Usefulness of Rat Skin as a Substitute for Human Skin in the *in Vitro* Skin Permeation Study

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**Abstract:** Sprague-Dawley (SD) rats are broadly used in preclinical studies for drug development, so a lot of information for the rats can be obtained especially from pharmacokinetic, pharmacological and toxicological studies. The purpose of this study was to clarify whether SD rat skin can be used to predict human skin permeability. *In vitro* permeation studies of the three model drugs, nicorandil, isosorbide dinitrate, and flurbiprofen, through human skin and SD rat skin were performed using Franz-type diffusion cells. The permeation rates of the three model drugs through human skin and SD rat skin were determined, and their variations were evaluated. The inter-individual variations in SD rat skin permeability of the three model drugs were much lower than that in human skin permeability, although the permeation rates of the three model drugs through the SD rat skin were about twice those through human skin. In addition, no difference in the skin permeability coefficients of the three model drugs was obtained between fresh SD rat skin and frozen SD rat skin. The markedly smaller variation in the permeability through SD rat skin compared with that through human skin indicated that *in vitro* permeation studies using SD rat skin would be especially useful for evaluating differences in the skin permeability of the three model drugs as well as for predicting human skin permeability.

**Key words:** human skin, inter-individual, rat skin, skin permeability, variation

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**Introduction**

*In vitro* skin permeation studies have been broadly used in the development of transdermal formulations. Ideally, the skin permeability of drugs should be determined in humans. However, human skin is not always available, and the use of human tissues and organs creates ethical problems. Additionally, sufficient quantities of good-quality human skin samples for permeation studies are not readily accessible to most investigators and are only available in limited amounts. Moreover, a large variation has been found among human skin specimens as a result of differences in age, race, and anatomical donor site [3, 4, 6, 9, 23, 25, 27, 29–31]. Therefore, the
large variation in human skin permeability is a point of concern and the most important problem in the use of human skin for the development of transdermal formulations [1, 3, 18, 28, 31, 36]. Furthermore, a variation in human skin permeability has been reported even among specimens (inter-individual variations) and within samples from one specimen or individual (intra-individual variations). Generally, the inter-individual variation in human skin permeability is higher than the intra-individual variation [1, 3, 31].

On the other hand, numerous animal models have been suggested as an alternative to human skin, including primate, porcine, mouse, rat, guinea pig, and snake skin [3, 6, 8, 9, 24, 26, 27, 32, 34]. Animal skins with a small variation in skin permeability may be much better suited than human skin for determining or estimating the skin permeabilities of drugs and for developing transdermal formulations [3, 18, 28]. The inter-individual variations in animal skin permeabilities may be similar to the intra-individual variations because the inter-individual and intra-individual variations are relatively small among skin specimens from inbred animal strains.

The skins of rodents (mice, rats, and guinea pigs) are the most commonly used animal models for in vitro and in vivo percutaneous permeation studies. The advantages of these animals are their small size and easy handling. Sprague-Dawley (SD) rats are broadly used for in vivo pharmacokinetic, pharmacological, and toxicological studies as parts of preclinical studies for drug development [14, 15, 19, 32, 35]. SD rats as well as hairless rats are often used for in vivo percutaneous absorption and in vitro skin permeation studies. However, few studies have examined the correlation between in vitro permeation in SD rat skins and human skins.

We investigated the in vitro permeation studies of three model drugs, nicorandil (NR), isosorbide dinitrate (ISDN), and flurbiprofen (FP), through human and SD rat skin were performed using Franz-type diffusion cells. The three model drugs were selected because of their different log $K_{ow}$ (logarithm of the octanol/water partition coefficient at 37°C). This study deals with both an old and new theme in this field. Although a number of researchers have investigated in vitro human and rat skin permeability, few reports have simultaneously examined the difference between human and rat skin permeability focusing on the variation in permeability. Therefore, the purpose of the present study was to evaluate the inter-individual and intra-individual variations in the in vitro human skin permeability, to examine the inter-individual variations in the in vitro SD rat skin permeability, and to clarify whether SD rat skin can be used to predict human skin permeability.

