Influence of Skin Thickness on the in Vitro Permeabilities of Drugs through Sprague-Dawley Rat or Yucatan Micropig Skin

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The purpose of this study was to clarify the influence of skin thickness on the in vitro permeabilities of 3 model drugs with different physicochemical properties (nicorandil (NR), isosorbide dinitrate (ISDN) and flurbiprofen (FP)) through Sprague-Dawley rat (rat) or Yucatan micropig (YMP) skin. Intact, dermis-split, stratum corneum-stripped or stratum corneum-stripped and dermis-split rat or YMP skin (rat skin thickness: approximately 0.4, 0.9 or 1.2 mm; YMP skin thickness: approximately 0.4, 0.9, 1.8 or 2.8 mm) were set in Franz-type diffusion cells to determine the permeation rate, lag time and resistance ratio of the viable epidermis and dermis against whole skin (Rrat/Rtot) of the drugs. The YMP skin permeabilities of the drugs decreased with an increase in the skin thickness, and significant differences were observed in the permeation rates and lag times between intact and dermis-split (0.4 mm) YMP skins. The decreases in the permeabilities of the drugs through the YMP skin were larger than those through the rat skin. The influence of resistances of ISDN and FP through the dermis-split rat or YMP skin was greater at 0.9 mm skin thickness than 0.4 mm skin thickness. The Rrat/Rtot values for the YMP skins were relatively large for lipophilic drugs (ISDN and FP), and these ratios increased with an increase in the dermis thickness. These results suggest that in vitro skin permeation studies must be done using dermis-split (0.4 mm) skin with the thinnest dermis for predicting in vivo human percutaneous absorption rate.

Key words Yucatan micropig; intact skin; stripped skin; split skin; skin permeability

In vitro skin permeation studies have been broadly used in the development of transdermal formulations. Animal skins are frequently used as an alternative to human skin in percutaneous absorption studies, because human skin is not always available and the use of human tissues and organs creates ethical problems.1—10 Numerous animal models, including primate, porcine, mouse, rat, guinea pig and pig, have been utilized for skin permeability studies.1,3,4,11—13 Animal skins with minimal variations in skin permeability may be much better than human skin for determining or estimating the skin permeabilities of drugs and for developing transdermal formulations.1,14,15

In our previous studies, the in vitro permeation of 3 model drugs was investigated through Sprague-Dawley rat (rat), Yucatan micropig (YMP) and human skin.16,17 The 3 model drugs were selected because of their different logKow values (the logarithm of the octanol/water partition coefficient at 37°C; Table 1).18 Nicorandil (NR), isosorbide dinitrate (ISDN) and flurbiprofen (FP) were used as hydrophilic, lipophilic and highly lipophilic drugs, respectively. The inter-individual variations in rat and YMP skin permeabilities for the 3 drugs were smaller than the human skin permeability.16,17 In addition, in vitro permeation studies using rat and YMP skin were particularly useful for evaluating differences in the skin permeabilities of the model drugs and for predicting human skin permeability.16,17

Guidelines for a standard experimental protocol for in vitro skin permeation studies were published by the Organization

Table 1. Chemical Structures and Physicochemical Parameters of the Model Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Molecular weight</th>
<th>Solubility in water (mg/mL)</th>
<th>logKow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicorandil (NR)</td>
<td></td>
<td>211.17</td>
<td>39.6</td>
<td>-1.02</td>
</tr>
<tr>
<td>Isosorbide dinitrate</td>
<td></td>
<td>236.14</td>
<td>1.34</td>
<td>1.34</td>
</tr>
<tr>
<td>Flurbiprofen (FP)</td>
<td></td>
<td>244.27</td>
<td>0.0277</td>
<td>3.86</td>
</tr>
</tbody>
</table>

a) Hatanaka et al.18 b) Solubility in water at 37°C. c) Logarithm of octanol/water partition coefficient at 37°C.
for Economic Cooperation and Development (OECD) in 2004. These guidelines state that trypsin-isolated stratum corneum (SC), heat-separated epidermis, dermis-split skin (split skin) and intact skin can be used, but the use of skin thicker than 1 mm should be avoided unless specifically required to investigate the test substance in skin layers. Careful consideration should also be paid to the skin preparation method used for in vitro permeation studies, because both the permeation and the lag time can be greatly affected. The lack of blood flow in in vitro permeation experiments causes the lower dermis to act as a barrier to the diffusion of these compounds that would not exist in vivo settings. Nonetheless, these skin preparations have been used until now to determine in vitro skin permeation.

The skin is a heterogeneous organ: the SC is lipophilic, while the lower layers, the viable epidermis and the dermis (VED), are relatively hydrophilic. The SC is considered to be the primary barrier layer among the different skin layers, because the permeation rate of drugs is much lower through intact skin than through stripped skin. Recently, in vitro human skin permeation studies have demonstrated that VED also acts as an important permeation barrier and that skin thickness influences the permeabilities of drugs. In addition, several investigators have reported that the in vitro skin permeation rates of lipophilic compounds were overestimated using epidermal membranes and were underestimated using intact skins, compared with in vivo perturbance absorption rates in humans. Their findings indicate that VED acts as an important permeation barrier as well as stratum corneum. Therefore, we suggested that in vitro skin permeation studies should be performed, ideally using split skin with various thicknesses. The use of split skin in in vitro permeation studies is challenging, because skin samples with a constant thickness are difficult to prepare using a dermatome. Therefore, few reports have examined the influence of skin thickness on the permeabilities of drugs with different physicochemical properties in in vitro permeation studies using split or stratum corneum-stripped and dermis-split (stripped/split) rat or YMP skin. Nevertheless, we attempted to dermatome the skin using an electric dermatome by a new method established in our previous studies.

