Methods for In Vitro Percutaneous Absorption Studies IV: the Flow-Through Diffusion Cell

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Abstract □ A flow-through diffusion cell system for percutaneous absorption studies has been developed. The results of initial studies with a limited number of compounds are reported. The cells were constructed from Teflon and contained a glass window in the bottom for viewing the receptor contents. A flow rate of at least 5 mL/h is required through the receptor (volume, 0.4 mL) for accurate results. The skin permeation of water, cortisone, and benzoic acid was determined in the flow-through cell and a standard static-diffusion cell. The absorption profiles and quantitative values obtained were similar for the two types of cells. The permeation of cortisone and benzoic acid applied in a petrolatum vehicle was determined in vivo in rats and with rat skin in the flow-through and static-diffusion cells. Good agreement was obtained between the results of the in vivo and in vitro procedures. The percutaneous absorption of a hydrophobic compound [3-phenyl-2-propenyl 2-aminobenzoate (cinnamyl anthranilate)] was enhanced with normal saline receptor solution in the flow-through cell when compared with results in the static cell. Maximum in vitro absorption was obtained with either cell using a 6% solution in water of the nonionic surfactant polyethylene glycol 20 oleyl ether (PEG-20 oleyl ether).

In vitro percutaneous absorption experiments are sometimes conducted with the principles of diffusion rigorously in mind. An aqueous solution of the penetrant is applied to one side of the skin and its diffusion is followed into identical aqueous fluid on the other side of the membrane, with stirring devices on both sides (two-chambered cell technique).[1] At present, skin absorption is often measured after application of a compound (in a small amount of vehicle) to the surface of the skin. Permeation is monitored by removal of aliquots from the stirred solution in the chamber below the skin (one-chambered static cell).[2,3] This technique more closely simulates the in vivo situation because the skin is exposed to ambient conditions and is not excessively hydrated as in the two-chamber procedure.

A further refinement in absorption measurements is the use of a perfusion fluid below the surface of the skin to take up the material penetrating the skin. Sampling is facilitated by collecting the effluent in vials in a fraction collector. The flow-through diffusion cell is a one-chambered cell with a number of advantages. Automatic sampling allows a saving of labor in addition to around-the-clock monitoring of the absorption profile. Sink conditions are easily maintained even in absorption studies that use large amounts of nonradiolabeled material. A more physiological assessment of percutaneous absorption might be obtained for compounds of limited water solubility if the cells mimic the effect of blood flow through the skin by taking up and carrying away these compounds.

The volume of the receptor is critical. To completely remove the material that has penetrated into the receptor in a given time, the volume of fluid pumped into the cell must be many times the volume of the receptor. This requires that the receptor volume be small so that the volume of effluent from the cell is manageable. This point has also been stressed by Barry[4] in a recent comparison of flow-through and static cell features.

A flow-through cell system has been designed and its initial evaluation with a limited number of compounds is reported. Percutaneous absorption studies with rat skin have been conducted as a function of flow rate. Absorption profiles of test compounds have been compared with a static-diffusion cell and with the results of in vivo experiments. Previous work has shown that the absorption of hydrophobic compounds is difficult to measure accurately by in vitro techniques.[5] The value of a flow-through system for the measurement of the absorption of compounds with limited water solubility is examined.

Experimental Section

The radiolabeled compounds used in the absorption studies to facilitate quantitation were from New England Nuclear and included [^{3}H]water, specific activity 1.0 mCi/g; [1,2-^{3}H]cortisone, specific activity 43.0 Ci/mmol; and [7-^{14}C]benzoic acid, specific activity 29.4 mCi/mmol. 3-[3-^{14}C]Phenyl-2-propenyl 2-aminobenzoate (cinnamyl anthranilate), specific activity 10.5 mCi/mmol was obtained from the Food and Drug Administration, Division of Cosmetics Technology. All compounds were determined to have a radiochemical purity of ≥97%. Female Osborne–Mendel rats 3–6 months of age were used. For diffusion-cell studies, full-thickness skin was excised from the animal and a 350-μm section was removed from the surface of the skin with a Padgett dermatome (Kansas City, MO).

