DEVELOPMENT OF A RAT MODEL TO INVESTIGATE CONTRIBUTIONS OF ANATOMIC AND PHYSIOLOGIC DETERMINANTS OF IN VIVO SKIN PERMEATION

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ABSTRACT

Title of Dissertation: Development of a Rat Model to Investigate Contributions of Anatomic and Physiologic Determinants of In Vivo Skin Permeation

Nicholas M. Fleischer, Doctor of Philosophy, 1991

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The skin is a heterogeneous, bi-directional impediment to chemical flux, in which the stratum corneum is a major, though not the sole, rate-limiting barrier layer to permeation. Systemic toxicity following dermal exposure to environmental chemicals and use of skin as a portal for systemic administration of drugs have led to extensive investigations of the inward flux of xenobiotics applied to the outer surface of skin. Those investigations mainly utilized in vitro experimental systems that were limited by the absence of normal physiologic functions. Heretofore, only a few, complex in vivo models have been reported for the study of contributions of cutaneous physiology, especially skin capillary permeability and blood flow, to the cutaneous permeation of xenobiotics.

The objective of the present research was to investigate an in vivo skin permeation model system that was sensitive to perturbations of skin capillary physiology and

stratum corneum. A "fuzzy" rat model system was devised that employed outward cutaneous migration of a systemically administered permeation probe, isoflurane. Specially devised, transdermal vapor collection devices were used to capture the outward flux of isoflurane through the skin. Isoflurane flux measurements, coupled with blood isoflurane concentrations, were used to calculate cutaneous permeability coefficients (Kp) of isoflurane, as an index of permeation, under various conditions of normal or perturbed cutaneous physiologic states. Physiologic perturbations were performed to test the sensitivity of the model system to detect effects of minoxidil-mediated vasodilation, phenylephrine-mediated vasoconstriction, and leukotriene D_4-mediated increased capillary permeability on the outward flux of isoflurane. Tape stripping and topical ether-ethanol application produced either physical removal or chemical disruption of the stratum corneum, respectively.

Minoxidil, leukotriene D_4, tape stripping of stratum corneum, and topical ether-ethanol experiments produced statistically significant increases (52 to 193%) in the Kp's, while phenylephrine had no significant effect on isoflurane permeation. These experiments have documented the characteristics of this model system for probing the individual contributions of anatomical and physiological function in outward skin permeation of the lipid-soluble probe, isoflurane.

DEVELOPMENT OF A RAT MODEL TO INVESTIGATE CONTRIBUTIONS OF ANATOMIC AND PHYSIOLOGIC DETERMINANTS OF IN VIVO SKIN PERMEATION

by

Nicholas Miklos Fleischer

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AUC	area under the curve
AV	arteriovenous
AVA	arteriovenous anastomoses
Cl_{FIT}	model-dependent derived clearance
Cl_{M-I}	model-independent derived clearance
Cmax	peak concentration
C_{ss}	steady-state concentration
DMSO	dimethyl sulfoxide
DSCD	dermal substance collection device
ECD	electron capture detector
EFAD	essential fatty acid deficiency
FEP™	brand of Teflon by DuPont
GC	gas chromatograph
H & E	hematoxylin and eosin
IPPSF	isolated perfused porcine skin flap
k_{10}	elimination rate constant from the central compartment
Kp	permeability coefficient
Kp_{eff}	effective permeability coefficient
LDF	laser Doppler flowmetry
LDV	laser Doppler velocimetry

LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
PTFE	polytetrafluoroethylene
PU	perfusion units
QC	quality control
SRS-A	slow reacting substance of anaphylaxis
TVCD	transdermal vapor collection device
UV	ultra-violet
V_1	volume of the central compartment

INTRODUCTION

1. Background/Objectives

A. Skin as a permeation barrier

A little over 100 years ago, the skin was believed to be a completely impermeable membrane. Fleischer (1877) stated that there existed a difference in the permeability characteristics of living and dead skin; dead skin being permeable and living skin being completely impermeable. We now know that skin is far from being a complete, effective barrier either for exogenous chemicals entering the skin and diffusing through it to the systemic circulation or for internalized chemicals migrating to the outermost layer of skin, the stratum corneum, and exiting the body. The transfer of xenobiotics across the skin is well known and has been extensively documented (Scheuplein and Bronaugh 1983; Chien 1987; Shroot and Schaefer 1987; Bronaugh and Maibach 1989; Hadgraft and Guy 1989; Scott, Guy and Hadgraft 1990). Percutaneous drug administration has become so sophisticated that we are now able to treat serious systemic diseases such as hypertension and heart disease and provide effective analgesia with medication applied to the skin. In the United