The purpose of this study was to clarify the influence of skin thickness on the in vitro permeabilities of 3 drugs with different physicochemical properties through rat or YMP skin. To accurately determine the permeation rate of drugs through rat or YMP skin, in vitro permeation experiments were performed using split rat and YMP skin of various thicknesses.

In addition, the goal in the present in vitro skin permeability study is to predict blood concentration of drugs after topical application of transdermal therapeutic system (TTS). If the blood concentration of drugs after topical application of TTS in human can be predicted from the in vitro skin permeation parameters through the animal skin, the development efficiency of TTS will be greatly improved. Thus, establishment of such a method is expected for predicting blood concentration of drugs after topical application of TTS.

**MATERIALS AND METHODS**

**Materials** NR, ISDN, and FP were purchased from Toronto Research Chemicals Inc. (North York, Canada), Alexis Corporation (San Diego, CA, U.S.A.) and Sigma-Aldrich (St. Louis, MO, U.S.A.), respectively. All other chemicals and solvents were of reagent grade and were obtained commercially.

**Rat Skin** The care of the rats and the present protocols complied with the “General Considerations for Animal Experiments” and were approved by the Ethics Committee for the Treatment of Laboratory Animals at Taisho Pharmaceutical.

The permeation profiles of NR, ISDN and FP through rat skin were determined using in vitro permeation experiments. Male rats (8 weeks old; Charles River Laboratories Japan Inc., Atsugi, Kanagawa, Japan) were used for the skin permeation studies. The rats were euthanized by cervical dislocation under anesthesia with isoflurane, and the dorsal region of the rat skin was carefully shaved. An intact rat skin section (approximately 3 cm in diameter or approximately 4×4 cm) was excised from the dorsal region of the rat skin at a point 7±1 cm from the rat’s ears. After the subcutaneous fat was removed from all the intact rat skin sections, the intact rat skin samples were prepared. The thickness of the intact rat skin samples was measured using a dial thickness gauge (Model H; Osaka MFG. Co., Ltd., Tokyo, Japan). The thickness of the intact rat skin samples was 1.18±0.04 mm.

The intact rat skin section (approximately 4×4 cm) was dermatomed to a nominal thickness of approximately 0.4 or 0.9 mm using an electric dermatome (Model B; Padgett Instruments, Inc., Kansas, MO, U.S.A.; Fig. 1). A corkboard (width×depth×height: 3 cm×10 cm×1 cm) was fixed to a styrene foam board (width×depth×height: 3 cm×10 cm×0.5 cm). The dermis side of excised intact rat skin section was fixed onto the styrene foam board using pins. The split rat skin sample with a thickness of 0.4 or 0.9 mm was made by the electric dermatome. The exact thicknesses (0.4, 0.9 mm) of the split rat skin samples were 0.41±0.02 mm and 0.85±0.04 mm, respectively. The excised intact and split rat skin samples were then frozen and stored at −80°C until use (maximum period of one month).

On the experiment day, the intact and split rat skin samples were thawed in a CO2 incubator at 32°C (relative humidity: 80±10%) for 15 min. To prepare the stratum corneum-stripped skin (stripped skin) and stratum corneum-stripped and dermis-split skin (stripped/split skin; approximately 0.4 or 0.9 mm thickness), tape stripping was performed 10 times using adhesive cellophane tape (30 mm width, Nichiban Co., Ltd., Tokyo, Japan) from rat skin before starting the skin permeation experiment. Those rat skin samples were immediately used in the skin permeation experiments.

**YMP Skin** Frozen YMP skin sets (female pigs: 5 months old) were obtained from Charles River Japan Inc. (Yokohama, Kanagawa, Japan). Each YMP skin set consisted of 16 skin sheets (sheet size: approximately 10×10 cm) was enclosed in a plastic bag identified by numbers. The frozen YMP skin sets were stored at −80°C until the permeation studies (maximum period of six months).

YMP dorsal skin sheets (L-1, L-5, R-1 and R-5) of two YMP skin sets were selected for the permeation studies. The day before the experiment, the YMP skin sheets were thawed in a CO2 incubator at 32°C (relative humidity: 80±10%) for 15 min. After removing the subcutaneous fat from all the YMP skin sheets, the intact YMP skin sheets were prepared.
The intact YMP skin sheets were dermatomed to a nominal thickness of approximately 0.4, 0.9 or 1.8 mm using an electric dermatome to obtain the split YMP skin sheets (Fig. 1). A corkboard (width x depth x height: 8 cm x 10 cm x 1 cm) was fixed to a styrene foam board (width x depth x height: 8 cm x 10 cm x 0.5 cm). The dermis side of excised YMP skin sheet was fixed onto the styrene foam board using pins. The split YMP skin sheet with a thickness of 0.4, 0.9 or 1.8 mm was made by the electric dermatome. Nine intact or split YMP skin samples (each 2.5 x 3 cm) were cut from each intact and split YMP skin sheet using a surgical knife. Those intact and split YMP skin samples were then measured using a dial thickness gauge. The exact thickness of the intact YMP skin samples was 2.75 ± 0.05 mm, and those of the split (0.4, 0.9, 1.8 mm) skin samples were 0.44 ± 0.03, 0.89 ± 0.07 and 1.75 ± 0.08 mm, respectively. The intact and split YMP skin samples were placed on a paper towel soaked with physiological saline and stored in a sealed container at 4°C for 16 h before starting the skin permeation experiment.

