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Dermal Microdialysis Sampling *in Vivo*

Joseph M. Ault,[1,4] Christopher M. Riley,[1] Noel M. Meltzer,[3,5] and Craig E. Lunte[2,6]

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Microdialysis sampling of the dermis in vivo was accomplished using a linear microdialysis probe. In contrast to previous studies using a commercial cannula-style microdialysis probe, the linear probe had no effect on the flux of drug through the skin *in vitro*. The extent of tissue damage *in vivo* due to probe implantation was evaluated by histological examination and microdialysis delivery studies. Tissue damage due to implantation of the linear probe was minimal with no bleeding or edema observed. Infiltration of lymphocytes into the tissue was observed beginning 6 hours after probe implantation with scar tissue beginning to form after approximately 32 hours. The infiltration of lymphocytes had no effect on the behavior of implanted microdialysis probes. Delivery of 5-fluorouracil was between 20 and 25% for six different probes implanted in six different animals demonstrating good probe-to-probe and implantation-to-implantation reproducibility. Constant delivery was maintained for at least 24 hours in all cases indicating that experiments of at least 24 hour duration are feasible. The dermal concentration of topically applied 5-FU cream, Efudex®, was continuously monitored by an implanted microdialysis probe demonstrating the feasibility of this technique as for monitoring skin drug levels *in vivo*. The dermal concentration of 5-FU following topical application was approximately 40-fold higher for in vitro excised skin than for in vivo intact skin.

KEY WORDS: dermal sampling in vivo; microdialysis sampling; 5-fluorouracil; transdermal drug delivery.

INTRODUCTION

The dermis can be an important site for the study of the disposition of drugs. Transdermal drug delivery can be advantageous relative to other routes because first-pass metabolism is reduced, patient compliance is good, and bioavailability problems inherent in gastrointestinal absorption are avoided (1-3). In addition, many drugs are active in the skin (4). Correlations between blood levels and pharmacological activity of these drugs have not been good. A method for sampling the dermis *in vivo* would provide better understanding of the processes involved.

Currently most studies of transdermal drug delivery are performed *in vitro* using a two compartment diffusion cell (5). There has been poor correlation between results obtained *in vitro* and the actual behavior of the drugs *in vivo* (6). Methods for observing transdermal delivery *in vivo* have relied on the appearance of the drug in blood. However, an increase in the plasma concentration of a drug is not necessarily due to an increased flux of the drug through the skin (7).

To actually measure the concentration of a drug in the skin currently requires that the skin be excised, homogenized, extracted, and then the extract analyzed. Such a procedure is long and prone to errors such as incomplete extraction or drug decomposition during sample preparation. In addition, it is possible to obtain only a single time point from each experimental animal. Preliminary results have demonstrated the ability of microdialysis probes to continuously monitor the concentration of drugs in the dermis *in vitro* (8). This has provided the first direct observation of the dermal flux of a percutaneously administered compound as no alternative method exists (1).

While the initial studies were promising, tissue damage was observed when using commercially available cannula-style microdialysis probes (8). A linear microdialysis probe based on previously described transverse designs (9-13) was developed to minimize tissue damage. This design is based on coating a dialysis membrane with biocompatable silicone, leaving a small uncoated active window across which dialysis occurs. This report describes the evaluation of this probe design both *in vitro* and *in vivo*. Parameters such as tissue damage and response to implantation, long-term stability, and reproducibility were investigated. Finally, the utility of this technique for continuous dermal drug monitoring *in vivo* was demonstrated by monitoring the concentration of 5-fluorouracil (5-FU) in the dermis of an awake, freely-moving fuzzy rat following topical application of 5-FU cream (Efudex®). A report has recently described the use of a canulla style microdialysis probe to monitor the concentration of methotrexate in the dermis of anesthetized rats (14).

MATERIALS AND METHODS

Chemicals

5-Fluorouracil (5-FU), potassium phosphate, phosphoric acid and modified Dulbecco phosphate buffered saline were obtained from Sigma Chemical Company (St. Louis, MO). Water for buffer preparation was processed through a Milli-Q water system (Waters Corp., Bedford, MA). The topical preparation of 5-FU, Efudex® 5% cream, was provided by Hoffmann-La Roche, Nutley, NJ.

Microdialysis System

The microdialysis system consisted of a Model 22 syringe pump (Harvard Apparatus, South Natick, MA) with 10 mL plastic syringes (Becton Dickinson and Company, Rutherford, NJ). All connections were made with polyethylene or silastic tubing. A perfusion flow rate of 2.0 μL/min was used for all experiments. Dialysate samples were collected directly in the 20 μL sample loop of the chromatographic system over 14.5 minute intervals. At a perfusion rate of 2.0

[1] Department of Pharmaceutical Chemistry and Center for Bioanalytical Research, University of Kansas, Lawrence, KS 66045, USA.
[2] Department of Chemistry and Center for Bioanalytical Research, University of Kansas, Lawrence, KS 66045, USA.
[3] Hoffmann La-Roche Pharmaceuticals, Nutley, NJ 07110, USA.
[4] Current address: DuPont Merck Pharmaceuticals, Experimental Station, Wilmington, DE 19880, USA.
[5] Current address: Astra-Merck, 725 Chesterbrook Blvd., Wayne PA 19087, USA.
[6] To whom correspondence should be addressed.

