Diffusion of [2-14C]Diazepam Across Hairless Mouse Skin and Human Skin

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The objectives of this study were to investigate the absorption of diazepam applied topically to the hairless mouse in vivo and to determine the diffusion of diazepam across isolated hairless mouse skin and human skin. [14C]Diazepam was readily absorbed after topical administration to the intact hairless mouse, a total of 75.8% of the 14C-label applied being recovered in urine and feces.

Diazepam was found to diffuse across human and hairless mouse skin unchanged in experiments with twin-chambered diffusion cells. The variation in diffusion rate or the flux for both human and mouse tissues was greater among specimens than between duplicate or triplicate trials for a single specimen. Fluxes for mouse skin (stratum corneum, epidermis, and dermis) were greater than for human skin (stratum corneum and epidermis): 0.35–0.61 μg/cm²/h for mouse skin vs 0.24–0.42 μg/cm²/h for human skin. The permeability coefficients for mouse skin ranged from 1.4–2.4 × 10⁻² cm/h compared with 0.8–1.4 × 10⁻² cm/h for human skin. Although human stratum corneum is almost twice the thickness of that of the hairless mouse, the diffusion coefficients for human skin were 3–12 times greater (0.76–3.31 × 10⁻⁶ cm²/h for human skin vs 0.12–0.27 × 10⁻⁶ cm²/h for hairless mouse) because of a shorter lag time for diffusion across human skin. These differences between the diffusion coefficients and diffusion rates (or permeability coefficients) suggest that the presence of the dermis may present some barrier properties. In vitro the dermis may require complete saturation before the diazepam can be detected in the receiving chamber.

[14C]Diazepam was not detected in the receiving chamber of the Franz cell apparatus in experiments with human skin. This indicated that the rate of diffusion was less than 0.09 μg/cm²/h. Since this diffusion technique more closely resembles topical administration to humans, these results appear to indicate that achieving therapeutic concentrations in humans may be difficult. In addition, the hairless mouse may not be a suitable model for predicting percutaneous absorption of diazepam in humans. J Invest Dermatol 88: 582–585, 1987

Diazepam (Valium, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is a benzodiazepine used as an anxiolytic, sedative, hypnotic, and anticonvulsant agent as well as a skeletal muscle relaxant [1,2]. Like many benzodiazepines, the biologic half-life of diazepam is long (20–50 h), it is lipid soluble, and several metabolites possess pharmacologic activity [3]. Anxiolytic effects can be anticipated at blood concentrations of 300–400 ng/ml diazepam [3]. These characteristics complicate the use of this type of drug topically. For example, the long biologic half-life does not assist in efficient delivery [4]. However, diazepam can be considered to be a good prototype for preliminary studies on the transdermal permeability of benzodiazepine drugs in general.

Objectives of this study were to determine: (1) whether radiolabeled diazepam is absorbed in vivo following topical administration to the hairless mouse, (2) the in vitro diffusion rates of diazepam across hairless mouse skin and human skin, and (3) whether diazepam was metabolized during diffusion.

MATERIALS AND METHODS

Diazepam was a gift of Hoffmann-La Roche Inc. (Nutley, New Jersey). [2-14C]Diazepam, sp act 54 mCi/nmol (99% purity), was purchased from the Amersham Corp. (Arlington Heights, Illinois). Methanol for use in high-performance liquid chromatography (HPLC) was purchased from Fisher Scientific (Itasca, Illinois). Water for HPLC was filtered through a Milli Q System (Waters Associates, Milford, Massachusetts). Female hairless mice, 6–9 weeks old, weighing 24–30 g, were purchased from Temple University (Philadelphia, Pennsylvania). Twin-chambered diffusion cells [5], designed with a magnetic stirring well in each chamber (2 ml per chamber) and a surface for diffusion of 0.79 cm², were made at the Research Resources Laboratory of the University of Illinois (Chicago, Illinois). An 8-cell Franz diffusion apparatus [5] with a 2.01-cm² surface for diffusion was purchased from the Crown Glass Company (Somerville, New Jersey).

In Vivo Mouse Experiments Diazepam was administered topically (1.0 μg/kg dose, 1 μCi in 0.05 ml 95% ethanol) over a 2-cm² area on the back of the neck with a Hamilton microsyringe (Reno, Nevada). The solvent was allowed to evaporate and the area was left uncovered for 24 h. Animals were housed in Nagle free-standing metabolism cages (Fisher Scientific) to aid in the collection of urine and feces.

The urine and feces were collected daily for 3 days [6] and the
radiolabel assayed by liquid scintillation counting (LSC, see below). For comparison, mice were also given the same dose of diazepam i.p. (in 0.05 ml propylene glycol) and their urines and feces were also collected and assayed for the 14C-label.