Skin Permeation Procedure The in vitro skin permeation study was performed using the same procedure as that outlined in Test Guideline 428 of the OECD. The permeation experiments were mainly performed using modified Franz-type diffusion cells (diffusional area: 0.95 cm², diameter: 1.1 cm). The Franz-type diffusion cells are usually applied for determination of permeation rates of the 3 model drugs through intact, split, stripped or split/skin skin. The skin samples were mounted on the diffusion cells, and the receptor cells were filled with 3.2 mL of distilled water or 0.1 M phosphate buffer (pH 7.4). One of the 3 drugs (NR, ISDN or FP) was dissolved in ethanol/water (3/1, v/v). A 5-μL aliquot of the drug solution (30 mg/mL) was then applied to the skin surface. The receptor cell medium was kept at 32°C and stirred using a Teflon stirrer driven at a constant motor speed of 150 rev/min throughout the skin permeation experiment. The diffusion cells were maintained in a CO₂ incubator at 32°C (relative humidity: 80 ± 10%).

In the intact and split skin experiments, samples of receptor fluid (1.0 or 2.0 mL) were removed and replaced with the same volume of fresh solution over an experimental period of 10 h for rat skin and 24 h for YMP skin. In the stripped and stripped/split skin experiments, samples of receptor fluid (1.0 mL) were removed and replaced with the same volume of fresh solution over an experimental period of 4 or 6 h for rat skin and 4 or 12 h for YMP skin. The receptor fluid samples were stored in a freezer at −80°C until the analysis of the drug concentration.

Sample Preparation Procedure. Sample Pretreatment and LC-MS/MS Conditions for FP and NR The concentrations of FP and NR in the samples were determined using LC-MS/MS with negative or positive ion electrospray ionization (ESI). Briefly, a 50-μl aliquot was added to 200 μL of organic solvent (acetonitrile/methanol [9/1, v/v]) containing an internal standard (IS) of FP (furosemide: 0.25 μg/mL) or NR (propranolol: 0.25 μg/mL) and vortex-mixed. After centrifugation at 3600×g at 4°C for 10 min, the resulting supernatant (5 μL) was directly injected into an LC-MS/MS system equipped with a CTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an HP1100 binary pump (Agilent Technologies, Palo Alto, CA, U.S.A.), and an API 3000 mass spectrometer equipped with a TurboIonSpray interface (Applied Biosystems, Foster, CA, U.S.A.).

Chromatographic separation was performed using a Shim-pack XR-ODS (2.2 μm, 30×3.0 mm i.d.; Shimadzu, Kyoto, Japan) at 50°C using a flow rate of 1.3 mL/min for the mobile phase with a linear gradient from a 98% mobile phase A (aqueous 0.1% formic acid) to a 98% mobile phase B (acetonitrile) for 1.0 min and holding at 98% B for 1.0 min. The total run time for a single injection was 2.0 min. An approximately 2/7 portion of the column effluent was directed to the LC-MS/
MS system, and mass spectrometric detection was performed using a multiple reaction monitoring (MRM) mode of transition unique to each compound: m/z 243→m/z 192 for FP, m/z 329→m/z 205 for furosemide (IS), m/z 212→m/z 136 for NR and m/z 260→m/z 116 for propranolol (IS). The retention times for FP, furosemide, NR and propranolol were approximately 1.2, 0.9, 0.6 and 0.8 min, respectively. The calibration curve range was from 0.003 to 10 μg/mL for FP and NR, and the accuracy and precision were calculated.

**Sample Pretreatment and HPLC Conditions for ISDN**

The concentration of ISDN in the samples was determined using an HPLC system equipped with a UV detector. Briefly, a 250-μL aliquot was added to 100 μL of organic solvent (acetonitrile/methanol [9/1, v/v]) and vortex-mixed. After centrifugation at 3600×g for 10 min at 4°C, the resulting supernatant (10 μL) was directly injected into an HPLC (LC-10A/Dvp system; Shimadzu, Kyoto, Japan) with a UV detector (SPD-10A/Dvp system; Shimadzu, Kyoto, Japan).

Chromatographic separation was performed using a Capcell Pak C18 UG120 (5 μm, 4.6×150 mm i.d.; Shiseido Co., Ltd., Tokyo, Japan) at 40°C. The mobile phase was 0.1% phosphoric acid solution/acetonitrile/methanol [9/1/0.1, v/v/v]) and vortex-mixed. After centrifugation at 3600×g for 10 min at 4°C, the resulting supernatant (10 μL) was directly injected into an HPLC (LC-10A/Dvp system; Shimadzu, Kyoto, Japan) at 40°C. The mobile phase was 0.1% phosphoric acid solution/acetonitrile/methanol [9/1/0.1, v/v/v]) and vortex-mixed. After centrifugation at 3600×g for 10 min at 4°C, the resulting supernatant (10 μL) was directly injected into an HPLC (LC-10A/Dvp system; Shimadzu, Kyoto, Japan).

The concentration of ISDN in the samples was determined using an HPLC system equipped with a UV detector. Briefly, a 250-μL aliquot was added to 100 μL of organic solvent (acetonitrile/methanol [9/1, v/v]) and vortex-mixed. After centrifugation at 3600×g for 10 min at 4°C, the resulting supernatant (10 μL) was directly injected into an HPLC (LC-10A/Dvp system; Shimadzu, Kyoto, Japan) with a UV detector (SPD-10A/Dvp system; Shimadzu, Kyoto, Japan).

The retention time was approximately 6 min. The calibration curve range was from 0.003 to 10 μg/mL for ISDN, and the accuracy and precision were calculated.