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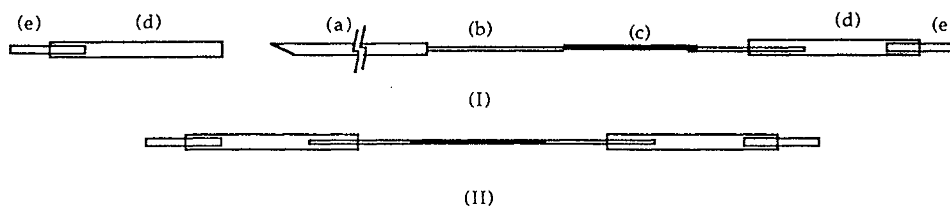

Figure 1. Diagram of the dermal microdialysis probe system illustrating the probe before (I) and after (II) the liquid circuit has been connected. (a) 25 gauge needle, (b) silicone coated dialysis membrane, (c) uncoated membrane (dialysis window), (d) silastic and (e) polyethylene tubing.

μL/min, this results in a 45% overfill of the sample loop. The valve was then switched to the inject position for 30 seconds. Therefore a microdialysis sample was injected every 15 minutes.

Linear Microdialysis Probe

The linear microdialysis probe was fabricated from a regenerated cellulose dialysis fiber (9000 M.W. cutoff, 150 μm I.D., Spectrum Medical Industries, Los Angeles, CA) as shown in Figure 1. The fiber was spray coated with silicone dispersion 236 (Dow Corning, Midland, MI) to prevent analyte penetration. An active dialysis window was created by placing a 5 mm length of stainless steel tubing over the center of the cellulose fiber during coating with the silicone. After the silicone had dried this tubing was removed to expose the active dialysis window. Silicone was selected over other polymers (i.e. Teflon®) or resins because of its flexibility when dry, uniformity of application, and ability to cure at room temperature. The beveled shaft of a 25 gauge stainless steel needle was glued to one end of the fiber to serve as an insertion guide during implantation. A 4.5 mm section of silastic tubing (0.02" i.d., 0.037" o.d.) was attached to the other end of the probe to which PE-10 tubing was then attached for the remainder of the liquid connection to the microdialysis system. After implantation, the insertion guide was removed and replaced by a second set of silastic and PE-10 tubing and connected to the LC sample loop.

Analysis of 5-FU

A column switching system was used for analysis of 5-FU consisting of two Model LC-6A pumps, a Model C-R5A integrator, a Model SPD-2 UV-VIS spectrophotometric detector, an FCV-2AH 6-port switching valve and a Model SCL-6A system controller (Shimadzu Corp., Kyoto, Japan) as described previously (8). A Panasonic model TR-120 MDPA (Matsushita Electronic Industry Co. Ltd., Osaka, Japan) video monitor and a model FDD-1A dual floppy drive (Shimadzu Corp., Kyoto, Japan) were used for data analysis and storage. A pellicular RP18 percolumn (30-40 μm, 0.2 × 2 cm, Upchurch Scientific Inc., Oak Harbor, WA) and three ODS Hypersil columns (5 μm, 4.6 × 15 cm, Keystone Scientific, State College, PA) were used for the chromatographic separation of 5-FU. A 20 μL injection loop attached to a C6W injection valve mounted on an EF60 electronic actuator (Valco Instruments Inc., Houston, TX) was used for on-line sample injection. The syringe pump, electronic actuator and chromatographic system were electronically connected using an interface box built in-house.

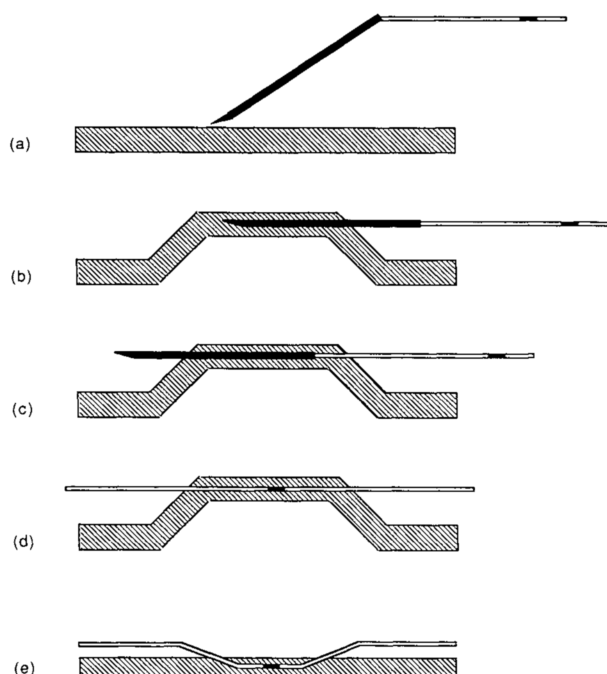

Figure 2. Diagram of the dermal microdialysis probe implantation scheme. The shaded region represents the skin while the black section along the probe represents the skin while the black section along the probe represents the exposed dialysis membrane.