The flow-through cells were machined from Teflon to enable the fashioning of a small receptor. The basic design for the cells and holding block is illustrated in Fig. 1. A circle of skin is placed on the ledge of the receptor, and the top is screwed tightly into place. The inside section of the top is free to rotate so that the top can be tightened without twisting the skin. For the cells used in this study (exposed skin area, 0.64 cm^2), the volume of the receptor was 0.4 mL. Normal saline or other fluid is pumped into the receptor through the right sidearm. The tube for each sidearm stops 1 mm short of reaching the inside receptor wall. The flowing fluid therefore enters and leaves the receptor through a hole that is the size of the diameter drilled for the sidearms of the cell. To facilitate mixing, the diameter of this hole for the inlet tube is essentially equal to the full depth of the receptor. The outlet tube hole is smaller so that as fluid is forced out of the cell, a slight backpressure is created. This ensures intimate contact of the fluid with the undersurface of the skin. A glass plug forms the bottom of the receptor so that the inside contents are visible, permitting verification that air bubbles are not present inside the receptor. The receptor fluid is deaerated before each experiment by vacuum filtration through a 0.5-μm filter (Millipore).

The skin surface temperature in the diffusion cells was maintained at 32°C by the heated aluminum holding block (Fig. 1). Water was pumped through the block from a 35°C water bath. A multichannel cassette pump (Manostat, New York) controlled the flow rate of receptor fluid to each cell. The effluent was collected in scintillation vials in a fraction collector. Ready-Solv MP scintillation fluid (Beckman) was then

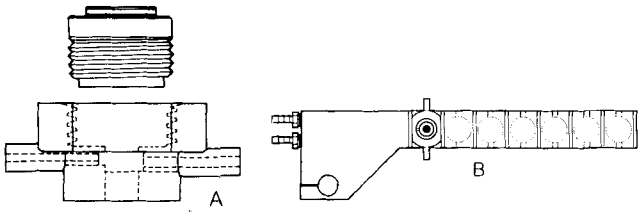

Figure 1—*Flow-through cell and holding block. Key: (A) cross section of diffusion cell. Skin is placed on ledge and cap is screwed on tightly. Receptor fluid is pumped into right sidearm through plastic tubing. Effluent from left sidearm is collected in a fraction collector. Glass plug is below the receptor (in dotted line square). (B) Aluminum holding block for cells. To maintain a physiological temperature, heated water from a water bath is pumped through the block, using ports at left. The hole at bottom left is for attachment to post on fraction collector.*

added directly to the vials and the radioactivity was determined in a Beckman LS9000 scintillation counter.

Static cell experiments were conducted as previously described.[3] Skin was clamped between the two parts of a one-chambered cell and placed in a 32°C water bath. The contents of the receptor were mixed with a magnetic stirring bar. Aliquots were removed from the receptor at various intervals for quantitation of the amount of absorbed material present. The surface area of exposed skin in these cells was also 0.64 cm^2.

Percutaneous absorption studies were conducted in vivo by previously published methods.[6] Hair was removed from the back of female rats by lightly shaving the area with an electric clipper. Radioisotopes, dissolved in a petrolatum vehicle, were applied to 2.0 cm^2 of skin. The site was protected by a nylon ring attached to the skin with a cyanoacrylate adhesive. The removal of this device was prevented by tying rubber tubing immediately behind the front legs and before the rear legs. The animals were placed in metabolism cages, and 24-h urine samples were collected each day for 5 d. At the end of day 1, each animal was briefly removed from its cage, its restraints were removed, and the application site was cleaned with soap and water. In separate animals, urinary excretion from an intraperitoneal injection was determined to correct for loss of radioactive material by other routes. The correction factor represents the fraction of absorbed compound excreted by the urinary route; the factors were 21.0% for cortisone and 75.3% for benzoic acid. In diffusion-cell experiments, performed for comparison with the in vivo studies, the skin surface was also cleaned after 24 h with soap and water. Absorption was measured for a total of 3 d in the benzoic acid studies and 5 d when cortisone was applied.

Statistical analysis was by the *t* test. The significance of the difference between absorption methods was determined using a two-tailed probability.

Results

Initially, experiments were performed to determine the proper flow rate for flow-through cell experiments. Tritiated water was applied to the surface of the rat skin in a water vehicle, and the steady-state absorption rate of the compound was determined at different normal saline flow rates. Since the volume of the cell receptors was 0.4 mL, an amount per hour greater than 10 times this volume (5 mL/h) was first used. The volume of effluent from this flow rate could be collected by a fraction collector in 20-mL scintillation vials. The absorption of tritiated water was determined at a slower (1 mL/h) and a faster (40 mL/h) rate. The absorption profiles (Fig. 2) show that almost identical permeation was obtained at the 5- and 40-mL/h rates. Absorption at the slower rate was not significantly different, but the variability, as indicated by the standard error bars, was much greater. The absorption at the 1-mL/h rate was lowest at the initial measurement and seemed to reach a steady state more slowly, and only near the end of the experiment. The 5-mL/h flow rate was used in subsequent experiments.