### Table 1. Chemical structures and physicochemical parameters of the model drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Structure</th>
<th>Molecular weight</th>
<th>Solubility in water (mg/ml)</th>
<th>Log $K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicorandil (NR)</td>
<td><img src="image" alt="Nicorandil" /></td>
<td>211.17</td>
<td>39.6</td>
<td>-1.02</td>
</tr>
<tr>
<td>Isosorbide dinitrate (ISDN)</td>
<td><img src="image" alt="Isosorbide dinitrate" /></td>
<td>236.14</td>
<td>1.34</td>
<td>1.34</td>
</tr>
<tr>
<td>Flurbiprofen (FP)</td>
<td><img src="image" alt="Flurbiprofen" /></td>
<td>244.27</td>
<td>0.0277</td>
<td>3.86</td>
</tr>
</tbody>
</table>

*a) Hatanaka et al. (1990) [11]. b) Solubility in water at 37°C. c) Logarithm of the octanol/water partition coefficient at 37°C.*
Materials and Methods

Reagents

Nicorandil (NR), isosorbide dinitrate (ISDN), and flurbiprofen (FP) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), Alexis Corporation (San Diego, CA, USA), and Sigma-Aldrich (St. Louis, MO, USA), respectively. All other chemicals and solvents were of reagent grade and were obtained commercially.

Human skin preparation

Permeation profiles of NR, ISDN, and FP through human skin were determined by in vitro permeation experiments. Frozen human abdominal skin specimens [from 3 male (46–52 years) and 3 female Caucasians (38–53 years); approximately 10 × 10 cm in size] were obtained from the non-profit Human and Animal Bridging Research Organization (Tokyo, Japan). The frozen skin specimens were stored at –80°C until the permeation studies.

The frozen human skin specimens were thawed in a CO₂ incubator at 32°C (relative humidity: 95%) for 15 min. The subcutaneous fat was removed from each human skin specimen, and then the specimen was dermatomed to a nominal thickness of 400 µm using an electric dermatome (Model B; Padgett Instruments, Inc., Kansas, MO, USA). Nine or less human skin samples (each 2.5 × 2.5 cm) could be obtained from one human specimen. In addition, the thickness of the human skin samples was measured, with the thickness ranging from 0.36 to 0.44 mm. The human skin samples were placed on a paper towel soaked with saline and stored in a sealed container at 4°C for 16 h before the start of the skin permeation studies.

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

Permeation profiles of NR, ISDN, and FP through rat skin were determined by in vitro permeation experiments. Male SD rats (8 weeks old) were used for the skin permeation studies. The SD rats were euthanized under anesthesia with diethyl ether, and the dorsal region of the SD rat skin was carefully shaved. One round section of SD rat skin (2.5 cm in diameter) was excised from the dorsal skin at a point 7 ± 1 cm from the rat’s ears. After the subcutaneous fat was removed using scissors, the excised SD rat skin samples were frozen and stored at –80°C until use (usually within 1 month). Fresh SD rat skins were also used immediately after excision for comparison. The thickness of the excised SD rat skin samples ranged from 1.1 to 1.3 mm. The damage in the stratum corneum of shaved SD rat skins was evaluated histologically. No damage was observed by shaving in the stratum corneum of both the fresh and frozen SD rat skin sections.

Inter-individual variations in SD rat skin permeability of the three model drugs were performed among 6 SD rat skin samples.

Skin permeation procedure (Franz-type diffusion cells)

The method used for the percutaneous absorption study was the same as that outlined in Test Guideline 428 of the Organization for Economic Cooperation and Development [20].