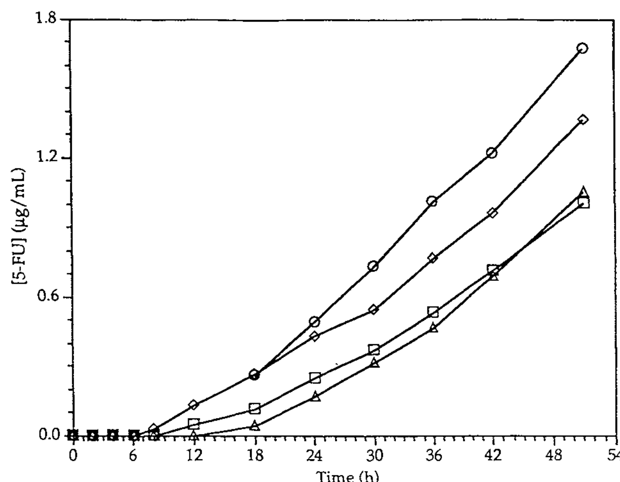

Figure 3. The Franz diffusion cell receptor concentration of 5-FU vs. time profile of topically applied Efudex® cream. Symbols are: □ and ◇ are probe implanted; ○ and △ are controls with no implantation.

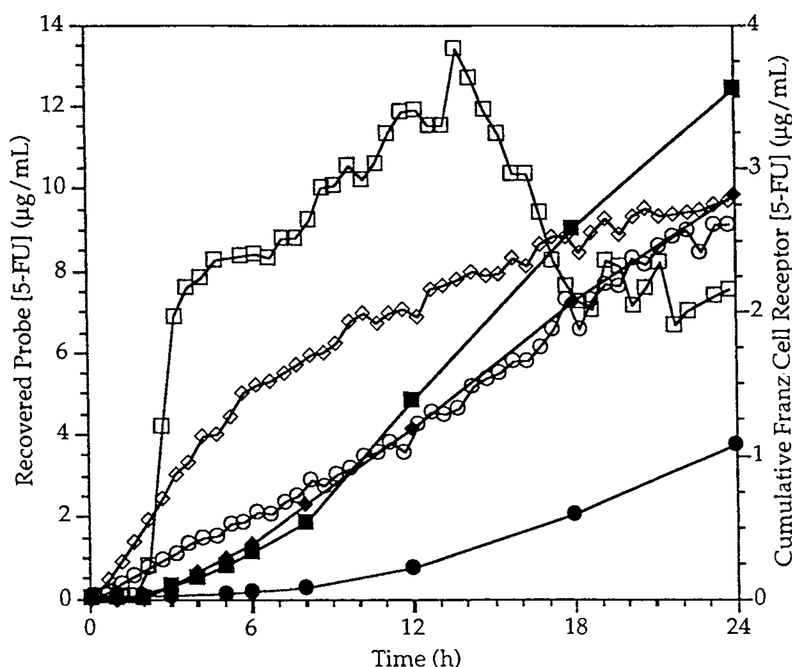

Figure 4. Comparison of the concentration versus time profile for 5-FU in the dermis and in the receptor cell for excised fuzzy rat skin mounted in a Franz diffusion cell. The open symbols correspond to the dermal concentrations determined by microdialysis while the closed symbols correspond to the cumulative concentration determined in the receptor cell. Data is from three different section of rat skin with the same symbol being used for the same skin section.

Detection of 5-FU was performed at 260 nm for all analyses. All columns were eluted with KH_2PO_4/H_3PO_4 (50 mM, pH 3.5) at a flow rate of 1.0 mL/min. The width of the switch window between the two columns was 1.1 minutes. Microdialysis samples were collected in the injection loop and injected directly into the chromatograph without any prior sample preparation.

Isolated Skin Preparation

Dorsal skin was excised from adult male, fuzzy rats weighing between 450 and 500 g. The excised skin was washed with Dulbecco phosphate buffered saline (DPBS), the excess connective or adipose tissue was removed, and the skin mounted on the Franz diffusion cell as previously described (8).

Probe Implantation

Implantation in Vitro. The linear microdialysis probe was implanted into the skin using a hemostat modified to hold the microdialysis probe. The insertion guide was firmly clamped with a hemostat and an entry puncture made in the skin (Figure 2a). The probe was inserted through the skin for an appropriate distance (15-20 mm) leaving the skin through an exit puncture (Figure 2b-d). The remainder of the insertion guide was then pulled through the skin, positioning the uncoated, active dialysis window beneath the site of topical 5-FU application (Figure 2e). The insertion guide was removed and connecting tubing attached to the ends of the implanted probe.

