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Technical Note

Comparisons of *in Vitro* Nitroglycerin (TNG) Flux Across Yucatan Pig, Hairless Mouse, and Human Skins

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INTRODUCTION

Hairless mouse skin has been widely used as an *in vitro* model for studying skin permeation (1–3). However, the use of mouse skin may overestimate enhanced permeation as much as sevenfold compared to human cadaver skin (4). Yucatan pig skin, on the other hand, was shown to be a better model for transdermal studies, most likely because of comparable lipid composition (5) and physiological and morphological characteristics (6) to those found in human skin. Hawkins and Reifenrath (7) reported that *in vitro* permeation of 10 compounds through whole pig skin correlated well with human *in vivo* permeation. *In vivo* comparison of several compounds also showed that miniature swine skins and human skins are comparable (8). In the present study, we determined which skin type, hairless mouse or Yucatan pig, is the more suitable model of nitroglycerin (TNG) permeation in human skin.

The miniature Yucatan pig offers the additional advantage for TNG study of being an excellent *in vivo* model of cardiovascular diseases in man, most notably atherosclerosis (9), while it is also an accepted model for human pulmonary, gastrointestinal, renal, immunological, and metabolic functions (10).

MATERIALS AND METHODS

Skin Preparation Techniques

Human Stratum Corneum

Preparation and Storage. Full-thickness human skin was obtained from the National Disease Research Interchange (NDRI), Philadelphia, PA, either as cadaver skin (normally from the abdominal area) or as surgical specimens (most often from reduction mammaplasty). The skin sections were received on dry ice, double-bagged. Upon receipt, the skin sections were stored frozen at −70°C until needed. Human stratum corneum-epidermis (SCE) was prepared using the method described by Bronaugh and Stewart (12).

Evaluation of SCE Integrity. SCE sections were removed from the freezer and allowed to thaw at room temperature. A dissecting microscope (Bausch & Lomb) was used for gross examination of the SCE sections throughout the cutting and mounting procedures described below. Smooth-tipped forceps were used to handle the SCE sections to avoid damaging the tissue. Circular pieces of SCE, approximately 1.7 cm^2 in area, were carefully cut using a cork borer and mounted in the flow-through diffusion cells (11). The donor tower was carefully tightened into place so as not to twist the skin. The diffusion cells were mounted on the aluminum heating block, and saline was pumped through the system at a rate of 2 ml/hr for approximately 30 min to allow the SCE sections to hydrate. At this time, SCE integrity was measured using a tritiated water procedure similar to that of Bronaugh *et al.* (12) with the exception that their procedure related the dose absorbed after 20 min to the true steady-state tritiated water permeability coefficient, while our experimentation indicated that at least 2 hr is required. Measurements made prior to 2 hr appear to underestimate the actual permeability coefficient. Our procedure utilized a 100-μl dose of tritiated water (New England Nuclear, 18 μCi/mmol), 0.300 μCi applied to each donor compartment. Effluent samples (2.0 ml) were collected hourly for 2 hr. Following sample collection, 15 ml of Ready-To-Use III scintillation cocktail (Eastman Kodak Company) was added to each vial. Samples were counted using an LKB 1214 liquid scintillation counter (Gaithersburg, MD) using the tritium auto window, color and chemiluminescence correction, and dpm conversion program. To determine the flux of tritiated water which had permeated the SCE, the mean dpm/hr values were calculated. A permeability coefficient (K_p) was determined for each SCE layer using the following formula:

$$K_p = (J/A)/C$$

J = flux as dpm/hr
A = diffusional area of each cell (0.340 cm^2)
C = concentration of tritium applied to donor as dpm/ml

SCE sections with K_p values within the range of 0.5 to 2.5 × 10^{-3} cm/hr are considered to be intact and suitable for fur-

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ther permeation experimentation (12). K_p values greater than 2.5×10^{-3} cm/hr generally indicate that the SCE section is physically damaged. Only those SCE sections with K_p values falling within the range of 0.5 to 2.5×10^{-3} cm/hr were used for further testing.

Pig Skin

Frozen skin sections from male Yucatan miniature pigs were obtained from Charles River Laboratories. The sections were approximately 4 × 6 in. and labeled by anatomical location. Upon receipt, the skin sections were stored at −70°C. When needed, the skin samples were allowed to thaw at room temperature. The epidermis and a portion of the dermis were removed using a Zimmer nitrogen-powered dermatome to a thickness of 0.5 to 1.0 mm. Circular pieces of approximately 1.7 cm^2 were cut for testing using a cork borer.

