal penetration.10 Penetration parameters such as diffusion and partition coefficients used in this model can be determined from in vitro penetration experiments to predict the skin penetration flux. Each layer of the skin assumes an inert and monolithic membrane for penetration. However, skin, especially VS, has biochemical activity. Skin enzymes are distributed around the basement layer of the epidermis3 and their distribution influences the penetration of drugs.3

Active transporters are present in the skin and may affect the percutaneous absorption of penetrants.3 One of the xenobiotic transporters, P-glycoprotein (P-gp), is expressed not only in human skin but in hairless mouse skin as well3 and is locally observed in dermis and hypodermis by immunohistochemical analysis.5 The other transporters, oligopeptide transporter, monocarboxylate transporter, organic anion transporting polypeptide, and organic cation transporter, were also expressed in the skin.6,7 A Zn transporter, Znt5/Slc30a5, participates in the mast cell-mediated delayed-type allergic response and may be involved in allergic contact dermatitis.8 These transporters may influence the dermatochemical distribution and elimination of chemicals in order to maintain the internal environment.

The biochemical and histological variations of the skin should be considered in transdermal penetration research. To investigate the effect of skin’s histological and biochemical homogeneity on the skin penetration of chemicals, we used hairless mouse skin in vitro to determine the percutaneous absorption from the SC side to the VS side (the forward direction) and from the VS side to the SC side (the backward direction). The model chemicals were selected with respect to their molecular weight, lipophilicity (Log $K_{ow}$), electric charge, and molecular structure. We also studied the percutaneous absorption of both stripped skin and intact skin, and we discuss the importance of the diffusive direction of chemicals in transdermal therapeutic systems.

**MATERIALS AND METHODS**

**Materials** Verapamil hydrochloride (VRP) was purchased from Sigma (St. Louis, MO, U.S.A.), and benzoic acid (BA), propranolol (PRP), $p$-hydroxybenzoic acid (pHBA), indomethacin (IM), caffeine (CF), progesterone (PS), testosterone (TS), prednisolone (PN), cyanocobalamin (VB$12$), allethrin (AT), and dexamethasone (DM) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Salicylic acid (SA) was purchased from Kanto Chemicals (Tokyo, Japan). Other chemicals were special reagent-grade and purchased from Wako Pure Chemical Industries, Ltd. The physicochemical properties of the chemicals are summarized in Table 1; some properties were obtained from the literature.9

**Penetration Experiments** Abdominal intact and stripped skin were excised from female hairless mice (Kud: Hr$^-$ strain, 7 weeks old, Kyudo Co., Saga, Japan). The SC of the stripped skin was removed completely by adhesive tape-stripping the skin 20 times (CF-24F, Nichiban Co., Tokyo, Japan). Skin samples were mounted on side-by-side permeation cells (KH-5P, Vidrex Co., Fukuoka, Japan). The receptor cell was filled with 5 mL of fresh solvent and the donor cell was filled with the chemical solution. The solvent was 40% polyethylene glycol (PEG) 400 solution for BA (saturated concentration, C$_{BA}$), SA (C$_{SA}$), pHBA (C$_{pHBA}$), TS (C$_{TS}$), AT (C$_{AT}$), PS (C$_{PS}$), PN (C$_{PN}$) and DM (C$_{DM}$) and phosphate buffer at pH 7.4 for CF (C$_{CF}$), IM (C$_{IM}$), VRP (500 µg/mL) and VB$_{12}$ (1000 µg/mL). Concentration of model chemicals in solvent was also summarized in Table 1. In the penetration experiments for BA, SA and pHBA, we also used buffer that adjusted the pH at 7.4 (phosphate buffer that adjusted the pH at 7.4 to 7.4 for BA, SA and pHBA).

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buffer), 5.2 (phosphate buffer), 4.2 (citric-phosphate buffer), 3.0 (citric-phosphate buffer), and 2.6 (citric-phosphate buffer) saturated with the chemicals. At predetermined time intervals, 200 μL of receptor solution was withdrawn for an HPLC assay. The steady-state penetration flux was evaluated from the linear portion of the plot of the cumulative amount penetrated against time. All animal experiments were conducted in accord with our institutional guidelines and were approved by the Animal Care and Use Committee, Kyushu Institute of Technology.