Following probe implantation the skin and probe were inspected visually for any damage and the skin containing the probe was then placed on the Franz diffusion cell. The section of skin containing the uncoated portion of the microdialysis probe (microdialysis window) was centered over the Franz cell receptor opening. The cap of the Franz diffusion cell was placed on top of the skin and carefully clamped as to not disturb flow through the connective tubing attached to the microdialysis probe. A control experiment was also performed in which the insertion guide was inserted and removed from the skin.

Implantation in Vivo. The linear microdialysis probe was implanted into the dermis of an anesthetized fuzzy rat as described above for implantation *in vitro*. The silastic connecting tubing from the probe was glued to the skin of the animal to secure the probe's position in the skin. The animal was then placed in a containment unit and allowed to recover from the anesthetic. Once implanted, the probe was contin-

Table I. Flux Through the Skin Determined from Microdialysis Sampling and the Franz Cell Receptor Compartment

Skin Sample	Microdialysis Sampling: Flux ($\mu g/cm^2 \cdot h$)[a]	r^2	Cumulative Receptor Concentration: Flux ($\mu g/cm^2 \cdot h$)[a]	r^2
1	2.38 ± 0.022	0.998	1.76 ± 0.025	1.000
2	0.98 ± 0.028	0.985	0.45 ± 0.064	0.980
3	1.60 ± 0.018	0.998	1.07 ± 0.037	0.999

[a] Values are the slope of the accumulated mass versus time data ± Std. Dev.

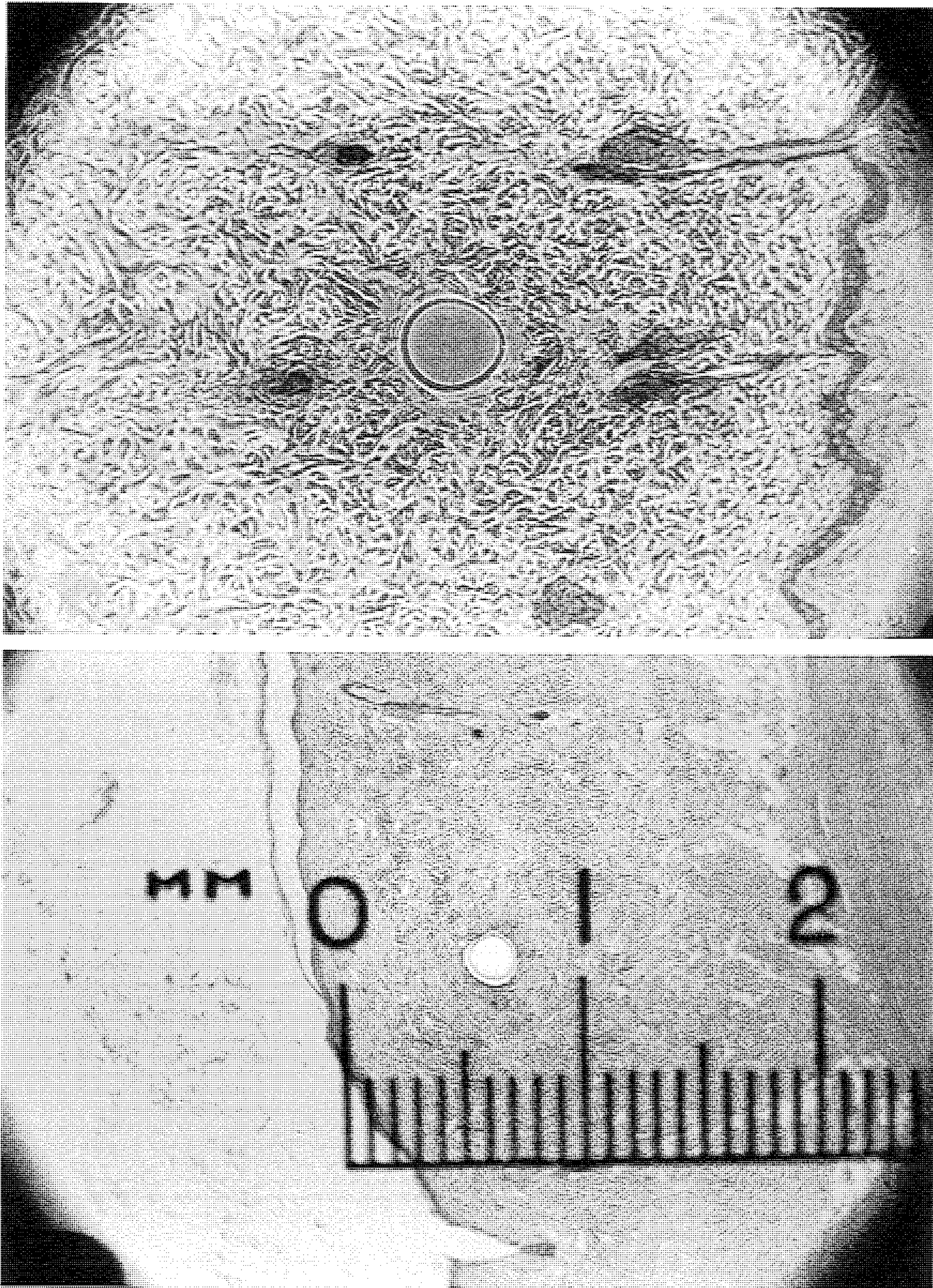

Figure 5. Photomicrographs of the excised tissue illustrating the effects of probe implantation on dermal tissue. Photographs a-d correspond to 6, 12, 24, and 32 hours after probe implantation respectively. The increased gray density at 12 h and beyond are lymphocytes infiltrating the implantation area. The dark gray/black areas at 24 and 32 h are differentiated tissue formation around the probe (scar tissue).