Mouse Skin

Excised dorsal skin of hairless male mice (SKh) was obtained from Charles River Laboratories. The skins were received frozen, stacked in a plastic container, and stored at −70°C until used. When needed, the skin was allowed to thaw at room temperature and sections were cut to approximately 1.7 cm^2 using a cork borer. One mouse skin would typically yield two or three suitable sections for testing.

Flow-Through Cell Testing

Permeation testing of nitroglycerin (TNG) across the three different skin types was performed at 32°C using the flow-through cell system (Vanguard International, Neptune, NJ) and the procedure described by Bronaugh and Stewart (11,12). This study utilized the smaller version of the flow-through cell (0.34-cm^2 exposure area, 0.14-ml receptor volume) discussed in the conclusion of Bronaugh's paper (11). A schematic of the flow-through cell and the flow-through cell apparatus appears in Fig. 1. The cells were connected to an ISMATEC eight-channel peristaltic pump which delivers degassed receiver fluid to the cell at a rate of 2.0 ml/hr, resulting in 14 receiver volume changes per hour. Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories) was used as the receiver fluid. Hepes buffer (Gibco Laboratories) was added to a final concentration of 25 m*M* and pH was adjusted to 7.0 using 1 *M* NaOH. Sample effluent was collected in tared scintillation vials. An Isco Retriever IV fraction collector (Isco, Lincoln, NB) was used to automatically change collection vials hourly for the first 6 hr, then every other hour for the remainder of the experiment through 24 hr. Samples were weighed and volumes were calculated using a Mettler AM100 electronic balance interfaced to an IBM PC equipped with Lotus Measure software. The samples were then analyzed for nitroglycerin concentration (μg/ml) by HPLC. Because of the large number of