**Data Analysis**

The permeation parameters for the 3 drugs were calculated by plotting the cumulative amount (μg/cm²) of drug that permeated through the skin against time. The steady-state permeation rate (J, μg/cm²/h) was calculated from the slope of the linear portion of the plot. The lag time (tlag) was obtained by extrapolation of the steady-state slope to its intersection with the time axis. The permeability coefficient (P) was calculated from the following Eq. 1:

\[
P = \frac{J}{C_0}
\]  

where \(C_0\) (μg/mL) is the concentration of NR, ISDN or FP in the solution applied to the skin. The permeability coefficients \(P_{tot}\) and \(P_{ved}\) were through whole skin and VED, respectively.

The barrier function of SC and VED against skin permeability was determined by the resistance (R) of each layer, calculated as the reciprocal of the permeability coefficient through the layer.21,28,29 The total resistance \(R_{tot}\) was then determined using the following Eq. 2:

\[
R_{tot} = R_{sc} + R_{ved} = \frac{1}{P_{tot}} = \frac{1}{P_{sc}} + \frac{1}{P_{ved}}
\]

where \(R_{sc}\) and \(R_{ved}\) are the resistances of SC and VED, respectively.

**Statistical Analysis**

All the data were shown as the mean±S.D. (n=3). The permeation rates and lag times in the intact rat skin were compared between split (0.4 mm) and split (0.9 mm) rat skin, between split (0.4 mm) and intact rat skin, between stripped/split (0.4 mm) and stripped/split (0.9 mm) rat skin or between stripped and split (0.4 mm) and stripped rat skin. The permeation rates and lag times in YMP skin were compared between split (0.4 mm) and split (0.9 or 1.8 mm) YMP skin, between split (0.4 mm) and intact YMP skin, between stripped and split (0.4 mm) and stripped/split (0.9 or 1.8 mm) YMP skin or between stripped/split (0.4 mm) and stripped YMP skin. The statistical analyses were performed using parametric Dunnett or Welch tests (the Bonferroni method for multiple comparisons was also applied). Probability (p) values less than 0.05 were considered statistically significant (for the Bonferroni method: p<0.025 for rat skin and p<0.017 for YMP skin). A linear regression analysis was used to evaluate the relationship between \(R_{ved}\) and skin thickness for each drug. The statistical analysis was performed using SAS software (ver. 8.2; ASA Institute Japan Ltd., Tokyo, Japan).

**RESULTS**

**Permeation Profiles of the 3 Model Drugs through Rat Skin. Intact and Split Rat Skin**

The influence of skin thickness on the permeabilities of the 3 model drugs was evaluated using intact (1.2 mm) and split (0.4, 0.9 mm) rat skin. Figure 2 shows the time course of the cumulative amounts of the drugs that permeated through the intact or split rat skin using the Franz-type diffusion cells. Table 2 summarizes the mean permeation rates and lag times of the drugs through intact or split rat skin. The skin permeation rate of ISDN was higher than those of NR and FP. The cumulative amounts of NR, ISDN and FP that permeated through the split (0.4 mm) rat skin over 10 h after the starting the skin permeation experiment were approximately 23%, 79% and 25% of the initial amounts applied on the skin, respectively. The permeation rates of NR, ISDN and FP through intact rat skin were approximately 0.84, 0.84 and 0.78 times lower than those through split (0.4 mm) rat skin, respectively. The permeation rates of all the drugs decreased with an increase in the skin thickness, although these differences were not significant. The lag times for the skin permeation of NR, ISDN and FP through split (0.9 mm) rat skin were approximately 3.6, 1.4 and 21.0 times longer than those through split (0.4 mm) rat skin, respectively. The lag times for the skin permeation of NR, ISDN and FP through intact rat skin were approximately 8.4, 4.4 and 110.0 times longer than those through split (0.4 mm) rat skin. Such significant differences in the lag times were observed between the intact and split (0.4 mm) rat skin for NR (p<0.05), ISDN (p<0.05) and FP (p<0.001). The lag times of all the 3 drugs increased with an increase in the skin thickness.

**Stripped and Stripped/Split Rat Skin**

Next, the influence of skin thickness on the permeabilities of the drugs was evaluated using split (1.2 mm) and stripped/split (0.4, 0.9 mm) rat skin. Figure 2 also shows the time course of the cumulative amounts of the drugs through stripped or stripped/split rat skin. In addition, Table 2 summarizes the mean permeation rates and lag times of the drugs through stripped or stripped/split skin. The cumulative amounts of NR, ISDN and FP that permeated through stripped/split (0.4 mm) rat skin over 1 h were approximately 80%, 91% and 76% of the initial amounts applied on the skin, respectively. The skin permeation rate of ISDN was higher than that of NR and FP in these in vitro stripped rat skin permeation experiments. The permeation rates of NR, ISDN and FP through the stripped rat skin were approximately 0.21, 0.13 and 0.09 times lower than those through stripped/split (0.4 mm) rat skin, respectively. Significant differences in the drug permeation rates were observed between stripped/split (0.4 mm) and stripped/split (0.9 mm) rat skin for NR (p<0.001), ISDN (p<0.001) and FP (p<0.025) and between stripped and stripped/split (0.4 mm)
Fig. 2. Time Course for the Cumulative Amounts of NR, ISDN and FP through Intact, Split, Stripped or Stripped/Split Rat Skin (Skin Thickness: 0.4, 0.9 or 1.2 mm)

Each value represents the mean±S.D. (n=3). Intact and split rat skin: (A), (B) and (C); stripped and stripped/split rat skin: (D), (E) and (F).