**BA Concentration in the Skin** The stripped skin sample was cut into pieces of 2.2×1.8 cm pieces, and each skin sample was embedded into Tissue-Tek OCT compound (Sakura Finetechical Co., Tokyo, Japan) at −20°C. The skin samples were sliced into 20-μm sections, and each two sliced skin sections from the surface of each skin sample was immersed into BA solution with buffer (pH 7.4, 4.2 and 2.6) and then equilibrated at 37°C for 24 h. The BA concentration in the supernatant was assayed by HPLC. We then calculated the skin/buffer partition coefficient $K_{\text{skin/buffer}}$ using the BA amount in each sliced skin section and the BA concentration in the solution.

**HPLC Analysis** All model chemicals were assayed in an HPLC assembled LC 10A system (Shimadzu Co., Kyoto, Japan) with four HPLC columns: a SUMIPACK-ODS A-212 (6×150 mm, Sumitomo Chemical Co., Tokyo, Japan) for BA and CF, a CAPCELL Pak C18 UG120 (4.6×250 mm, Shimadzu GLC, Osaka, Japan) for SA and pHBA, a TSK gel ODS 80Ts (4.6×150 mm, Tosoh Co., Tokyo, Japan) for VRP, DM, IM, PS, TS and PN, and an L-column ODS (4.6×150 mm, Chemical Evaluation and Research Institute, Tokyo, Japan) for AT. The column oven temperature was maintained at 40°C. The other assay conditions are summarized in Table 2.

### RESULTS AND DISCUSSION

**Directional Difference of Skin Penetration** We carried out the skin penetration experiments of the chemicals VRP, BA, AT, DM, IM, CF, PS, TS, PN, and VB$_{12}$, which have different molecular weights, lipophilicity, and electric charge, across stripped skin. The directional differences in the skin penetration profiles are shown in Fig. 1. The cumulative amount penetrated of model chemicals is influenced by their physicochemical properties and chemicals with high molecular weight, high lipophilicity, and nonelectric charge have the lower penetration across stripped skin. These results in Fig. 1 are agreed with the VRP profile from the backward direction (Fig. 1a), and the BA profile shows the reverse (Fig. 1b). The skin penetrations of AT and DT show the same profile between the

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**Table 1. Physicochemical Properties of the Chemicals Used**

| Drug | Molecular weight (g/mol) | $pK_a$ | Log $K_{ow}$ | Electric charge at pH 7.4 | Concentration in solvent (μg/mL) |
|------|--------------------------|--------|-------------|---------------------------|---------------------------------|
| BA   | 122                      | 4.19   | 1.87        | −1                        | $3.38\times10^4\pm5.27\times10^2$ |
| SA   | 138                      | 2.97   | 2.26        | −1                        | $6.20\times10^4\pm4.67\times10^3$ |
| pHBA | 138                      | 4.57   | 1.58        | −1                        | $2.47\times10^4\pm3.24\times10^3$ |
| CF   | 194                      | 10.4   | −0.07       | +1                        | $2.96\times10^4\pm1.87\times10^2$ |
| TS   | 288                      | −      | 3.32        | 0                         | $5.91\times10^5\pm4.93\times10^5$ |
| AT   | 302                      | −      | 4.96        | 0                         | $1.20\times10^5\pm3.56$         |
| PS   | 315                      | −      | 3.87        | 0                         | $1.58\times10^6\pm4.13\times10^5$ |
| IM   | 358                      | 4.17   | 4.27        | −1                        | $4.43\times10^4\pm9.11$         |
| PN   | 360                      | −      | 1.42        | 0                         | $2.13\times10^4\pm6.15\times10^2$ |
| DM   | 392                      | −      | 0.72($^a$)  | 0                         | $1.20\times10^4\pm7.05\times10^2$ |
| VRP  | 491                      | 8.6($^a$) | 5.69($^a$) | +1                        | 500                             |
| VB$_{12}$ | 1355                  | −      | −4.30       | 0                         | 1000                             |

$^a$ Values are from Kasim et al. 2004. BA: benzoic acid, SA: salicylic acid, pHBA: $p$-hydroxybenzoic acid, CF: caffeine, TS: testosterone, AT: althelrin, PS: progesterone, IM: indomethacin, PN: prednisolone, DM: dexamethasone, VRP: verapamil hydrochloride and VB$_{12}$: vitamin B$_{12}$.