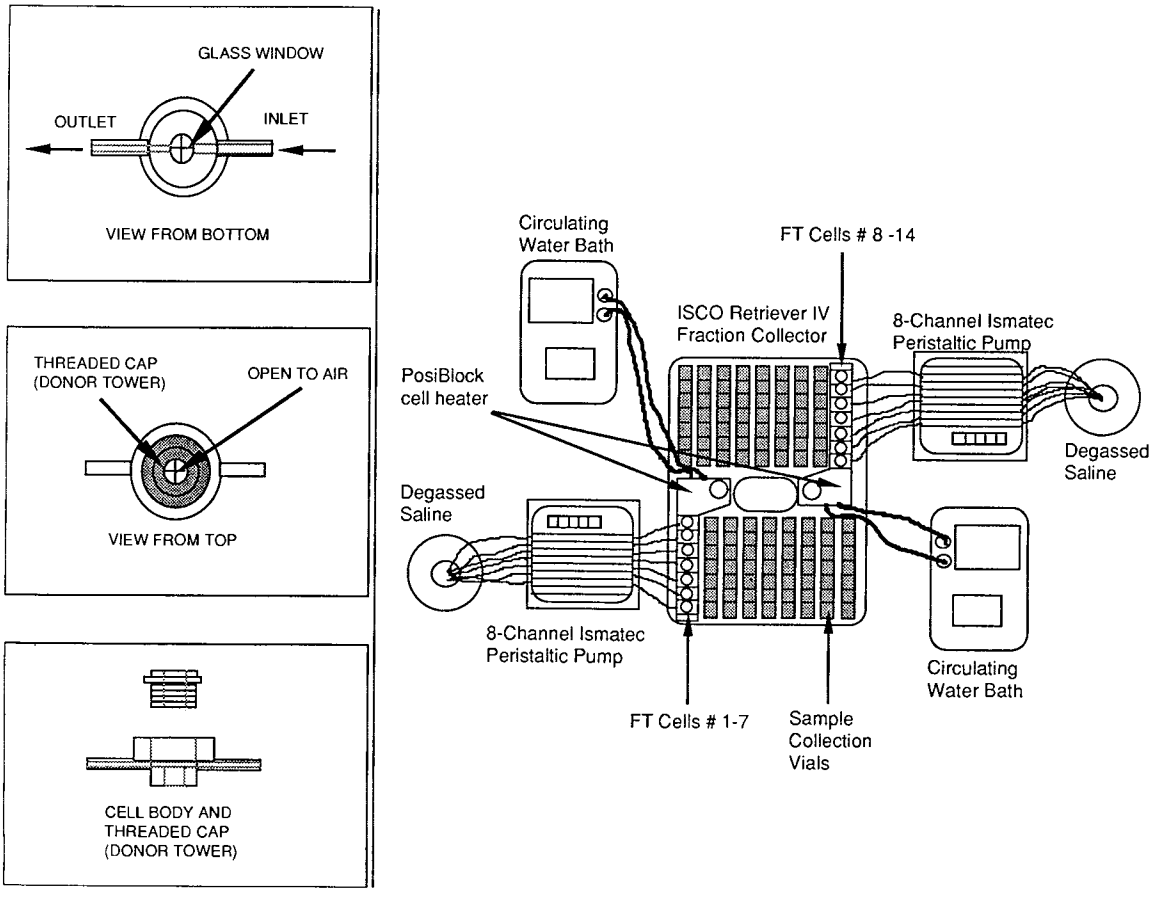

Fig. 1. A schematic of the flow-through cell and the flow-through cell apparatus.

samples, only a single TNG determination was performed on each sample.

Application of the Nitroglycerin Test Systems to Skin Sections

The Nitro-Dur delivery system consists of a single-layer polymer matrix of nitroglycerin in combination with an adhesive attached to an occlusive backing material. Sections of the test system were cut using a cork borer to a size of 1.7 cm^2 and applied to skin sections before mounting them into the permeation cells.

The Transderm-Nitro system, consisting of a pouch containing a nitroglycerin ointment, was dissected and tested using its component parts. Nitroglycerin ointment was removed through a slit in one end and placed into a vial. A 1.7-cm^2 section of membrane (ethylene vinyl acetate and adhesive laminate) was applied to the skin section and mounted in the diffusion chamber. The nitroglycerin ointment was then reapplied in the correct proportion by weight (20 mg/0.34 cm^2).

Analytical

The HPLC assay of nitroglycerin employed reverse-phase isocratic separation using 50:50 methanol:water as the mobile phase at a flow rate of 1.5 ml/min (Waters Model 510 pump) at 3000 PSI. The analytical column used was a 15-cm × 4.6-mm-ID Supelcosil LC-8, 5-μm particle size. Samples were injected using a Gilson Model 231 autoinjector. Absorbance of nitroglycerin was monitored at 210 nm using a Kratos Spectroflow Model 757 UV/VIS detector. Run times were typically 12 min, with the TNG peak appearing at approximately 4.5 to 5.0 min. Extended run times were necessary due to unidentified late-eluting peaks (9–10 min), possibly from components leaching from the tygon pump and connection tubing.

Nitroglycerin concentrations (μg/ml) and effluent sample volumes (ml) were incorporated into a Lotus 1-2-3 spreadsheet and the cumulative amount of drug transferred (μg/cm^2) at each time point was calculated. Release rates were calculated by dividing the cumulative amount of drug transferred as μg/cm^2 by the total time elapsed.

RESULTS AND DISCUSSION

Figure 2 displays the rate profiles produced by the Transderm-Nitro patch components when tested across each of the three skin types. As expected, nitroglycerin penetration through human stratum corneum and pig skin are zero-order processes producing a steady-state delivery profile. Delivery across human stratum corneum remains constant from approximately 8 to 24 hr at a rate of 16 to 17 μg/cm^2/hr. The release rate through Yucatan pig skin, though less than that through human stratum corneum, remains constant from about 6 to 24 hr at a rate of 10 to 11.6 μg/cm^2/hr. Delivery across hairless mouse skin is much greater than delivery through either pig skin or human stratum corneum. Although hairless mouse skin delivers TNG at a flux rate that appears to increase with time, approaching more than 31 μg/cm^2/hr at the 24th hour, we cannot conclude that the delivery profile deviates from steady state because of data variability.