States, the pharmacologic agents available in transdermal systems for administration through the skin include nitroglycerin for the prevention and treatment of angina, clonidine for the control of hypertension, scopolamine for the prevention of motion sickness, estradiol for estrogen replacement therapy, and fentanyl for the management of chronic pain. Numerous other agents are under investigation for using this novel route of drug administration some of which include analgesics, antihistamines, beta-adrenergic blockers, and steroids.

B. Bi-directional skin permeation

The transfer of xenobiotics, with the exception of inert gases, from inside the body, through the skin, to the outside surface is an area that has been only preliminarily investigated. Some nonvaporous chemicals have been detected in sweat (Johnson and Maibach 1971) or appendages, such as hair (Ishiyama, Nagai and Toshida 1983) and nails (Suzuki, Hattori and Asano 1984). Mass spectrometric studies have shown that at least 300-400 chemicals vaporize from the skin surface (Sastry et al. 1980). These chemicals range from elemental gases and water to more complex hydrocarbons, ketones, and alcohols. Outward oxygen flux has been described by several investigators (Versmold and Severinghaus 1982; Wilson, Maibach and Severinghaus 1982). Adamczyk and coworkers (1966)

presented a method for analyzing the excretion of carbon dioxide, helium, and argon by the human skin from several places on the body. Understanding the outward flux of gases across the skin has led to development of a technique of transcutaneous monitoring which is used clinically to evaluate the partial pressure of oxygen in blood.

Stoelting and Eger (1969) reported the first demonstration of the outward permeation of anesthetic vapor across the skin. They analyzed the concentration over time of ether or halothane in a sealed glass chamber which had been placed over the hand, forearm, and part of the arm in patients undergoing elective surgery. It was reported that the percentage of the anesthetic-blood concentration lost through skin was 0.21 and 0.25% for ether and halothane, respectively, after 60 minutes of anesthesia. They postulated that the amount of ether or halothane lost through skin was insignificant except after very long periods of administration.

Several investigators have reported on the deposition in skin sites of internally administered substances. Epstein and coworkers (1972) described the regional variation in griseofulvin concentration in the stratum corneum after oral administration. Additionally, they presented an outward flux for griseofulvin of 74 $ng/cm^2/24$ hours which they attributed to a washing-out of the stratum corneum by sweat. Lutz and coworkers (1977) and Tuey and Matthews (1980) described the

relatively large concentration of ^{14}C-labeled chlorinated biphenyls (analyzed for total radioactivity by oxidation to $^{14}CO_2$ and liquid-scintillation analysis) in the skin of rat and mouse after intravenous injection.

Peck and coworkers (1981) proposed the concept of continuous outward transepidermal chemical migration of non-volatile drugs. They suggested that a skin placed collection device could be devised to monitor chemical intake and compliance with therapeutic regimens and developed a mathematical solution for the amount of drug that would accumulate in such a device. A prototype device was described (Peck et al. 1982) and demonstrated to have the ability to collect several model chemicals which had outwardly migrated through the skin of animals and humans (Peck et al. 1988). Their experiments used a variety of animal species (rhesus monkey, "fuzzy" rat and human) and tested compounds with widely ranging physicochemical characteristics with molecular weights ranging from 18 to 5000 daltons and octanol/water partition coefficients ranging from 0.003 to 6,760.

The similarity of the permeability rate of theophylline through skin, whether observed from the stratum corneum inward or from the dermal side outward, was shown by Peck and coworkers (1987). Additionally, they reported an in vitro effective permeability coefficient for theophylline across full thickness "fuzzy" rat skin of 0.00032 ± 0.00013 (mean ± SD) cm/hr, which was nearly identical to that reported

for a chemically similar compound, caffeine, (0.00031 cm/hr) across full thickness Osborne-Mendel rat skin (Bronaugh et al. 1982). Their observed in vivo effective permeability coefficient was about 3 times higher than the in vitro value derived from diffusion cell experiments using full thickness rat skin; they attributed the non-viable characteristics of their in vitro skin permeability studies to explain the difference.