The permeation experiments were mainly performed using modified Franz-type diffusion cells (0.95 cm², diameter=1.1 cm) [7]. Franz-type diffusion cells are usually applied for determination of permeation rates of the three model drugs through skin. The skin was thawed in a CO₂ incubator at 32°C (relative humidity: 95%) for 30 min before the start of the skin permeation experiment and was then mounted on the diffusion cells. The receptor cell was filled with 3.2 ml of distilled water or 0.1 M phosphate buffer (pH7.4). One of the three model 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 applied to the skin surface. The receptor cell medium was kept at 32°C and stirred with a Teflon stirrer driven at 150 rev min⁻¹ by a constant speed motor throughout
the skin permeation experiment. The diffusion cells were maintained in a CO₂ incubator at 32°C (relative humidity: 95%). The receptor fluid (1.0 ml) was sampled at 1, 2, 3, 4, 5, 6, 8, 10, and 24 h after the start of the skin permeation experiment, and the same volume of fresh phosphate buffer was added to the receptor cell to maintain a constant volume. The receptor fluid samples were stored in a freezer at −80°C until analysis of the drug concentration.

Skin permeation procedure (side-by-side diffusion cells)

The side-by-side diffusion cells were used to compare the permeability coefficients (P) of the three model drugs through fresh and frozen rat skin. Water or buffer solution was selected as a donor solvent because they have no influence on skin permeability.

SD rat skin was thawed in a CO₂ incubator at 32°C (relative humidity: 95%) for 30 min before the start of the skin permeation experiment and was sandwiched between the two halves of the diffusion cells, with each half cell containing a volume of 3.0 ml and an effective diffusion area of 0.95 cm² (diameter=1.1 cm) [18, 28]. The receptor cell (dermis side of the skin) was filled with 3.0 ml of distilled water or 0.1 M phosphate buffer (pH 7.4), whereas the donor cell (stratum corneum side of the skin) was filled with 3.0 ml of NR, ISDN, or FP aqueous suspension or 0.1 M phosphate buffer (pH 7.4) solution. The receptor cell and donor cell medium were maintained at 32°C (relative humidity: 95%) in a CO₂ incubator. Both cell mediums were stirred using a Teflon stirrer driven at 150 rev min⁻¹ by a constant speed motor throughout the permeation experiment. A 1.0-ml aliquot of the receptor fluid was sampled at 1, 2, 3, 4, 5, 6, 8, 10, and 24 h, and the same volume of distilled water or fresh phosphate buffer was added to the receptor cell to maintain a constant volume. The receptor fluid samples were stored in a freezer at −80°C until analysis.

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 liquid chromatography-tandem mass spectrometry (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 (I.S.) of FP (furosemide; 0.25 µg/ml) or NR (propranolol; 0.25 µg/ml) and vortex-mixed. After centrifugation at 3,600 × 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 API 3000 mass spectrometer equipped with a TurboIonSpray interface (Applied Biosystems, Foster, CA, USA), and an API 3000 mass spectrometer equipped with a TurbolIonSpray interface (Applied Biosystems, Foster, CA, USA).

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 98% mobile phase A (aqueous 0.1% formic acid) to 98% mobile phase B (acetonitrile) in 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 transitions unique to each compound, m/z 243 → m/z 192 for FP, m/z 329 → m/z 205 for furosemide (I.S.), m/z 212 → m/z 136 for NR, and m/z 260 → m/z 116 for propranolol (I.S.). 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 high-performance liquid chromatography (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 3,600 × g for 10 min at 4°C, the resulting supernatant (10 µl) was directly injected into an HPLC (LC-10ADvp system; Shimadzu, Kyoto, Japan) with a UV detector (SPD-10ADvp system; Shimadzu).

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 (60/40, v/v) at a flow rate of 1.0 ml/min. The wavelength
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of the UV detector was set at 210 nm. The retention time was approximately 6 min. The calibration curve range was from 0.3 to 30 µg/ml for ISDN, and the accuracy and precision were calculated.