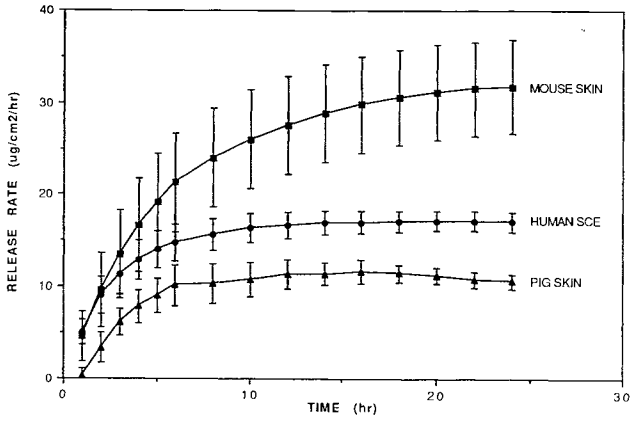

Fig. 2. Transderm-Nitro delivery system. Plot of mean ± SD nitroglycerin release rates as μg/cm^2/hr vs time (hr) across human stratum corneum (n = 6), hairless mouse skin (n = 7), and Yucatan pig skin (n = 7). Lines drawn for clarity.

The rate profiles produced by the Nitro-Dur patch across the three skin types are seen in Fig. 3. Zero-order delivery was observed across both human stratum corneum and pig skin. Nitroglycerin delivery across human stratum corneum was at a steady rate of 15 to 16 μg/cm^2/hr from 6 to 24 hr. Nitroglycerin penetration through Yucatan pig skin from the Nitro-Dur system was at a steady rate of 12 to 15 μg/cm^2/hr from 10 hr on. The release rate across hairless mouse skin steadily increased from 25.5 μg/cm^2/hr at 8 hr to 37.3 μg/cm^2/hr at 24 hr, and again, a large variability is observed in the mouse data. Hairless mouse skin appears to present an increasing rate of TNG penetration over time, with a 24-hr flux rate significantly greater than that seen with human stratum corneum and Yucatan pig skin. The release rate profiles for the three skin types tested with each delivery system were compared by performing one-way analyses of variance at each of four time points: 4, 12, 18, and 24 hr. If significant differences were noted by the F test ($P < 0.05$), then Duncan's multiple comparisons procedure with a significance level of $\alpha = 0.05$ was used to quantify the differ-

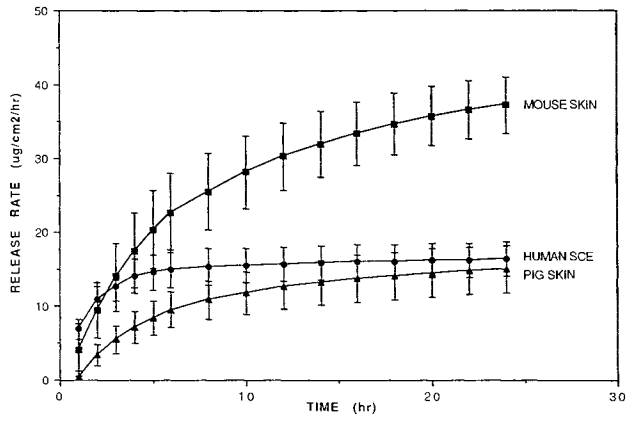

Fig. 3. Nitro-Dur delivery system. Plot of mean ± SD nitroglycerin release rates as μg/cm^2/hr vs time (hr) across human stratum corneum (n = 6), hairless mouse skin (n = 7), and Yucatan pig skin (n = 7). Lines drawn for clarity.

ences. The analysis of variance calculations were performed using the GLM procedure of the Statistical Analysis System (SAS). The results from the analyses of variance indicate that differences between skin type with respect to release rate are statistically significant. For both delivery systems tested, the release rate through mouse skin was significantly greater than through either Yucatan pig skin or human stratum corneum at 12, 18, and 24 hr. No significant difference in release rate was observed between pig skin and human stratum corneum for the Nitro-Dur system. In the case of the Transderm-Nitro system, however, the release rate of nitroglycerin through human stratum corneum was significantly greater than the release rate through Yucatan pig skin.

In conclusion, mouse skin was found to be an unacceptable model for predicting permeation through human skin because it exhibits significantly higher release rates from two nitroglycerin delivery systems that provide zero-order nitroglycerin delivery across human skin. This phenomenon may be caused by differences in epidermal lipid composition between the mouse and the human as determined by Gray and Yardley (5), whereas pig skin is similar to human skin. Total lipid content of rat epidermis was reported to be 20%, compared to 8% for the pig and 10% for man. Also, data from hairless mouse skin are more variable than from human stratum corneum or Yucatan pig skin. Accordingly, Yucatan pig skin appears to be a suitable model of human skin for *in vitro* permeation testing of transdermal nitroglycerin systems.

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