Conner and coworkers (1991), using caffeine as a model compound, demonstrated the applicability of Fick's law of diffusion to the phenomenon of chemical egress outward through the skin. They also pointed out that, at least for caffeine collection through the skin, egress through the shunt pathway of sweat ducts, produced under non-thermal-stress conditions, does not contribute to a significant extent. Murphy and coworkers (1990) reported on the collection of theophylline and its metabolite, caffeine, through the skin of pre-term infants, who were receiving theophylline therapy. They concluded that the transcutaneous collection method correlated with plasma concentrations consistent with a diffusion process.

C. Objective

The primary objective of this research was the development and investigation of an in vivo animal model

system for examining the contributions of skin blood flow, capillary permeability, and diffusion through viable epidermis and stratum corneum to the outward migration of a selected probe chemical. The individual contributions to skin permeation of the aforementioned skin components are still not entirely understood. Apart from model development, two principle questions were posed to test model performance: Can skin blood flow or capillary permeability be perturbed by vasoactive chemicals to the extent of producing a significant change in the efflux of the probe? Is the stratum corneum a significant barrier to permeation of a volatile, lipophilic chemical?

2. Anatomy and physiology of the skin

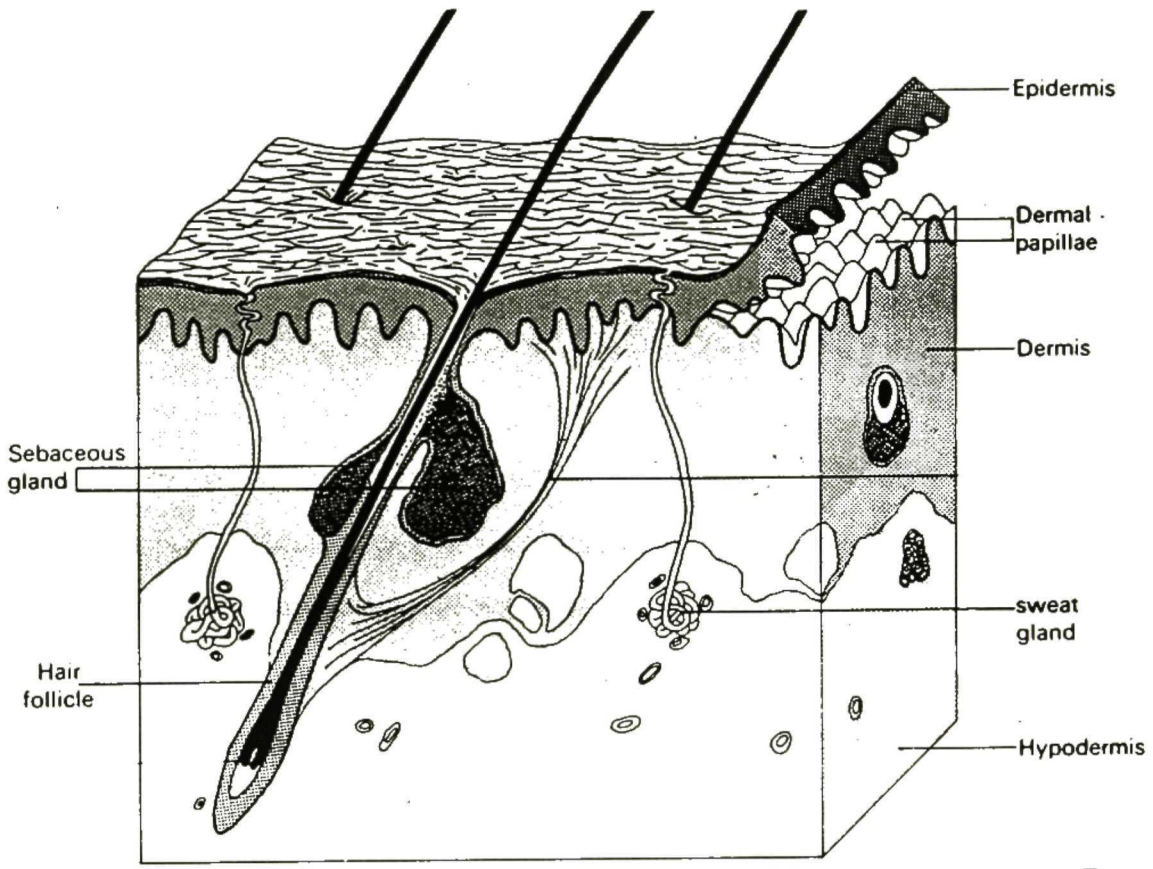
A. Anatomy

The skin is one of the major organs in the body, and, as it comprises nearly 10% of the body weight, it is also one of the largest organs in the body. It protects the mammalian organism from the outside environment and retains the substances necessary for life from escaping to the environment. The skin of humans is also involved with metabolism, heat regulation, and sensory functions. As all other organs in the body, the skin receives its metabolic requirements by way of the circulatory system.