**Table 2. Analytical Conditions of the Model Chemicals Used**

| Drug | Wave length (nm) | Mobile phase | Flow rate (mL/min) |
|------|-----------------|--------------|-------------------|
| BA   | 220             | 35% Acetonitrile/65% (0.048 M NaH$_2$PO$_4$−0.041 M H$_3$PO$_4$) | 1.0 |
| SA   | 270             | 25% Methanol/75% 0.1 M KH$_2$PO$_4$ (pH 7.0) | 1.0 |
| pHBA | 270             | 10% Methanol/90% 0.1 M KH$_2$PO$_4$ (pH 7.0) | 0.8 |
| CF   | 263             | 30% Methanol/70% distilled water | 1.0 |
| TS   | 254             | 65% Methanol/35% distilled water | 1.0 |
| AT   | 225             | 80% Acetonitrile/20% distilled water | 1.0 |
| PS   | 254             | 70% Methanol/30% distilled water | 1.0 |
| IM   | 254             | 70% Methanol /30% 0.05% phosphate aqueous solution | 1.0 |
| PN   | 254             | 50% Methanol/50% distilled water | 1.0 |
| DM   | 254             | 58% Methanol/42% distilled water | 1.0 |
| VRP  | 260             | 50% Methanol/50% 50m M NaH$_2$PO$_4$ (pH 2.2) | 1.0 |
| VB$_{12}$ | 280        | 12% Acetonitrile/88% 10 m phosphate buffer (pH 7.4) | 1.0 |

Abbreviations are indicated in the footnote of Table 1.
forward and backward directions (Figs. 1c, 1d) and the other chemicals IM, CF, PS, TS, PN, and VB also have the same results (data not shown). It has been reported that the percutaneous absorption of rhodamine 123, flurbiprofen, and itaconazole also has a directional difference across the stripped skin.4,5) These findings may indicate that the molecular weight and lipophilicity are not responsible for the directional difference of skin penetration within the range of the present experiments.

**Effect of Transporter on Skin Penetration** VRP is known to be a substrate of organic cation transport system11,12 that is represented by organic cation transporters (OCT; SLC22A1, 2 and 3) and of P-gp (ABCB1) that is a member of ATP-binding cassette family.13) Organic cation transport system acts as an influx transport for essential nutrients on the cell membrane and, on the other hand, P-gp acts as an efflux transport for xenobiotic substances. Ito et al has reported that P-gp enhanced the penetration flux for the forward direction to eliminate its substrates from skin to blood.5) Thus, our results in Figs. 1a and 1b may indicate that the percutaneous absorption of VRP and BA is mediated by the influx transporter and the efflux transporter, respectively. Thus, we investigated co-penetration experiments of VRP and BA with propranolol (PRP), the substrate of both organic cation transport system and P-gp, and we added PRP at the same concentration to each donor solution (500 µg/mL for VRP and 30 mg/mL for BA) to measure the skin penetration flux. The penetration profiles of VRP with PRP for the forward direction agreed with that for the backward direction (Fig. 2a), whereas the forward profile of BA was different from its backward profile (Fig. 2b). Thus, PRP acts as a competitive inhibitor only for the backward direction of VRP and, on the other hand, it reduced the cumulative amount of BA to a half in both directions (Figs. 1b, 2b) because a chemical potential of BA in the donor solution relatively decreased. Therefore, the directional difference of the VRP penetration profile was caused mainly by the transport of organic cation transport system in the skin and transporters did not influence the penetration profile of BA.

**Effect of a pH Gradient throughout Stripped Skin** We conducted penetration experiments with BA with a pH gradient between the donor solution (pH 4.2, BA saturated in 40% PEG400) and the receptor solution (pH 9.0, 40% PEG400). The natural pH values of the SC surface and the deeper layer in vivo are about 5.0 and 7.4, respectively.14) In vitro, the pH values are easily changed by solution contacting the skin surface.15) We also conducted an experiment using the same pH

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**Fig. 1. Penetration Profiles of VRP (a), BA (b), AT (c) and DM (d) across Stripped Skin of Hairless Mice**

The forward and backward directions represent the skin penetration from the SC side to the dermis side and from the dermis side to the SC side, respectively. Each data point is the mean±S.D. of three experiments.
values for the donor and receptor solutions. The pH values selected were 2.6, 4.2 (pK$_{a}$ of BA) and 7.4. The steady-state flux is shown in Fig. 3. There was no significant difference in the penetration flux between the forward and backward directions under the same pH. The steady-state fluxes of BA at pH 4.2 were the highest values for the forward and backward directions. These findings show that the pH gradient throughout stripped skin is important for the directional difference of BA penetration, and they may indicate that there is an interaction between BA and skin tissue at pH 4.2.