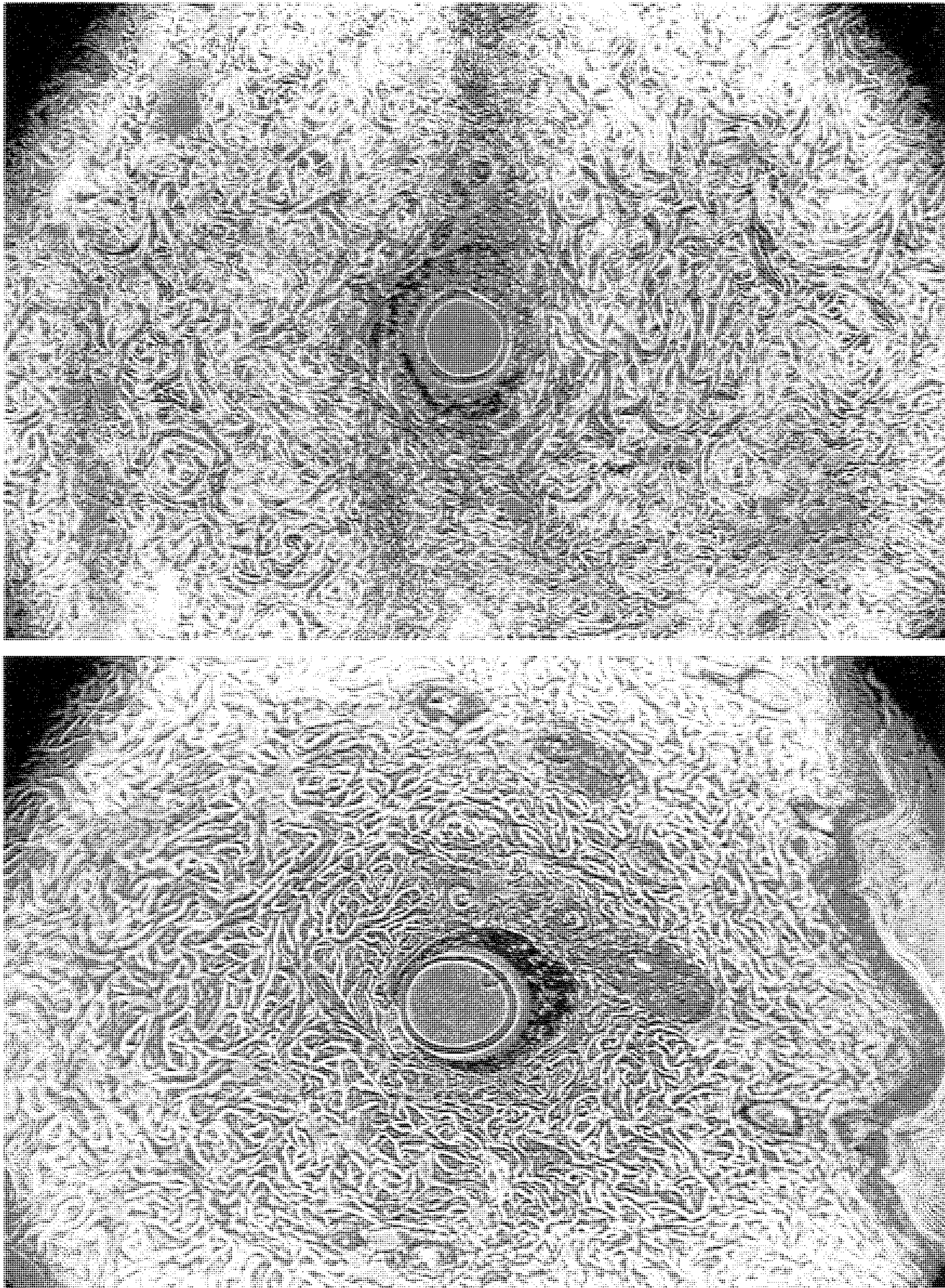

Figure 5. Continued.

uously perfused with either buffer or buffer containing 5-FU until the end of the experiment.