**Histological study**

Fresh and frozen SD rat skins were immediately fixed in 10% neutral buffered formalin and embedded in paraffin after excision and thawing, respectively. In addition, the frozen and fresh rat skins were fixed in 10% neutral buffered formalin and embedded in paraffin at 10 and 24 h, respectively, after the start of the permeation experiment using the side-by-side diffusion cells.

Human skin (No. 1) was immediately fixed in 10% neutral buffered formalin and embedded in paraffin after thawing and at 0, 10, and 24 h after the start of the permeation experiment using the Franz-type diffusion cells.

For conventional histological examinations, paraffin sections (5 µm) were made from formalin-fixed samples and stained using hematoxylin-eosin (HE). The stained samples were analyzed using light microscopy (Axioplan 2; Carl Zeiss, Jena, Germany) [4, 13, 27].

**Data analysis**

The permeation parameters for the three model 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. The permeability coefficient (P) was calculated from the following equation (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.

**Statistical analysis**

All data are shown as the mean ± SD. The variation coefficient (CV%) was calculated using the following equation (2):  

\[ CV% = \frac{SD}{mean} \times 100 \]  

The permeation rate between male human skin and female human skin, and the permeability coefficient (P) between fresh SD rat skin and frozen SD rat skin was compared using the Student’s t-test. A value of \( P<0.05 \) was considered statistically significant. Linear regression analysis was used to evaluate the relationship of the log P between fresh SD rat skin and fresh hairless rat skin or the relationship between the permeation rate of human skin and frozen SD rat skin. Statistical analysis was performed using the SAS software (SAS Institute Japan Ltd., Tokyo, Japan).

**Results**

**Inter- and intra-individual variation human skin permeability**

The inter-individual variations in human skin permeability were evaluated among specimens from 6 humans. Figure 1A shows the time course for the cumulative amounts of the three model drugs (NR, ISDN, and FP) that permeated through excised human skin using the Franz-type diffusion cells. Table 2 summarizes the mean permeation rates and their CV% of the three model drugs through the human skin. The permeation rate of ISDN was higher than those of NR and FP in the in vitro human skin permeation studies. The permeated amount of ISDN over 10 h after the start of the skin permeation experiment was about 70% of the amount applied to the skin. Therefore, the permeation rate of ISDN decreased over time after a lag-time period. The permeation rates of the three model drugs through male human skin were compared with those through female human skin. No significant difference was observed in the permeation rates of the three model drugs between male and female human skin.

The inter-individual variations in human skin permeability in males, females, or both males and females were evaluated using the CV% of the permeation rates. The CV% of the permeation rates for the three drugs in males ranged from 4.2 to 55.7%, and the CV% of the permeation rates for the three model drugs in females ranged from 19.4 to 80.5%. In addition, the CV% of the permeation rates of the three model drugs in both males and females ranged from 22.0 to 65.0%. The CV% of NR in males, females or both males and females was higher than those of ISDN and FP. The CV% of the three model drugs in both males and females was in the order
Next, the intra-individual variations in human skin permeability were evaluated within three samples from one specimen from each of the 3 males (No. 1, No. 2, and No. 3) and 3 females (No. 4, No. 5, and No. 6). Table 3 shows the CV% of the human skin permeation rates for the three model drugs. The CV% of the permeation rates for the three model drugs in males ranged from 5.3 to 55.5%, and the CV% of the permeation rates for the three model drugs in females ranged from 9.7 to 40.4%. The CV% of the permeation rates for NR (No. 3), ISDN (No. 5), or FP (No. 6) was more than 30% in males or females.

The intra-individual variations in human skin permeability of the three model drugs were compared with the inter-individual variations. The CV% of the inter-individual variations for the three model drugs in the 6 humans (3 males plus 3 females) ranged from 22.0 to 65.0%, while the CV% of the intra-individual variations for the three model drugs in the 6 humans ranged from 5.3 to 55.5%. Thus, the inter-individual variations in human skin permeability of NR were slightly higher than the intra-individual variations, although the inter-individual variations in human skin permeability of ISDN and FP were similar to the intra-individual variations.