Skin consists of SC and VS (the viable epidermis and dermis). The basement layer of the viable epidermis (the hydrophilic layer) becomes the SC (the lipophilic layer) after dehydrating from the cell and, thus, there may be a lipophilicity gradient throughout the viable epidermis. The pH of the solution changes the lipophilicity of BA. The pH gradient may thus cause the skin distribution of BA solubility. We investigated the skin/buffer partition coefficient $K_{\text{skin/buffer}}$ at the pH values of 7.4, 4.0 and 2.6 (Fig. 4). $K_{\text{skin/buffer}}$ had a constant value throughout the stripped skin at pH 7.4 and, in contrast, decreased with the increase in the distance from the skin surface at pH 4.2. At the depth of 40 µm, the values of $K_{\text{skin/buffer}}$ at pH 4.0 were 4.3 and 2.1 times larger than those at pH 7.4 and pH 2.6, respectively. Therefore, the high BA solubility around the surface of viable skin (ca. 40 µm) at pH 4.2 may cause the directional difference in skin penetration.

**Effect of Molecular Structure**

We also studied the effect of the molecular structure of the chemicals on the directional difference of BA penetration. We selected SA and pHBA, with structures similar to that of BA; SA and pHBA have a hydroxyl group on the ortho- and para- to carboxyl group of BA, respectively. There was no directional difference in the SA penetration flux (Fig. 5a). The pHBA penetration flux, in contrast, was larger for the forward direction than for the backward direction when the pH of the donor solution was different from the pH of receptor solution (Fig. 5b). SA tends to form intramolecular hydrogen bonding between the hydroxyl group and carboxyl group and stabilizes in solution. pHBA and BA form an intermolecular hydrogen bonding between the skin components lipid and protein. The interaction of pHBA and BA with skin tissue may cause the directional difference.

**Penetration across Intact Skin**

Transdermal therapeutic systems (TTs) usually focus on enhancing the SC penetration delivered drugs across intact skin. To research the mechanism...
underlying the directional differences in the skin penetration, we investigated the skin penetration experiments with stripped skin without SC from hairless mice, and then we examined the skin penetration profile of VRP and BA across intact skin with SC. Figure 6 shows no difference in these chemicals’ penetration profiles between the forward and backward directions. This indicates that the intact skin has no directional difference for chemical penetration. SC is a rate-limiting barrier for transdermal absorption because the ratio of the SC diffusion coefficient to that of the VS is generally less than 1/100. SC protects the internal environment and also maintains the pH gradient in skin when acid or alkaline solution is in contact with the skin surface.

Our findings revealed that the directional difference in chemical penetration at intact skin was able to ignore. Therefore, we concluded that intact skin with SC could be used in the diffusion-partitioning model based on Fick’s law of diffusion on TTS research. However, the thickness of the SC varies with age, anatomical site and disease’s conditions of each patient affecting the skin penetration flux of drugs from a TTS device. We need to pay attention to assume the penetration profiles of drugs on clinical application.

CONCLUSION

We investigated the percutaneous absorption of 12 chemicals in the forward direction and backward direction in hairless mouse skin in vitro. We found that VRP, BA and pHBA penetration across the stripped skin had a directional difference. The penetration flux of VRP for the backward direction was 3.2 times larger than that for the forward direction. The flux values of BA and pHBA for the forward direction were 2.1 and 4.6 times larger than those for the backward direction, respectively. This directional difference is caused by the organic cation transport system for VRP, the skin distribution of BA solubility, and the intermolecular hydrogen bonding between pHBA/BA and skin tissue in the stripped skin. There was no significant difference in the skin penetration profile of chemicals across intact skin for both the forward direction and backward direction. Therefore, intact skin can be used in the diffusion-partitioning model based on Fick’s law of diffusion on TTS research.

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