Table 2. Permeation Rates and Lag Times of NR, ISDN and FP through Intact, Split, Stripped or Stripped/Split Rat Skin (Skin Thickness: 0.4, 0.9 or 1.2 mm)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Skin type</th>
<th>Skin thickness (mm)</th>
<th>Permeation rate (μg/cm²/h)</th>
<th>Lag time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>Split</td>
<td>0.4</td>
<td>3.53±0.642</td>
<td>0.32±0.33</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>3.32±0.546</td>
<td>1.14±0.80</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>1.2</td>
<td>2.95±0.663</td>
<td>2.68±1.31*</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>327±21.6</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>120±13.7***</td>
<td>0.08±0.08</td>
</tr>
<tr>
<td></td>
<td>Stripped</td>
<td>1.2</td>
<td>70.0±7.69***</td>
<td>0.24±0.01**</td>
</tr>
<tr>
<td>ISDN</td>
<td>Split</td>
<td>0.4</td>
<td>18.5±3.06</td>
<td>0.16±0.28</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>18.4±3.74</td>
<td>0.23±0.11</td>
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<tr>
<td></td>
<td>Intact</td>
<td>1.2</td>
<td>15.6±1.58</td>
<td>0.71±0.18*</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>413±42.0</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>130±11.7***</td>
<td>0.07±0.06</td>
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<tr>
<td></td>
<td>Stripped</td>
<td>1.2</td>
<td>52.4±5.50***</td>
<td>0.32±0.06***</td>
</tr>
<tr>
<td>FP</td>
<td>Split</td>
<td>0.4</td>
<td>4.66±1.11</td>
<td>0.03±0.03</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>4.31±0.411</td>
<td>0.63±0.24</td>
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<tr>
<td></td>
<td>Intact</td>
<td>1.2</td>
<td>3.61±1.30</td>
<td>3.31±0.64***</td>
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<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>175±27.4</td>
<td>0.01±0.01</td>
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<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>36.8±1.06*</td>
<td>0.35±0.04**</td>
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<tr>
<td></td>
<td>Stripped</td>
<td>1.2</td>
<td>15.9±0.737*</td>
<td>0.77±0.16***</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=3). *p<0.05, **p<0.01 and ***p<0.001 compared with split (0.4 mm) or stripped/split (0.4 mm) rat skin for each drug using Dunnett tests. #p<0.025 compared with split (0.4 mm) or stripped/split (0.4 mm) rat skin for FP using Welch tests (the Bonferroni method for multiple comparisons was applied).
skin for NR ($p<0.001$), ISDN ($p<0.001$) and FP ($p<0.025$). Thus, the permeation rates of the 3 drugs decreased with an increase in the skin thickness.

The lag times for the skin permeation of NR, ISDN and FP through stripped rat skin were approximately 6.0, 32.0 and 77.0 times longer than that through stripped/split (0.4 mm) rat skin, respectively. Significant differences in the lag times of the drugs were observed between stripped/split (0.4 mm) rat skin for FP ($p<0.01$) and between stripped and stripped/split (0.4 mm) rat skin for NR ($p<0.01$), ISDN ($p<0.001$) and FP ($p<0.001$). The lag times of the 3 drugs increased with increase an in the skin thickness.

**Permeation Profiles of the 3 Model Drugs through YMP Skin. Intact and Split YMP Skin** The influence of skin thickness on the permeabilities of the 3 model drugs was evaluated using split (0.4, 0.9, 1.8 mm) and intact (2.8 mm) YMP skin. Figure 3 shows the time course of the cumulative amounts of the drugs that permeated through the intact or split YMP skin. Table 3 summarizes the mean permeation rates and lag times of the drugs through intact or split YMP skin. The cumulative amounts of NR, ISDN and FP that permeated through the split (0.4 mm) YMP skin over 10 h were approximately 22%, 74% and 21% of the initial amounts applied on the skin, respectively. The permeation rate of ISDN was higher than those for NR and FP in these YMP skin permeation experiments. The permeation rates of NR, ISDN and FP through intact YMP skin were approximately 0.44, 0.49 and 0.36 times lower than those through split (0.4 mm) YMP skin, respectively. Thus, an increase in the skin thickness decreased the drug permeation rates. In addition, the permeation rates were significantly different between the intact and split (0.4 mm) YMP skin for NR ($p<0.05$), ISDN ($p<0.01$) and FP ($p<0.05$).

The lag time for the skin permeation of FP through split (0.9 mm) YMP skin was approximately 1.6 times longer than that through split (0.4 mm) YMP skin, and the lag times for the skin permeation of NR, ISDN and FP through intact YMP skin were approximately 3.6, 10.1 and 4.3 times longer than those through split (0.4 mm) YMP skin, respectively. Significant differences in the lag times were observed between split (0.4 mm) and split (1.8 mm) YMP skin for ISDN ($p<0.05$) and FP ($p<0.001$) and between split (0.4 mm) and intact YMP skin for NR ($p<0.01$), ISDN ($p<0.01$) and FP ($p<0.001$). The lag times of the 3 drugs increased with an increase in the skin thickness.

**Stripped and Stripped/Split YMP Skin** The influence of skin thickness on the permeabilities of the drugs was evaluated using stripped (2.8 mm) and stripped/split (0.4, 0.9, 1.8 mm) YMP skin. Figure 3 also shows the time course of the cumulative amounts of the drugs that permeated through stripped
or stripped/split YMP skin. In addition, Table 3 summarizes the mean permeation rates and lag times through stripped and stripped/split YMP skin. The cumulative amounts of NR, ISDN and FP that permeated through the stripped/split (0.4 mm) YMP skin for NR (\(\approx 0.05\) or 0.14% for split (0.4 mm) YMP skin for each drug using Dunnett tests. \(p<0.017\) and \(p<0.003\) compared with split (0.4 mm) or stripped/split (0.4 mm) YMP skin for each drug using Welch tests (the Bonferroni method for multiple comparisons was applied).