Table 2. Permeation rates of NR, ISDN, and FP through human skin and SD rat skin (Inter-individual variation)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>N/a</th>
<th>Drug</th>
<th>Permeation rate (µg/cm²/h)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Male</td>
<td>3</td>
<td>NR</td>
<td>4.29 ± 2.39</td>
<td>55.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ISDN</td>
<td>9.91 ± 2.67</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FP</td>
<td>2.20 ± 0.01</td>
<td>4.2</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td></td>
<td>NR</td>
<td>2.51 ± 2.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ISDN</td>
<td>8.99 ± 1.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FP</td>
<td>2.01 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.7</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td></td>
<td>NR</td>
<td>3.40 ± 2.21</td>
<td>65.0</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td>ISDN</td>
<td>9.45 ± 2.08</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FP</td>
<td>2.16 ± 0.72</td>
<td>33.2</td>
</tr>
<tr>
<td>SD rat</td>
<td>Male</td>
<td>6</td>
<td>NR</td>
<td>5.72 ± 1.48</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ISDN</td>
<td>14.7 ± 1.91</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FP</td>
<td>4.33 ± 0.83</td>
<td>19.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD (n=3–6). a)Number of humans and SD rats. b)No significant difference in the permeation rates of the three model drugs between male and female human skin (P<0.05, t-test).

Fig. 1. Time course for the cumulative amounts of NR, ISDN, or FP through human (A) and SD rat (B) skin (skin permeation experiment using Franz-type diffusion cells). Each value represents the mean ± SD (n=6 for human skin and SD rat skin).
Influence of freezing on skin structure and permeability

Figure 2 shows the time course for the cumulative amounts of the three model drugs that permeated through the fresh and frozen SD rat skins using the side-by-side diffusion cells.

Table 3. CV% of the permeation rates for NR, ISDN, and FP through human skin (Intra-individual variation for three males and three females)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Drug</th>
<th>Permeation rate (µg/cm²/h)</th>
<th>CV%</th>
<th>Permeation rate (µg/cm²/h)</th>
<th>CV%</th>
<th>Permeation rate (µg/cm²/h)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>NR</td>
<td>5.45 ± 0.29</td>
<td>5.3</td>
<td>5.89 ± 1.26</td>
<td>21.4</td>
<td>1.54 ± 0.85</td>
<td>55.5</td>
</tr>
<tr>
<td></td>
<td>ISDN</td>
<td>11.6 ± 0.85</td>
<td>7.4</td>
<td>11.3 ± 3.22</td>
<td>28.5</td>
<td>6.84 ± 0.78</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>FP</td>
<td>2.38 ± 0.40</td>
<td>16.8</td>
<td>2.35 ± 0.40</td>
<td>16.8</td>
<td>2.20 ± 0.44</td>
<td>19.8</td>
</tr>
<tr>
<td>Female</td>
<td>NR</td>
<td>1.35 ± 0.13</td>
<td>9.7</td>
<td>1.34 ± 0.27</td>
<td>20.1</td>
<td>4.84 ± 1.07</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>ISDN</td>
<td>7.89 ± 1.48</td>
<td>18.8</td>
<td>8.09 ± 2.17</td>
<td>26.8</td>
<td>11.0 ± 2.56</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>FP</td>
<td>1.28 ± 0.24</td>
<td>18.9</td>
<td>1.47 ± 0.57</td>
<td>38.6</td>
<td>3.27 ± 1.32</td>
<td>40.4</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD (n=3).