Hairless Mouse Skin

After allowing 2 weeks for 14C-labeled metabolites with a long biologic half-life to be excreted, the mice used for the in vivo experiments were sacrificed by cervical dislocation. The skin from the back was removed and trimmed of fat and other extraneous tissues. The whole skin, comprising stratum corneum and epidermis (SCE) and dermis (D), was used for the diffusion experiments.

Human Skin

Samples of whole human cadaver skin were removed from the midline region of the chest less than 48 h after death. The subcutaneous fat was trimmed and the tissue treated according to the methods of Swarbrick et al.[7] before the removal of the SCE from the dermis. The SCE was then dried in a desiccator maintained at approximately 25% relative humidity for 2–3 days. The dried samples were wrapped in aluminum foil and stored in the refrigerator at 5°C and rehydrated by immersing the tissue in water at room temperature for 1 h before use.[7]

Diffusion Experiments Using Twin-Chambered Diffusion Cells

Whole mouse skin (SCE and D) or human skin (SCE) was placed between the two chambers of the twin-chambered cells. Approximately 50 μg of diazepam (0.23 μCi), dissolved in ethanol (0.05 ml), was added to the donor (stratum corneum) side of the cell containing 2 ml of saline. Saline 2 ml was added to the receiving chamber and the cells were incubated in a 35°C waterbath. Two 0.05-ml samples were taken from the receiving chamber through the sampling port at 0, 0.5, 1, 2, 3, 4, 5, and 6 h. Each sample was replaced with an equal volume of saline. Samples were assayed for the 14C-label by a liquid scintillation counter (see LSC below). The sensitivity of this method will detect a diffusion rate of 0.10 μg/cm²/h.

Diffusion Experiments Using the Franz Cell Apparatus

Approximately 50 μg of diazepam (0.1 μCi), dissolved in 0.05 ml ethanol, was placed on the stratum corneum surface (~2 cm²) and the ethanol allowed to evaporate. The receiving chamber was kept at 35°C by a circulating water bath. Two 0.05-ml samples of the saline solution in the receiving chamber were taken at 0, 0.5, 1, 2, 3, 4, 5, and 6 h through the sampling side arm. Each sample was replaced by an equal volume of saline. Samples were assayed for the 14C-label by LSC. The sensitivity of this method will detect a diffusion rate of 0.09 μg/cm²/h.

Analysis of Samples by HPLC

Samples from the donor and receiving sides of the twin-chambered diffusion cells were stored at −20°C until analyzed. Freshly thawed samples (1-ml aliquots) were extracted using a C-18 Bond Elut column (Analytichem International, Harbor City, California). The 14C-label was eluted from the Bond Elut column with three 1-ml aliquots of methanol and analyzed by HPLC. A 0.1-ml aliquot was analyzed with a Waters Associates liquid chromatograph, model 501 pump, U6K injector, with a μBondapak C18 radial compression column. The mobile phase was 65% methanol/35% water (Milli Q filtered) at a flow rate of 2 ml/min [8]. The HPLC eluate was monitored by an UV absorbance detector, model 440, operated at 254 nm. The eluate was collected in 20-ml liquid scintillation vials at 30-4 intervals in preparation for LSC analysis.

Liquid Scintillation Counting

The samples from diffusion cells and the eluate fractions from the HPLC were placed in 20-ml liquid scintillation vials and 10 ml of Budget-Solve scintillation cocktail (Research Products International Corp., Mount Prospect, Illinois) added. For urine samples, a 0.1- to 0.2-ml aliquot was placed in similar vials and 0.3 ml of glacial acetic acid was added before the addition of 10 ml of cocktail.

Fecal samples were collected and weighed daily for each animal. A 24-h sample was placed in a 20-ml vial to which 10 ml of deionized distilled water and 2–3 glass beads were added. The vials were mixed using a Vortex Genie mixer (Fisher Scientific), for 5–10 min or until a homogeneous suspension was prepared. Two 0.2-g samples were weighed into clean 20-ml scintillation vials and 0.3 ml of glacial acetic acid was added followed by 10 ml of scintillation cocktail. All samples were measured for the 14C-label with a Tri-Carb 4000 series liquid scintillation counter (85–95% efficiency) from Packard Instrument Company, Inc. (Downers Grove, Illinois).

Calculation of Diffusion Rates or Flux (J)

Diffusion rates or flux (J) were determined from the slope of the diffusion curves [7] and expressed as the amount of drug passing across a square centimeter of skin surface over time (μg/cm²/h). The permeability coefficients (P) were determined by dividing the observed value for flux by the initial drug concentration in the donor cell (25 μg/cm²). This value cannot be derived for experiments with the Franz cell because the donor solvents are allowed to evaporate. A linear regression program was used to determine the equation of the line for the linear portion of the diffusion curve [9]. Following the determination of the lag time (τ), skin thickness (h) the diffusion coefficients (D) were calculated using D = h²τ/6[10].