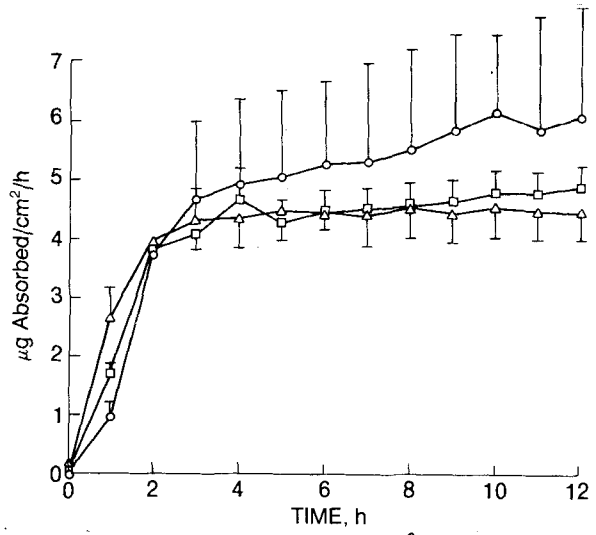

Figure 2—*Effect of flow rate on permeability of [^{3}H]water. The absorption of water through rat skin was determined at three flow rates. Key: (○) 1.0; (□) 5.0; (△) 40 mL/h.*

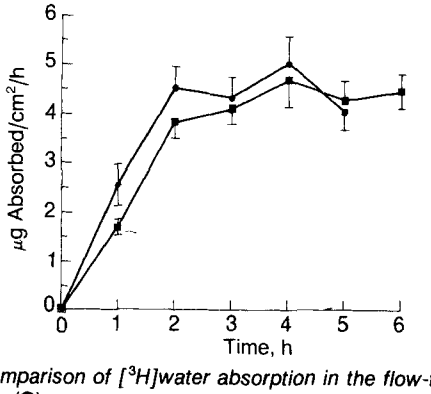

Figure 3—*Comparison of [^{3}H]water absorption in the flow-through (■) and static cells (●).*

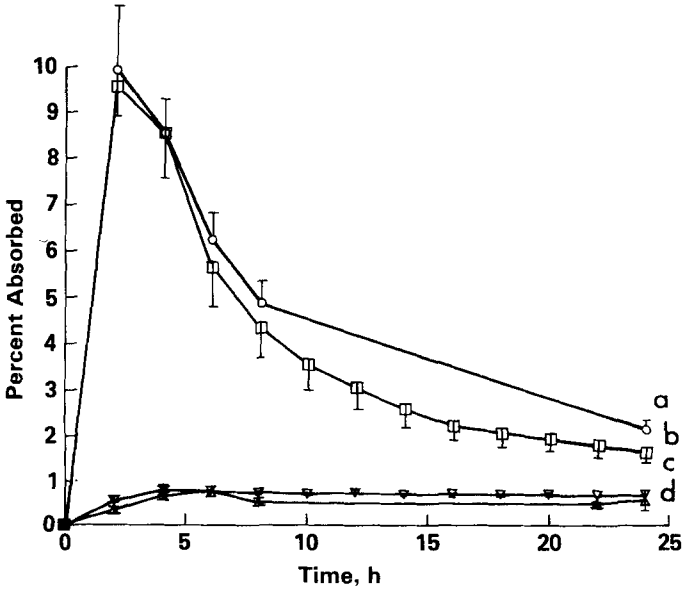

Figure 4—*Comparison of cortisone and benzoic acid absorption in flow-through and static cells. Compounds were applied to the rat skin membrane in an acetone vehicle. Key: flow-through cell–(c) cortisone; (b) benzoic acid; static cell–(d) cortisone; (a) benzoic acid.*

Table I—Comparison of Flow-Through and Static-Diffusion Cell Absorption[a]

Compound	Flow Cell	Static Cell
Water	4.3 ± 0.4 (5)	4.4 ± 0.2 (5)
Cortisone	8.5 ± 0.9 (5)	6.3 ± 0.8 (8)
Benzoic acid	45.9 ± 7.6 (5)	48.6 ± 3.8 (6)

[a] Values are the mean ± SEM of the number of determinations in parentheses. [1,2-^{3}H]Cortisone and [7-^{14}C]benzoic acid were applied in an acetone vehicle, [^{3}H]water in a water vehicle. For cortisone and benzoic acid, absorption is expressed as the percentage of the applied dose absorbed in 24 h. For water, the steady-state rate of absorption of the radiolabeled molecule is given ($\mu g/cm^2 \cdot h$). The values obtained for each compound in the two types of cells were not significantly different when compared by the two-tailed t test, $p < 0.05$.