Table 4. Comparison of the permeability coefficients of NR, ISDN, or FP through fresh and frozen SD rat skin

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fresh SD rat Skin</th>
<th>Frozen SD rat Skin</th>
<th>Fresh Hairless rat skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeability coefficient (cm/s) x 10⁻⁷</td>
<td>Log P (cm/s)</td>
<td>Permeability coefficient (cm/s) x 10⁻⁷</td>
</tr>
<tr>
<td>NR</td>
<td>1.20 ± 0.89</td>
<td>-6.92</td>
<td>1.51 ± 0.80ᵣ</td>
</tr>
<tr>
<td>ISDN</td>
<td>53.6 ± 15.2</td>
<td>-5.27</td>
<td>63.9 ± 7.64ᵣ</td>
</tr>
<tr>
<td>FP</td>
<td>342 ± 148</td>
<td>-4.47</td>
<td>258 ± 104ᵣ</td>
</tr>
</tbody>
</table>

The permeability coefficients are expressed as the mean ± SD (n=3). Log P represents the mean (n=3). The permeability coefficients of the three model drugs are calculated from the slope of the linear portion of the graph corresponding to the time from 8 to 24 h. ᵣNo significant difference in the permeability coefficients of the three model drugs between fresh and frozen SD rat skin (P<0.05, t-test). ᵣᵣᵣThe data for the fresh hairless rat was quoted from the results of Hatanaka et al. (1992)[12].

Fresh skin permeability was compared with the frozen skin permeability in the present study. The permeability coefficients (P) of the three model drugs through fresh and frozen rat skins were calculated to examine the influence of freezing on skin permeation. Table 4 summa-
rizes the permeability coefficients (P) of the three model drugs through fresh and frozen rat skins. The permeability coefficients of the three model drugs were calculated from the slope of the linear portion of the graph corresponding to the time from 8 to 24 h. For the three model drugs, the permeability coefficients (P) of fresh rat skin ranged from $1.20 \times 10^{-7}$ to $342 \times 10^{-7}$ cm/s, while those through frozen SD rat skin ranged from $1.51 \times 10^{-7}$ to $258 \times 10^{-7}$ cm/s. No significant difference was observed in the permeability coefficients (P) of the three model drugs between fresh and frozen rat skins. The log P values of NR, ISDN, and FP through fresh rat skin were $-6.92$, $-5.27$, and $-4.47$ (unit for P is cm/s), while those through frozen rat skin were $-6.82$, $-5.19$, and $-4.59$, respectively.

No influence of freezing was observed in the skin permeability of the three model drugs through the frozen SD rat skin.

**Histology of the human and rat skin**

Figure 3 shows morphological observations of the human skin sections (No. 1) immediately after thawing (A) and at 0 h (B), 10 h (C), and 24 h (D) after the start of the permeation experiment. The scale indicates 10 µm. Original magnification, ×200.

Figure 4. Hematoxylin and eosin staining of fresh and frozen SD rat skin sections. Fresh SD rat skin sections at 0 h (A), 10 h (B), and 24 h (C) after the start of the permeation experiment. Frozen SD rat skin sections immediately after thawing (D) and at 10 h (E) and 24 h (F) after the start of the permeation experiment. The scale indicates 10 µm. Original magnification, ×200.
ments using the Franz-type diffusion cells. No obvious changes were observed in the stratum corneum at 0, 10, and 24 h. However, obvious dilatations were observed in the dermal connective tissues at 10 and 24 h.

Figure 4 shows the frozen rat skin sections immediately after thawing (0 h) and 10 and 24 h after the start of the permeation experiments and the fresh rat skin sections at 0, 10, and 24 h after the start of the permeation experiments using the side-by-side diffusion cells. No obvious changes were observed in the stratum corneum morphology of the fresh and frozen rat skin sections at 0, 10, and 24 h. Although vacuolations of the epidermis and dilatations of the dermal connective tissues were frequently observed in both the fresh and frozen rat skin sections at 10 and 24 h, the changes in epidermis and dermis morphology in frozen SD rat skin sections at 0, 10, and 24 h were similar to those in fresh SD rat skin sections. Therefore, the changes in skin morphology in frozen SD rat skin sections at 0, 10, and 24 h were similar to those in fresh SD rat skin sections.