The skin consists of three layers (Figure 1). The outermost layer, the epidermis, is a tightly bound, stratified squamous epithelium covered by a thin layer (the non-viable stratum corneum) of keratin and lipid. Keratin, a physically tough, insoluble protein, provides the largest part of the protective quality of skin. The epidermis varies in thickness over most of the body from 70 to 1,120 microns (1 micron = 0.0001 cm) but on the palms and soles can attain a thickness of 800 to 1,400 microns. The dermis, which lies under the epidermis is primarily a collagenous tissue. It also imparts a certain amount of protection from physical injury and is the site of sensory nerve endings and of blood vessels which are an integral part of body temperature regulation. The thickness of the dermis varies from 1,000 to 2,000 microns over most areas but may be greater than 5,000 microns over the back. The innermost layer of skin is a fibro-fatty tissue, often referred to as the subcutaneous tissue or hypodermis. This layer also buffers from trauma and temperature fluctuations (Stenn 1983). Interspersed in varying quantities throughout the skin are two skin appendages, the pilosebaceous apparatus, and the eccrine sweat gland. The pilosebaceous apparatus includes the hair follicle, sebaceous gland, and in some areas an apocrine sweat gland. The skin appendages originate from the deep dermis or subcutaneous areas and traverse the skin layers to the outermost surface of the epidermis.

FIGURE 1. STRUCTURE OF SKIN

Schematic representation of the layers of the skin and skin appendages. (Modified from Wheater, Burkitt and Daniels 1979)


Epidermis
Dermal papillae
Dermis
Sebaceous gland
sweat gland
Hair follicle
Hypodermis