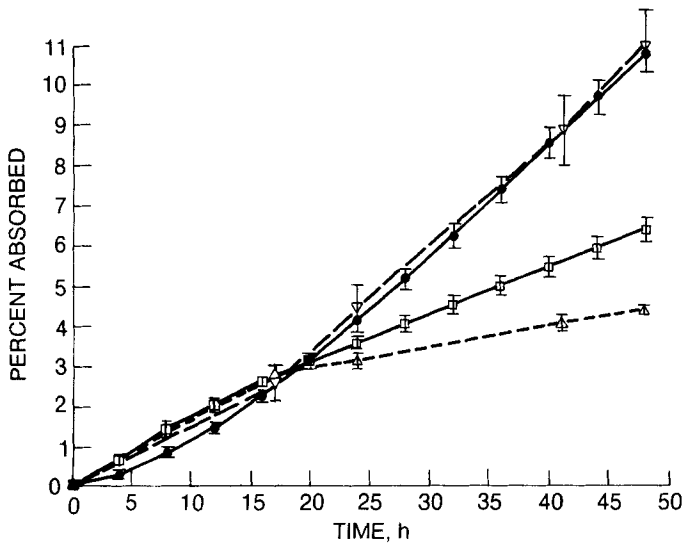

Figure 5—*Effect of cell type and receptor fluid on absorption (cumulative amount permeating the skin) of cinnamyl anthranilate when applied to the rat skin membrane in a petrolatum vehicle. Receptor fluid was either normal saline solution or 6% PEG-20 oleyl ether in water. Static cell data were abstracted from ref 4. Key: Flow-through cell-(□) normal saline; (●) 6% PEG-20 oleyl ether; static cell-(Δ) normal saline; (▽) 6% PEG-20 oleyl ether.*

The amount of absorption of three compounds in the flow-through cell was compared with values obtained in the standard static-diffusion cell. Similar absorption profiles were achieved with either cell for tritiated water (water vehicle, Fig. 3) and for cortisone and benzoic acid (acetone vehicle, Fig. 4). Almost identical steady-state absorption rates were obtained for tritiated water (Table I). The percentage of the applied dose absorbed for benzoic acid and cortisone did not differ significantly in the two cell types.

Permeation data from the diffusion cells were compared with values for the absorption of benzoic acid and cortisone in living rats. Petrolatum was used as the vehicle in this series of experiments because we have often used it in the past for in vivo measurements. In both the in vivo and in vitro studies, the site of application was washed with soap and water at the end of 24 h. Good agreement is seen in the permeation data from the three procedures (Table II).

Flow-through and static cell data were compared for a hydrophobic compound (cinnamyl anthranilate) that had previously given us unsatisfactory results with the static cell.[5] A 6% solution of PEG-20 oleyl ether (Volpo 20, Croda Inc., New York) increased absorption of the compound in the static cell and gave values more similar to in vivo results.[5] Cinnamyl anthranilate was evaluated in the flow-through cell, using saline or a solution of the nonionic surfactant as the receptor fluid. With normal saline receptor fluid, absorption was significantly increased by the use of the flow-through cell (Fig. 5). However, the amount of absorption was less than the similar values obtained with either type of cell when 6% PEG-20 oleyl ether was the receptor fluid.

Discussion

The design of this flow-through cell is the result of a number of modifications of the cell originally used by Marzulli.[7] The opening of the inlet sidearm into the receptor was constructed so that it was almost as large as the depth of the receptor (for proper mixing). A group of cells were made from plexiglass so that the contents of the receptor could be seen. Uptake of test compounds by this porous material created problems, as the radioactive material from one experiment contaminated succeeding experiments by leaching out from the cells into the receptor fluid. The final design of Teflon with a window in the bottom provided a view of the receptor contents in a cell made of a machinable, inert material.

The flow rate was critical up to a certain point. A minimum flow rate is required for good mixing and for removing absorbed compound from the receptor in a rapid manner. But flow rates above this minimum (in our case ~5 mL/h) resulted in no change in absorption rate (Fig. 2). An exception to this might be with the use of compounds that have limited solubility in the receptor fluid (as with cinnamyl anthranilate, discussed below). Crutcher and Maibach[8] found that absorption of testosterone and testosterone propionate varied with the flow rates utilized in their system, probably because they had not yet reached the critical rate of perfusing their relatively large surface area of exposed skin (4.5 cm^2). The two compounds have very limited water solubility, another possible explanation for the increase in absorption with increase in flow rate.