Inter-individual variation in frozen SD rat skin permeation

The inter-individual variations in frozen SD rat skin permeability were evaluated. Frozen SD rat skin can be used instead of fresh SD rat skin. Figure 1B shows the time course for the cumulative amounts of the three model drugs through the excised SD rat skin using the Franz-type diffusion cells. Table 2 summarizes the mean permeation rates and their CV% of the three model drugs through the frozen SD rat skin. The permeation rate of ISDN was higher than those of NR and FP in the in vitro rat skin permeation studies. The amount of ISDN that permeated over a period of 10 h after the start of the skin permeation experiment was about 65% of the amount applied to the skin. Therefore, the permeation rate of ISDN decreased over time after a lag time period. The CV% of the permeation rates of the three model drugs ranged from 13.0 to 25.9%, which was much smaller than that in human skin permeability. The CV% of the three model drugs was in the order of ISDN<FP<NR.

Discussion

Evaluating the in vitro human skin permeability of drugs using human skin is a reasonable step in the development of transdermal formulations. In this study, we used human abdominal skins from 6 humans and selected three model drugs with different partition coefficients [11, 16, 18, 26, 32]. The log Kow values of NR, ISDN, and FP are –1.02 (low), 1.34 (intermediate), and 3.86 (high), respectively. NR, ISDN, and FP are used as hydrophilic, lipophilic, and highly lipophilic drugs, respectively. The stratum corneum is a membrane with a lipid pathway and a pore pathway, which serve as the main routes for lipophilic and hydrophilic drugs, respectively [16, 18].

The CV% of the inter-individual variations for the three model drugs in the 6 humans ranged from 22.0 to 65.0%. Southwell et al. [31] evaluated the in vitro human skin permeation of 3 compounds using 8 human skins and reported that the CV% ranged from 39 to 71%. In addition, Schäfer-Koring et al. [28] evaluated the in vitro human epidermis permeation of 7 drugs and reported that the CV% ranged from 90 to 163%. These reports and related studies suggest that the variation in the in vitro human skin permeability would be comparatively large, which is similar to the findings of the present study [1, 3, 18, 28, 31, 36].

The permeation rates of the three model drugs through male human skin were compared with those through female human skin. No significant difference was observed in the permeation rates of the three model drugs between male and female human skin. In addition, the CV% of the inter- and intra-individual variations in the skin permeability of the three model drugs in males was compared with those in females. However, no clear sex difference was found in the CV% of the inter- and intra-individual variations. Few studies have examined for sex difference in the CV% of the inter- and intra-individual variations in human skin permeability. Qvist et al. [25] reported that inter- and intra-individual variations in human skin permeability of drugs may depend on various factors such as the skin site selected, age of the donor and lifestyle.

Sex difference in the CV% of the inter- and intra-individual variations in the human skin permeability of
test drugs might be clarified if the same human skin specimen is used for testing.

The CV% of the intra-individual variations of human skin permeation ranged from 5.3 to 55.5% in this study and was smaller than the CV% of the inter-individual variations. However, a permeation study using skin obtained from the same donor would have practical limitations in addition to ethical problems. The results of the present in vitro human skin permeation studies suggest that human skin might not be suitable for evaluating the in vitro skin permeability of drugs.

We examined whether SD rat skin would be useful as an alternative to human skin. Frozen SD rat skin was prepared and used. The influence of freezing on skin structure and permeability was examined.

The changes in skin morphology in frozen SD rat skin sections at 0, 10, and 24 h were similar to those in fresh SD rat skin sections. Therefore, regarding the skin permeability within 24 h, the permeability coefficients (P) of NR, ISDN, and FP through frozen SD rat skin were compared with those through fresh SD rat skin. In this study, no significant differences were observed in the permeability coefficients (P) of NR, ISDN, and FP between frozen and fresh SD rat skins.

Bronaugh et al. [5] and Harrison et al. [10] reported that no differences between fresh and frozen human skin for water permeability, while Babu et al. [2] reported that freezing skin slightly influences the permeability of drugs through rat skin. However, the methods used for freezing of fresh SD rat skins and thawing of frozen SD rat skins in this study were different from those used by Babu et al.