### Table 3. Permeation Rates and Lag Times of NR, ISDN and FP through Intact, Split, Stripped or Stripped/Split YMP Skin (Skin Thickness: 0.4, 0.9, 1.8 or 2.8 mm)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Skin type</th>
<th>Skin thickness (mm)</th>
<th>Permeation rate ((\mu g/cm^2/h))</th>
<th>Lag time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>Split</td>
<td>0.4</td>
<td>4.01±0.381</td>
<td>1.92±0.29</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>3.88±1.15</td>
<td>1.54±1.40</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>1.8</td>
<td>2.39±0.647</td>
<td>4.72±1.92</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>2.8</td>
<td>1.76±0.199*</td>
<td>6.89±0.48**</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>137±11.0</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>45.4±14.0**</td>
<td>0.24±0.09</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>1.8</td>
<td>15.0±0.200**</td>
<td>0.46±0.14*</td>
</tr>
<tr>
<td></td>
<td>Stripped</td>
<td>2.8</td>
<td>7.37±3.15**</td>
<td>1.17±0.23***</td>
</tr>
<tr>
<td>ISDN</td>
<td>Split</td>
<td>0.4</td>
<td>12.4±1.57</td>
<td>0.34±0.44</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>13.3±2.90</td>
<td>0.36±0.14</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>1.8</td>
<td>8.05±1.63</td>
<td>1.19±0.47**</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>2.8</td>
<td>6.04±0.753**</td>
<td>3.45±0.23***</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>147±25.2</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>56.2±6.30</td>
<td>0.05±0.05</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>1.8</td>
<td>20.1±4.01*</td>
<td>0.51±0.15</td>
</tr>
<tr>
<td></td>
<td>Stripped</td>
<td>2.8</td>
<td>12.5±2.44*</td>
<td>1.63±0.27*</td>
</tr>
<tr>
<td>FP</td>
<td>Stripped/split</td>
<td>0.4</td>
<td>4.16±1.27</td>
<td>1.86±0.42</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>0.9</td>
<td>3.78±1.10</td>
<td>2.89±0.63</td>
</tr>
<tr>
<td></td>
<td>Split</td>
<td>1.8</td>
<td>2.45±0.386</td>
<td>7.38±0.52**</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>2.8</td>
<td>1.49±0.0868*</td>
<td>8.08±0.62***</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.4</td>
<td>89.8±10.2</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>0.9</td>
<td>14.0±0.929*</td>
<td>0.38±0.22</td>
</tr>
<tr>
<td></td>
<td>Stripped/split</td>
<td>1.8</td>
<td>6.75±0.279*</td>
<td>1.14±0.38</td>
</tr>
<tr>
<td></td>
<td>Stripped</td>
<td>2.8</td>
<td>2.86±0.363*</td>
<td>3.70±0.52**</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. \((n=3)\). \(p<0.05, **p<0.01\) and \(***p<0.001\) compared with split (0.4 mm) or stripped/split (0.4 mm) YMP skin for each drug using Dunnett tests. \(p<0.017\) and \(p<0.003\) compared with split (0.4 mm) or stripped/split (0.4 mm) YMP skin for each drug using Welch tests (the Bonferroni method for multiple comparisons was applied).

The lag times for the skin permeation of NR, ISDN and FP through stripped YMP skin were approximately 35.8, 81.5 and 26.4 times longer than those through stripped/split (0.4 mm) YMP skin samples. The lag times for the skin permeation of the 3 drugs were significantly different between stripped/split (0.4 mm) and stripped/split (1.8 mm) YMP skin for NR \((p<0.05)\) and between stripped and stripped/split (0.4 mm) YMP skin for NR \((p<0.001)\), ISDN \((p<0.017)\) and FP \((p<0.017)\). The lag time of the 3 drugs was increased with an increase in the skin thickness.

**Relationship between Resistance and Skin Thickness**

Table 4 summarizes the permeability coefficients of the 3 model drugs through each skin layer of rat or YMP skin. The permeability coefficient \(P_{tot}\) of the drugs decreased with an increase in the skin thickness. In addition, the permeability coefficient \(P_{ved}\) of the drugs decreased remarkably with skin thickness. The relationships were evaluated between the \(R_{ved}\) \((1/P_{ved})\) of the 3 drugs and skin thickness.

Figure 4 shows the \(R_{ved}\) for the 3 drugs plotted against the mean thickness of the stripped and stripped/split rat or YMP skin. The correlation coefficients \((r)\) for the 3 drugs ranged from 0.938 to 0.984 for rat skin and from 0.980 to 0.998 for YMP skin. A good correlation was obtained between the \(R_{ved}\) of the 3 drugs through stripped or stripped/split rat or YMP skin and the skin thickness.

**DISCUSSION**

The goal in the present *in vitro* skin permeability study is to predict blood concentration of drugs after topical application of TTS in humans. Initially, we thought that *in vivo* human percutaneous absorption rate should be predicted using *in vitro* human skin permeation study. The OECD guidelines also specify a similar approach. However, variations in the *in vitro* human skin permeability were remarkably larger than those in the *in vitro* animal skin permeability.1,14–17 Then, we
reexamined whether in vitro human skin permeability could be predicted from in vitro animal skin permeability. Our previous studies have shown that in vitro YMP skin permeability was markedly similar to the in vitro human skin permeability, however, the goal in the present study is to predict blood concentration of drugs after topical application in humans from in vitro skin permeation study using YMP skin. Therefore, we confirmed in the present study that the in vitro permeation study using thickness-adjusted YMP skin could quantitatively predict the in vivo percutaneous absorption rate of drugs in humans.