Animal Containment System

The animal containment system consisted of a large plastic container, a two channel liquid swivel (Harvard Apparatus, South Natick, MA) and a spring animal tether. The liquid swivel was mounted at the top center of the containment system using a laboratory clamp attached to a metal rod which was in turn attached to the sides of the bowl using two additional laboratory clamps. The tether was attached to the liquid swivel and to the animal by clipping a fishing clip

attached to the end of the tether through a hole in the collar around the rodent's neck. The rodent collar was made by sleeving a bar wrap tie with PE tubing.

Histology

Probes were implanted and perfused with buffer for 0, 1, 6, 12, 24, 30 and 72 hours, after which the animal was sacrificed and the tissue excised. The tissue was placed in a 10% buffered formalin solution and fixed for approximately 7 days. Tissue processing and staining with a Hemotoxylin/Eosin stain were performed at Lawrence Memorial Hospital, Lawrence KS. The tissue and probe were dehydrated and rehydrated during this procedure exposing the tissue and probe to various concentrations of xylene, methyl and isopropyl alcohol at elevated temperatures for varying periods of time. After staining and mounting the slides were inspected visually and photographs taken of the appropriate region of the skin.

Probe Delivery and Recovery Studies *in Vivo*

For delivery studies, the perfusion syringe was filled with a 2μg/mL solution of 5-FU in DPBS. Perfusion at a rate of 2.0 μL/min was begun 1 to 6 hours after probe implantation and was continued for 24 hours. The concentration delivered was calculated as the difference between the concentration initially in the perfusate and the concentration remaining in the dialysate following perfusion. Probe delivery (D) is defined as the percentage of 5-FU lost by the perfusate and described by the equation:

$$D = \left(\frac{C_p - C_d}{C_p}\right) \times 100\% \quad (1)$$

where C_p is the initial concentration of drug in the perfusate and C_d is the concentration remaining in the dialysate following perfusion.

For recovery studies, the probe was perfused with DPBS at a flow rate of 2.0 μL/min. after several blank dialysates had been collected, Efudex® cream was applied to the surface of the skin in a 1 cm circle. 5-FU diffused through the skin to the probe where it was recovered in the dialysate for analysis. The concentration of 5-FU determined in the dialysate represents the average dermal concentration during the 14.5 minute sampling interval.

RESULTS AND DISCUSSION

Franz Diffusion Cell Experiments

Franz diffusion cell experiments were performed to assess damage to the skin caused by implantation of the linear microdialysis probe. In previous studies, increased flux of drug through the skin was observed following implantation of a commercially available cannula-type microdialysis probe (8). The use of the linear microdialysis probe resulted in minimal tissue damage based on accumulation of 5-FU in the receptor cell. Skin samples in which an insertion guide was inserted and then removed or a probe was implanted exhibited no difference with respect to drug flux through the skin compared to two control skin samples as illustrated in Figure 3.

To asses the ability of microdialysis sampling to monitor drug concentrations in the skin, the microdialysis results were compared to the receptor compartment concentration of the Franz diffusion cell. The results for three different skin samples are shown in Figure 4. While the typical steady increase in receptor compartment concentration was observed, the dermal concentration as determined by microdialysis increased until a relatively steady plateau was achieved. Each of the skin samples behaved differently but there was qualitative agreement between the microdialysis concentrations and the receptor compartment concentrations. Thus, higher microdialysis (dermal) concentrations corresponded to a faster increase in the receptor compartment concentration.

A more useful comparison can be made by directly comparing the flux of drug through the skin as determined from sampling in the skin by microdialysis versus in the receptor compartment. According to Fick's laws of diffusion under steady state conditions, the flux of drug through the skin can be determined independently for each technique. Steady state flux can be determined empirically from a plot of the accumulated mass per unit surface area versus time (13). This is readily applied to the Franz cell where the receptor is of fixed volume (5.5 mL) and surface area (0.626 cm^2), and diffusion occurs in a single dimension perpendicular to the surface of the skin and receptor. The linear probe system is more complex since diffusion into the probe is three dimensional and no fixed "receptor" exists from which cumulative levels can be sampled. An approximate solution is to assume that the surface area is the area of the microdialysis window (0.056 cm^2) and that the probe "receptor" volume is the sample volume (20 μL). Although a concentration gradient around the probe and directional differences of diffusion into the probe can exist, the concentration recovered by the probe is an average over the entire surface area without directional distinction. Both techniques exhibited a linear plot after an 8 hour period to establish steady-state flux and detectable levels of 5-FU in the receptor compartment. The two methods gave consistent results for the calculation of the flux of 5-FU through the skin (Table I). The flux determined by microdialysis sampling is somewhat higher in this case because the data was not corrected for difference in recovery in the skin sample.

Histology

Histology studies were performed at various time points to investigate skin response to probe implantation. Issues such as inflammatory response, physical disruption, and probe shape were addressed by histological examination of full thickness fuzzy rat skin following probe implantation and perfusion *in vivo* (Figure 5b). Immediately after probe implantation there was no evidence of either edema or substantial tissue disruption. Infiltration of lymphocytes to the site of the probe was first observed 6 hours after implantation (Figure 5a) and increased during the entire study period. Noticeable changes in the cells surrounding the implanted probe occurred approximately 32 hours after probe implantation (Figure 5d). This cellular change was seen as an elongation of cells surrounding the probe.