The accuracy of data from the flow-through cell was initially determined by comparing the results to those obtained in the standard static diffusion-cell system. Good agreement was found between both types of cells for both absorption profiles (Figs. 3 and 4) and numerical comparisons (Table I) for the absorption of water, cortisone, and benzoic acid. This is evidence of good mixing and rapid removal of absorbed material from the flow-through cell receptor.

Diffusion-cell absorption values were then compared with results from in vivo studies in rats. The in vivo absorption of cortisone and benzoic acid in a petrolatum vehicle was similar to that obtained in the two types of diffusion cells (Table II). The in vivo and in vitro absorption of benzoic acid is slightly lower than reported previously[6] when the skin was not washed at 24 h. Because of the desquamation of stratum corneum cells in vivo, more accurate results are probably obtained with this washing, as unabsorbed material can be lost as the cells begin to "flake off" after ~1 d. Good agreement between in vivo and in vitro absorption values has been reported for a number of compounds.[2,6,9]

The relative rates of penetration of benzoic acid and cortisone were dependent on the experimental conditions. With the acetone vehicle and a 24-h time course, benzoic acid absorption was much greater than that of cortisone (Table I). When the petrolatum vehicle was used and the in vitro experiments were conducted for up to 5 d, benzoic acid penetration was less than

Table II—Comparison of In Vivo and In Vitro Absorption[a]

Compound	In Vivo	In Vitro	
		Flow Cell	Static Cell
Cortisone	19.6 ± 1.3 (4)	20.1 ± 1.1 (6)	22.8 ± 2.7 (5)
Benzoic acid	37.0 ± 2.8 (8)	28.3 ± 3.0 (6)	35.5 ± 5.2 (5)

[a] Percentage applied dose absorbed (corrected). Values are the mean ± SEM of the number of determinations in parentheses. Compounds were applied in a petrolatum vehicle. The values obtained for each compound by the three methods were not significantly different from each other when compared by the two-tailed t test, $p < 0.05$.

two-fold greater than that of cortisone. In the longer experiment, the cortisone partially "caught up" with benzoic acid in that it continued to partition out of its reservoir in the skin. Almost all of the benzoic acid absorption occurred in the first 24 h.

The absorption of hydrophobic compounds can present problems in an in vitro diffusion-cell system due to a lack of solubility of the penetrant in the receptor solution.[5] The percutaneous absorption of two such compounds, cinnamyl anthranilate and 1-(3-ethyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)ethanone (acetyl ethyl tetramethyl tetralin), differed substantially from in vivo values obtained in rats. A marked increase in absorption without apparent damage to the skin was obtained, using a 6% solution in water of the nonionic surfactant PEG-20 oleyl ether as the receptor fluid. This effect is probably the result of increased partitioning of the compounds from the skin into the receptor fluid. For cinnamyl anthranilate, the percentage of the applied dose absorbed after 5 d was: in vivo, 45.6; in vitro (saline), 5.8; in vitro (6% PEG-20 oleyl ether), 27.9.[5] The continual replacement of receptor fluid in a flow-through cell might also result in an increased partitioning of hydrophobic compounds from skin. After 48 h, a significant increase in cinnamyl anthranilate absorption was obtained compared with the static cell with saline used as the receptor fluid in both cells (Fig. 5). The results obtained with the 5-mL/h flow rate were not increased further by a flow rate of 40 mL/h (data not shown). Perhaps a still greater increase in flow rate would have resulted in enhanced absorption, but the large volume of effluent produced would be undesirable. Maximum absorption of cinnamyl anthranilate was obtained using the 6% PEG-20 oleyl ether receptor fluid with either type of diffusion cell. The increased partitioning from the skin resulting from the use of the surfactant solution appears greater than the effect on partitioning of continual replacement of saline in the flow-through system.

The flow-through cell (saline receptor fluid) was not able to replace the use of the nonionic surfactant solution in the measurement of the absorption of cinnamyl anthranilate. Its value in the study of the permeation of this compound was the automatic sampling provided.

Recently, we have designed a flow-through cell (available from Crown Glass Co., Sommerville, NJ) with a smaller receptor volume but identical in other ways. The exposed skin surface area is 0.32 cm^2 and the volume of the receptor is 0.13 mL. With these cells, the flow rate can be reduced to ~1.5 mL/h (data not shown). This low flow rate is ideal when sampling over a period of days and is desired because of the reduction in the volume of effluent produced. The reduced skin surface area allows smaller pieces of skin to be used—a benefit with human skin, which is usually in short supply.

References and Notes

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