Bronaugh et al. analyzed the capillary clearance of drugs from the dermis in human skin and showed that a diffusion thickness of 0.2 mm was preferable and suitable for estimating in vivo absorption. In addition, van de Sandt et al. and Cnubben et al. reported that the in vitro skin permeation rates of lipophilic compounds (propoxur and ortho-phenylphenol) were overestimated using human epidermal membranes and underestimated using intact human skins, compared with in vivo percutaneous absorption rates in humans. Sato et al. and Kawamata and Tojo tried to predict the plasma concentration of nicorandil or GTS-21 after topical application of nicorandil (gel) or GTS-21 (TTS) in hairless rats from the in vitro intact hairless rat skin permeation parameters. However, they reported that some differences were observed between the observed (in vivo) and the calculated plasma concentration of drugs. Thus, the blood concentration of drugs after topical application in rat cannot be completely predicted by the in vitro skin permeation studies using the intact rat skins. Therefore, split skin, which is thicker than the epidermis, was selected for our in vitro permeation studies. The percutaneous absorption rates and blood concentration of drugs after topical application of TTS in rat or human can be well predicted by the in vitro skin permeation studies using the optimal thickness of split rat or human skin.

However, human skin is not always available and the use of human tissues and organs creates ethical problems as well as large variation in in vitro human skin permeability. Our previous studies have shown that rat and YMP skins with minimal variations in skin permeability are possibly much better than human skin for determining or estimating the skin permeabilities of drugs and for developing transdermal formulations.
Therefore, we selected rat and YMP skins for the in vitro skin penetration studies. Although several researchers have already investigated the in vitro rat and YMP skin permeabilities, few studies have focused on the prediction of in vivo percutaneous absorption rate or blood concentration of drugs after topical application of TTS in human from the in vitro rat or YMP skin permeation parameters. In vitro skin permeation studies using optimal thickness of rat or YMP skin may be performed for predicting the in vivo percutaneous absorption rate in human. Therefore, the in vitro permeation experiments were performed using split rat and YMP skins with various thicknesses for accurately determining the permeation rate of drugs.

On the other hand, we then dermatomed the skin using an electric dermatome by a new method established in our previous studies. In the new method, a styrene foam board of 0.5 mm thickness was placed exactly between the skin and corkboard. The styrene foam board has a role of cushion in this method. The variation in the skin thickness was suppressed to prepare a uniform skin sample. The thickness of rat or YMP skin was kept constant. Although skin samples were sometimes observed in these skin samples. Then, the in vitro permeation studies were performed using split (0.4 mm) skin with the thinnest dermis.

In the present study, we selected 3 model drugs as hydrophilic (NR), lipophilic (ISDN) and highly lipophilic (FP) drugs with molecular weights of around 200. In addition, in vitro skin permeation experiments were performed using intact, split, stripped or stripped/split rat or YMP skin (Figs. 2, 3). As Fick’s first law of diffusion describes steady-state diffusion rate through a membrane, where , , , and are the steady-state permeation rate, the drug concentration in the applied solution to skin, the diffusion coefficient of drugs in the membrane, partition coefficient of drugs between the stratum corneum and vehicle and the diffusion path length across the membrane, respectively. We used fixed concentrations ( ) of the 3 model drugs and changed the skin thickness ( ). The influence of skin thickness on the permeability of the 3 drugs with different physicochemical properties was evaluated using permeation rates and lag times. The permeation rates of the 3 drugs through rat or YMP skin decreased with an increase in the skin thickness. In addition, the lag time of the 3 drugs through rat or YMP skin increased with an increase in the skin thickness. The decrease in the permeability of the 3 drugs through intact or stripped YMP skin was larger than that through intact or stripped rat skin. The changes were the greatest for the permeability of FP (highly lipophilic drug) through intact or stripped rat or YMP skin. These in vitro skin permeation results showed that the drug permeability through rat or YMP skin was greatly influenced by the skin thickness.

Wilkinson et al. reported a relationship between skin thickness and the physicochemical properties of caffeine (log ), propoxur (log ) and testosterone (log ) in human skin permeation studies. The permeability of these drugs through split human skin (approximately 0.5 mm) decreased with an increase in the skin thickness (intact skin: approximately 1 mm). The maximum flux and lag time of caffeine (hydrophilic drug) through split human skin were 2.4 and 0.8 times that through intact human skin, respectively. Thus, the skin thickness influenced the permeability of the hydrophilic drug. In addition, the maximum flux and lag time of testosterone (highly lipophilic drug) through split hu-
man skin were 10.1 and 0.3 times that through intact human skin, respectively. Henning et al.\(^{27}\) reported the permeability coefficients and lag times of flufenamic acid (log \(K_{eq}=3.9\)) in lipophilic vehicles through intact (2.02 mm) or split (0.55 mm) human skin from *in vitro* permeation studies. The permeability coefficient and lag time of flufenamic acid through split human skin were 5.2 and 0.1 times that through intact human skin, respectively. The influence of dermis thickness on the permeability of lipophilic drugs was observed with an increase in the thickness of human skin. These results using human skin correspond with those in YMP skin in the present study.\(^{20,27}\) The influence of skin thickness on the *in vitro* permeability of hydrophilic and lipophilic drugs through human skin is similar to that through YMP skin.\(^{20,27}\) The reduced influence of thickness in the intact rat skin was due to rat skin being thinner than either YMP or human skin. However, blood concentration of drugs after topical application in human cannot be quantitatively predicted from their *in vitro* experimental data,\(^{20,27}\) because they did not clarify the optimal skin thickness by changing the human skin thickness to predict *in vivo* human percutaneous absorption rate. Therefore, prediction of blood concentration of drugs after topical application in human must be predicted by the *in vitro* skin permeation studies using the optimal thickness of split rat and YMP skin.