Examination of the skin sections showed that probe implantation did not result in significant physical damage to the

Table II. Comparison of Recovery and Delivery of 5-FU in Vitro

Probe	Recovery[a]	Delivery[a]
1	55.1 ± 0.3	49.2 ± 1.5
2	40.3 ± 7.5	43.2 ± 3.0
3	34.5 ± 1.5	28.9 ± 0.6
4	39.8 ± 3.8	39.2 ± 0.5
5	48.7 ± 1.7	49.8 ± 1.4

[a] Values are % ± Std. Dev. with n = 5.

skin. For example, no pocket containing extracellular fluid or blood was apparent around the probe. The small space seen between the probe and the tissue in Figure 5 was attributed to shrinking of the probe membrane as a result of dehydration during tissue processing. Prior to lymphocyte infiltration, there was no evidence of cellular attachment to the probe membrane. The cells surrounding the probe took on the shape of the membrane but did not adhere to it. At later time points, after extensive lymphocyte infiltration, attachment of cells to the membrane was seen. This is particularly evident in the 24 hour slide (Figure 5c) in which cells were still attached to the membrane after it had shrunk during fixation.

Delivery Studies *in Vivo*

Delivery studies were conducted to monitor the performance of the linear probe *in vivo* and to assess the effect of changes in the skin on probe performance (15). Delivery of drug through the probe is a function of the permeability of both the dialysis membrane and the skin and therefore should reflect changes in either parameter (16). The delivery is also a measure of recovery. As seen in Table II, the delivery and recovery are identical for a given microdialysis probe and compound. While it is very difficult to determine directly the recovery of a microdialysis probe in vivo it is relatively straightforward to determine the delivery. In most cases it is therefore most convenient to determine the delivery of a compound in vivo and use this as the recovery. Probes exhibited consistent delivery of a known concentration of 5-FU for over 24 hours in six different fuzzy rats (Figure 6). All six probes exhibited a delivery between 20 and 25 percent for 5-FU. This probe-to-probe and implantation-to-implantation reproducibility of deliver indicated that the probes performed in a predictable manner and were delivering drug to a similar environment. Although lymphocyte infiltration began after 6 hours, no changes in delivery were observed during the first 24 hour after implantation. This indicated that the infiltration of lymphocytes did not change the behavior of the microdialysis experiment. Therefore, consistent results can be achieved for at least the first 24 hours after probe implantation. For delivery studies extended beyond 24 hours (Figure 6 inset), a rapid increase in probe delivery and variability occurred after 30 hours in two cases (rats 1 & 2) and after 60 hours in another case (rat 3). The other three probes failed for mechanical reasons at var-

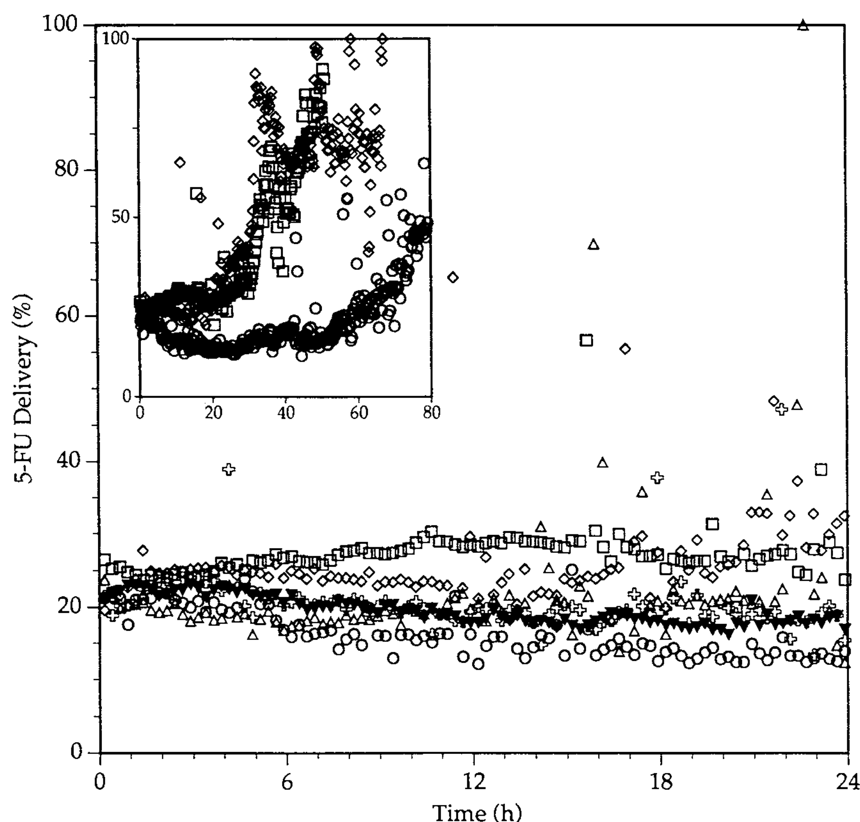

Figure 6. Profiles of the delivery of 5-FU versus time for 6 individual fuzzy rats *in vivo* for 24 h time periods. Inset, the profile of the delivery of 5-FU vs. time for 3 fuzzy rats *in vivo* extended for time periods greater than 24 h.