RESULTS

Absorption Across Hairless Mouse Skin In Vivo

Following topical administration of [2-14C]diazepam, recovery of the 14C-label in urine and feces after 3 days was 54.8 ± 1.5% and 21.0 ± 2.3%, respectively. Total recovery of the label in urine and feces after 3 days was 75.8 ± 2.3% (Table I). After i.p. administration, the recovery of the 14C-label in urine and feces was 40.3 ± 6.4% and 47.2 ± 3.6%, respectively. The relative bioavailability of the 14C-label applied topically compared with i.p. administration was greater than 86%. This indicates that [14C]diazepam was readily absorbed through the hairless mouse skin.

Diffusion Across Hairless Mouse Skin in Vitro

The diffusion rates of [2-14C]diazepam using twin-chambered diffusion cells and Franz diffusion cells were found to be similar to one another for each mouse tissue. The values ranged from 0.35–0.61 μg/cm²/h (n = 3) (Table II). Permeability coefficients were found to range from 1.4–2.4 × 10⁻² cm/h. The lag times ranged from 0.50–1.14 h determined from the x-intercept values of regression lines that best fitted the diffusion curves (Fig 1). The diffusion coefficients ranged from 0.12–0.27 × 10⁻⁶ cm²/h and were found not to be significantly different for twin-chambered cells compared to Franz diffusion cells.

High-performance liquid chromatography analysis of the 14C-label in the donor and receiving chambers of the twin-chambered diffusion cell yielded a single radioactive peak with a retention time of 6.0 min, identical to diazepam. A typical HPLC chromatogram of [14C]diazepam together with the radioactive data from eluate samples analyzed by LSC is presented in Fig 2.

Diffusion Across Human Skin In Vitro

The diffusion rate of [2-14C]diazepam using human skin and twin-chambered diffusion cells ranged from 0.24–0.42 μg/cm²/h (n = 3) (Table III). The permeability coefficients ranged from 0.8–1.4 × 10⁻² cm²/h.

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Recovery of 14C-Label (n = 4)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>40.3 ± 6.4</td>
<td>87.9 ± 2.8</td>
</tr>
<tr>
<td>Feces</td>
<td>47.2 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Washes</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*The application site of topical administration was washed 3 times with 1% Tween 80 using cotton swabs. The swabs were placed in liquid scintillation vials, cocktail was added, and the vials counted. NA = not applicable.
The lag times ranged from 0.13–0.64 h. and the diffusion coefficient ranged from 0.76–3.31 × 10⁻⁶ cm²/h (Fig 3). Only diazepam was found following HPLC and LSC analysis of the contents of the receiving chamber. The diffusion rates in experiments with the Franz cell could not be determined for the amounts of [¹⁴C]-label detected were below the sensitivity of our assay (0.09 µg/cm²/h).

DISCUSSION

[²-¹⁴C]Diazepam was readily absorbed through the intact skin of the hairless mouse. The relative bioavailability of topically applied [¹⁴C]diazepam compared with i.p. administration was greater than 86%. These results suggest that most of the drug applied to the skin was absorbed over the 24-h treatment period. However, we were unable to determine from these experiments whether the absorbed material was diazepam or its metabolites.

Diazepam was found to diffuse unchanged across both human SCE and whole mouse skin. The diffusion rate of diazepam across hairless mouse skin in vitro was found to be similar for the twin-chambered and Franz diffusion cells (range 0.35–0.61 µg/cm²/h). The calculated permeability coefficients for diazepam were greater than reported for progesterone and other compounds [11,12]. In general, the diffusion rates across mouse skin for both diffusion systems were greater than across human skin (range 0.24–0.42 µg/cm²/h) using twin-chambered cells. The whole mouse skin (~0.44 mm) used in these experiments was approximately 7 times thicker than human skin (SCE ~ 0.06 mm, [13]).

Since the stratum corneum is considered the major barrier to diffusion, only the thickness of this layer was used in calculating the diffusion coefficients [14]. However, the calculated diffusion coefficients for human skin were 3–12 times greater than for mouse skin (0.76–3.31 × 10⁻⁶ cm²/h for human vs 0.12–0.27 × 10⁻⁶ cm²/h for hairless mouse). The thickness of human stratum corneum (~17 μm) is approximately twice that of the hairless skin.