Our results suggest that the methods of freezing and thawing of skins influence the skin morphology and permeability and that freezing does not affect the skin permeability within 24 h after the start of the permeation experiments in this study. Therefore, we used frozen SD rat skin in the drug permeation experiments.

However, a reduction in the metabolic enzyme activity may occur after skin freezing. This possible reduction in enzyme activity might be considered when performing in vitro skin permeation studies using frozen skin, since numerous metabolic enzymes are present in the skin [21].

In general, SD rat skin and hairless rat skin are used for skin permeation studies. Therefore, we compared the log P values of fresh SD rat skin and fresh hairless rat skin using side-by-side cells. The data for the hairless rat was quoted from the results of Hatanaka et al. [11, 12]. For three model drugs, a good relationship in log P was found between the fresh SD rat skin and the fresh hairless rat skin (r=0.992), which indicated that fresh SD rat skin could be used instead of fresh hairless rat skin in the permeability studies.

We examined whether frozen SD rat skin would be useful as an alternative to human skin. The inter-individual variations in the skin permeability of the three model drugs through frozen SD rat skin were examined. The permeation rates of NR, ISDN, and FP through frozen SD rat skin were 5.72, 14.7, and 4.33 µg/cm²/h, respectively; these values were 1.7, 1.6, and 2.0 times higher than those observed for 6 human skins, respectively.

Figure 5 shows the relationship between the permeation rates of the three model drugs through human skin and frozen SD rat skin. We found a good relationship between the permeation rates of human skin and frozen SD rat skin (r=0.999). The differences in permeation...
rates of the three model drugs are most likely caused by the thickness of the stratum corneum and the species difference in skin barrier function [9, 16, 27].

On the other hand, the CV% of the permeation rates for NR, ISDN, and FP through frozen SD rat skin was 25.9, 13.0, and 19.2%, respectively. These CV% values were remarkably smaller than those of human skin. The different thicknesses of the stratum corneum and the non-uniformity of the skin structure in humans might cause variations in drug permeability.

In addition, we evaluated if and how the physicochemical properties of the three model drugs were related to the CV% of the inter-individual variations in the human skin and SD rat skin permeability. The CV% of the permeation rates for the three model drugs through human and SD rat skin was in the order of ISDN<FP<NR.

Akomeah et al. [1] reported that the skin permeation rate of lipophilic drugs generally shows less variation than that of hydrophilic drugs. The high variations in hydrophilic drug permeation compared with lipophilic drugs may suggest that the in vitro skin permeation of drugs becomes more sensitive to inter- and intra-individual variations in skin lipid content, appendageal density, and imperfections (pores, cracks) as the hydrophilic nature of the drug increases. In addition, Morimoto et al. [17] reported that functional groups like -OH, -NH₂, and -COOH in drugs decrease the diffusivity of these drugs in the membranes. In this study, the lipophilicities of the test drugs were in the order of FP>ISDN>NR. However, FP contains -COOH, but no significant groups are found in ISDN. Therefore, the CV% would be ranked in the order of ISDN<FP<NR.

These studies showed that the inter-individual variations in frozen SD rat skin permeability were remarkably small, although the inter-individual variations in human skin permeability were large. Since it is easy to handle SD rat skin for in vitro skin permeation studies and the permeation rate through SD rat skin was well correlated with that for human skin, frozen SD rat skin might be a useful alternative to human skin for permeation studies.

In conclusion, the inter-individual variations in SD rat skin permeability were compared with those of human skin permeability. Our findings demonstrated that the inter-individual variations of the permeation rates for the three model drugs through the SD rat skin were remarkably smaller than those through human skin and that the permeation rates were well correlated with each other. It might be reasonable and practical to evaluate skin permeability using SD rat skin and to predict human skin permeability based on SD rat skin permeability.

References