The skin is not a homogenous membrane but is instead composed of different successive layers. The total resistance of such a membrane composed of consecutive layers is the sum of the resistances of each single layer. Therefore, the barrier function of SC and VED on the skin permeability is evaluated by determining the resistance in each layer, calculated from the reciprocal of the permeability coefficient through the layer.\(^{21,28,29}\) The resistances \(R_{tot}\), \(R_{ac}\) and \(R_{ved}\) are the resistances of the total (SC and VED), SC and VED layers, respectively. To clarify the relationship between resistance (\(R_{ved}\) or \(R_{tot}\)) and skin thickness, the resistances of the 3 drugs through rat or YMP skin was plotted against skin thickness.

High correlations (\(r \geq 0.938\)) were obtained for the relationship between the \(R_{ved}\) of the 3 model drugs through the stripped or stripped/split rat or YMP skin and the skin thickness (Fig. 4). In addition, the slopes of each drug for the stripped and stripped/split YMP skin were larger than those for the stripped and stripped/split rat skin. The slope of FP for the stripped and stripped/split YMP skin was the largest among the 3 drugs. Our findings suggest that the permeability of FP through stripped or stripped/split YMP skin is influenced with an increase in the dermis thickness. The resistance of drugs through intact skin can be attributed to not only SC but also VED. In addition, the VED (stripped skin) is composed of 2 layers, the viable epidermis and the dermis. Behl et al.\(^{35}\) reported that the dermis contributes to the majority of the total resistance through stripped skin. Ngawhirunpat et al.\(^{14}\) reported that the change in the permeability through stripped skin might be a consequence of the change in dermis thickness.

Next, the barrier ratio was considered for \(R_{ved}/R_{tot}\) and \(R_{ved}/R_{ved}\). The \(R_{ved}/R_{tot}\) values of the 3 drugs in split (0.9 mm) rat and YMP skin were from 2.5- to 4.5-fold and from 2.7- to 5.9-fold compared to split (0.4 mm) rat and YMP skin, respectively (Table 4). The influence of the resistance of ISDN and FP through the split rat or YMP skin was greater at 0.9 mm skin thickness than 0.4 mm skin thickness.

The \(R_{ved}/R_{tot}\) of NR for the intact and split (0.4 and 0.9 mm) rat skin ranged from 1.1 to 4.1% and was not altered by an increase in the skin thickness. The \(R_{ved}/R_{tot}\) of ISDN and FP for the split (0.4, 0.9 mm) and intact rat skin ranged from 4.4 to 29.9% for ISDN and from 2.6 to 20.7% for FP. These values increased with an increase in the skin thickness. The \(R_{ved}/R_{tot}\) of ISDN was similar to that of FP. The \(R_{ved}/R_{tot}\) values for the 3 drugs could be classified in the order of ISDN=FP>NR.

In addition, the \(R_{ved}/R_{tot}\) of the 3 drugs for intact and split (0.4, 0.9, 1.8, 2.8 mm) YMP skin ranged from 2.9 to 27.0% for NR, from 8.5 to 48.8% for ISDN, and from 4.3 to 52.5% for FP. These values increased with an increase in the skin thickness. The \(R_{ved}/R_{tot}\) values for the 3 drugs could be categorized in the order of FP>ISDN>NR. The values of \(R_{ved}/R_{tot}\) for each drug through intact YMP skin were larger than that through intact rat skin.

These experiments showed that the permeability of the 3 drugs through rat or YMP skin was influenced with an increase in the dermis thickness in *in vitro* skin permeation experiments conducted over 24 h. The influence of the dermis thickness on drug permeability was more pronounced for intact YMP skin than for intact rat skin. The influence of dermis thickness on the permeability of ISDN and FP was also observed in split (0.9 mm) rat and YMP skin. A remarkable influence of the dermis thickness on the permeability of ISDN and FP (lipophilic drugs) was observed with an increase in the skin thickness.

Drugs are generally taken up by cutaneous capillaries at a depth of 0.2 mm from the skin surface. Accordingly, one should reduce the influence of dermis thickness in *in vitro* skin permeation studies to reflect blood concentration of drugs after topical application in humans. Therefore, predictions of percutaneous absorption rate after topical application in humans would depend on *in vitro* skin permeation studies using split (0.4 mm) skin with the thinnest dermis.

**CONCLUSION**

The influence of skin thickness on the permeability of the 3 model drugs with different physicochemical properties was evaluated using *in vitro* rat and YMP skin permeation studies. Our findings showed that the *in vitro* permeability of the 3 drugs through rat or YMP skin decreased with an increase in the dermis thickness, with the YMP skins showing larger changes than the rat skins, and remarkable changes were observed for ISDN and FP (lipophilic drugs). In addition, the influence of the dermis thickness on the *in vitro* permeability of drugs through the YMP skin was similar to that through human skin.\(^{20,27}\)

In the *in vitro* skin permeation studies, one should reduce the influence of the dermis thickness. The present results suggest that prediction of percutaneous absorption rate of drug after topical application in humans can be well done by the *in vitro* skin permeation studies using split (0.4 mm) skin with the thinnest dermis.

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REFERENCES