Table II. Diffusion Rates, Permeability Coefficients, and Diffusion Coefficients for Whole Mouse Skin With Twin-Chambered and Franz Diffusion Cells

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Diffusion Cell¹</th>
<th>Flux (µg/cm²/h)</th>
<th>Permeability Coefficient (10⁻² cm²/h)</th>
<th>Lag Time (hours)</th>
<th>Diffusion Coefficient (10⁻⁶ cm²/h²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1—Trial 1</td>
<td>TC</td>
<td>0.35</td>
<td>1.4</td>
<td>0.50</td>
<td>0.27</td>
</tr>
<tr>
<td>Trial 2</td>
<td>TC</td>
<td>0.39</td>
<td>1.6</td>
<td>0.77</td>
<td>0.18</td>
</tr>
<tr>
<td>2—Trial 1</td>
<td>TC</td>
<td>0.46</td>
<td>1.8</td>
<td>0.81</td>
<td>0.17</td>
</tr>
<tr>
<td>Trial 2</td>
<td>TC</td>
<td>0.44</td>
<td>1.8</td>
<td>0.83</td>
<td>0.16</td>
</tr>
<tr>
<td>3—Trial 1</td>
<td>TC</td>
<td>0.53</td>
<td>2.1</td>
<td>0.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Trial 2</td>
<td>TC</td>
<td>0.61</td>
<td>2.4</td>
<td>0.90</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>0.41</td>
<td>NA²</td>
<td>1.14</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>0.48</td>
<td>NA²</td>
<td>1.12</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>0.53</td>
<td>NA²</td>
<td>0.91</td>
<td>0.15</td>
</tr>
</tbody>
</table>

¹ Twin-chambered (TC) cells have a 0.79-cm² surface area for diffusion. Franz (F) cells have a 2.05-cm² surface area for diffusion.
² See [13] for the thickness of stratum corneum (0.9 × 10⁻³ cm).
³ Skin tissue was not the same as #2 used for twin-chambered cells due to insufficient amount of skin.
⁴ NA = Not applicable with Franz diffusion cells.

Figure 1. Diffusion of diazepam across isolated hairless mouse skin. Twin-chambered diffusion cells (open circles) and Franz cells (closed circles) (range as bars).

Figure 2. High-performance liquid chromatography and LSC data. A HPLC record showing the UV-absorbance of diazepam (dotted line). The eluate was collected in liquid scintillation vials at 30-s intervals and the dpm per sample plotted on the same scale (solid line).
Table III. Diffusion Rates, Permeability Coefficients, and Diffusion Coefficients for Human Skin With Twin-Chambered* Diffusion Cells

<table>
<thead>
<tr>
<th>Tissue I.D. No.</th>
<th>Flux (µg/cm²/h)</th>
<th>Permeability Coefficient (10⁻⁶ cm/h)</th>
<th>Lag Time (hours)</th>
<th>Diffusion Coefficient (10⁻⁶ cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1—Trial 1</td>
<td>0.29</td>
<td>1.2</td>
<td>0.64</td>
<td>0.76</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.30</td>
<td>1.0</td>
<td>0.24</td>
<td>2.01</td>
</tr>
<tr>
<td>Trial 3</td>
<td>0.24</td>
<td>0.8</td>
<td>0.19</td>
<td>3.31</td>
</tr>
<tr>
<td>2—Trial 1</td>
<td>0.28</td>
<td>0.9</td>
<td>0.13</td>
<td>1.73</td>
</tr>
<tr>
<td>3—Trial 1</td>
<td>0.42</td>
<td>1.4</td>
<td>0.43</td>
<td>1.12</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.38</td>
<td>1.2</td>
<td>0.35</td>
<td>1.37</td>
</tr>
</tbody>
</table>

*Twins-chambered (TC) cells have a 0.79-cm² surface area for diffusion.

In future experiments we plan to study the effects of the dermis on the diffusion rates and coefficients of diazepam with hairless mouse skin. The effects of accelerants on diazepam diffusion will be investigated to improve the flux so as to approach therapeutic plasma concentrations.

We wish to thank Dr. Geoffrey W. J. Lee for valuable discussions in the preparation of this manuscript.


mouse (~9 µm) and the human skin appeared to have a shorter lag period than whole mouse skin; both factors greatly influence this calculation [10,13]. The longer lag time for whole mouse skin indicates that the presence of the dermis may present some barrier properties.

Repeated trials of a specimen of both human and mouse tissues varied less than the individual specimens (Tables II, III). Because of the individual differences, data were not pooled but their range of values are reported.

One unexpected finding was the lack of diffusion or a diffusion rate less than the sensitivity of our assay (0.09 µg/cm²/h) across human skin using Franz diffusion cells. This is probably due to the thickness of the human stratum corneum and possibly to the physical state of the applied diazepam. Exposure to the atmosphere in the Franz cell will have removed solvent and the drug is likely to be precipitated on the surface. In the twin-chambered cells the drug remains in solution. Since the Franz cell more closely resembles the topical application of drugs to humans [5,15], it would appear to indicate that topical application in humans may prove to be difficult. Accelerants or penetration enhancers may be required in order to keep diazepam in solution to increase absorption and reach efficacious plasma concentrations [3,16]. In addition, the hairless mouse may not be a suitable model for predicting the percutaneous absorption of diazepam in humans.