ious times after 30 hours but before any observed change in their delivery behavior. The observed change in delivery roughly correlated to the formation of scar tissue around the dialysis membrane. A decrease in delivery was anticipated due to slower diffusion of 5-FU through the scar tissue resulting in an accumulation around the probe. The observed increase in delivery indicates that either 5-FU transport through or removal from the dermis has increased. It is not completely clear at this time whether this response is a result of the microdialysis probe implantation or of the long-term continuous delivery of 5-FU to the dermis. For example, long term delivery of 5-FU may be inducing enzymes which increase the metabolic clearance of 5-FU. Alternatively, long term probe implantation may lead to scar tissue formation which is more permeable to 5-FU than normal dermal tissue.

A 24 hour window during which the microdialysis behavior is predictable and consistent is sufficient for most transdermal drug delivery studies. Alternatively, by allowing a 72 hour period prior to the start of the transdermal delivery experiment, long term experiments are possible as a new steady-state condition has been reached. It is the period between 32 and 72 hours during reach the microdialysis behavior is changing that should not be used.

Recovery Studies *in Vivo*

The utility of microdialysis sampling of the skin was demonstrated by monitoring the flux of drug through the skin *in vivo*. Following topical application of Efudex® cream the dermally implanted microdialysis probes successfully monitored the levels of 5-FU in the skin of fuzzy rats for up to 12 hours (Figure 7). The concentration of 5-FU in the skin rapidly reach relatively constant levels although more variation was observed than in the in vitro skin experiments. Table III summarizes the data for monitoring 5-FU in the dermis of six different rats following topical application of 5-FU cream.

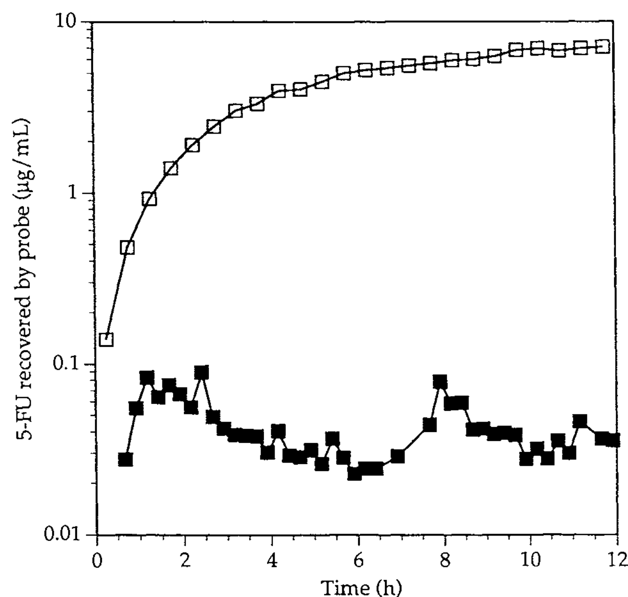

Figure 7. Representative recovery versus time profile for topically applied 5-FU (Efudex® cream) from a freely moving fuzzy rat ■ and excised rat skin in a diffusion cell □.

Table III. Steady-state Concentrations of 5-FU in the Dermis in Vivo and in Vitro

	5-FU concentration[a] μg/mL	Number of samples averaged
Rat 1[b]	0.033 ± 0.008	36
Rat 2	0.082 ± 0.022	34
Rat 3	0.38 ± 0.30	32
Rat 4	0.17 ± 0.14	26
Rat 5	0.11 ± 0.02	13
Rat 6	0.057 ± 0.014	12
Skin 1	9.24 ± 1.39	17
Skin 2	5.45 ± 1.27	18
Skin 3	2.46 ± 0.86	18

[a] Values are concentration ± Std. Dev.
[b] Rat refers to in vivo experiments and Skin refers to in vitro experiments.

The steady-state concentrations of 5-FU in the skin during the previously described diffusion cell experiments is also shown in Table III. Although the same 5-FU preparation was used, the steady-state concentrations of 5-FU determined in the dermis during the in vivo experiments were consistently approximately 40-fold lower than the concentrations determined in the dermis in the in vitro experiments. In addition, the steady-state levels during the in vitro experiments were consistently less variable than the in vivo experiments. It is likely that the high clearance of 5-FU and intact vasculature of the in vivo system far more effectively removes 5-FU than can be accomplished in the excised skin preparation. This results in both the lower concentrations and greater variability in concentration for the in vivo experiments than for the diffusion cell experiments. However, it is very likely that experiments in vitro do not accurately reflect the situation in vivo. Microdialysis sampling in vivo provides a technique for directly monitoring the dermal concentration of a drug, whether administered topically or systemically, which is superior to current in vitro methods.

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