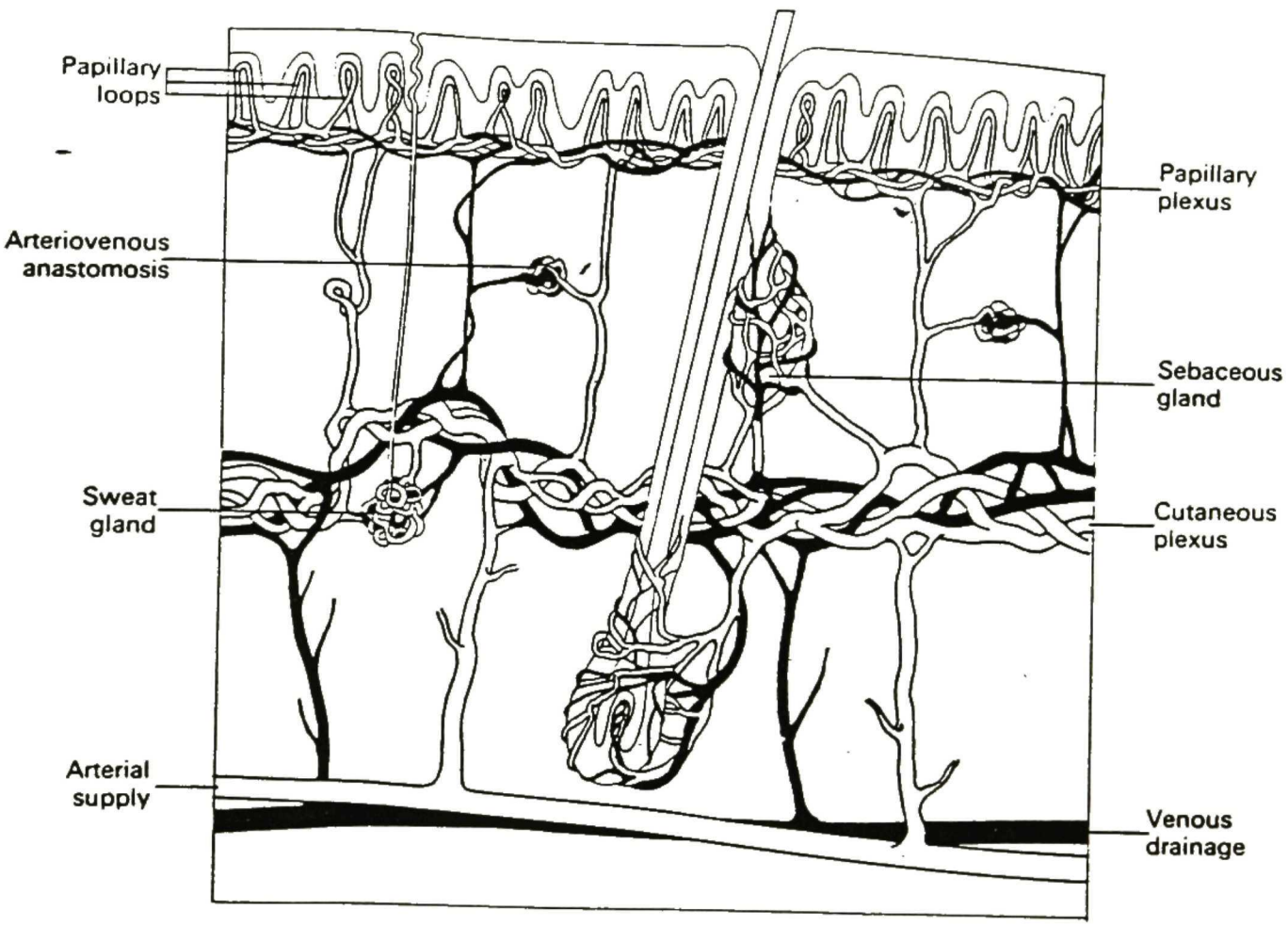
B. Blood flow

A schematic representation of the circulation within the skin is shown in figure 2. The blood flow to the skin far exceeds the metabolic demands of that tissue. This excess vascular capacity functions in thermal regulation. Capillaries carry substances to the outermost viable layer of the dermis and present them for potential transepidermal loss. In addition, these capillaries serve to carry away substances which have been percutaneously absorbed.

Shunting of blood flow to or from dermal capillaries occurs through large vascular communications directly between the arteries and the venous plexuses called arteriovenous anastomoses (AVA). In humans, AVA are mainly located on the surfaces of the hands and feet, the lips, the nose, and the ears, which are the body areas most often exposed to extreme cooling. Hales and coworkers (1978) indicated that the predominance of AVA in the rat are found in its tail and there are very few, if any found on the dorsum. Their studies measured the change in temperature in the rectum, body skin, left ear, right ear, mid-tail, and tip of the tail after warming the spinal cord to perturb the thermoregulatory reflex in the central nervous system. They observed a substantial increase in the temperature of both the mid-tail and tip of the tail but virtually no changes on the other sites and attributed the increase to sympathetic action on AVA's.

FIGURE 2. CIRCULATION IN THE SKIN

Schematic representation of the circulation within the skin. (Adapted from Wheater, Burkitt and Daniels 1979)


Papillary loops
Arteriovenous anastomosis
Sweat gland
Arterial supply
Papillary plexus
Sebaceous gland
Cutaneous plexus
Venous drainage

Blood flow through the skin is more variable than in other parts of the body. This high variability arises from the marked responses to the rate of internal, not local, metabolic activity and to the temperature of the surroundings. Within the capillaries, however, blood does not flow at a continuous rate but flows intermittently, characterized as a phenomenon called vasomotion. Vasomotion is the result of intermittent contraction of the metarterioles and precapillary sphincters. Even though blood flow through the capillaries is intermittent, flow through a tissue bed, such as skin, can be thought of as averaged blood flow because of the large number of capillaries present.

C. Variability of permeation

It is known that, at least in humans, the rate and extent of percutaneous absorption of compounds varies from anatomical site to site, with large variability between sites. Feldmann and Maibach (1967) measured the in vivo absorption of radiolabeled cortisol from eleven different skin sites using normal volunteers. They observed that the amount absorbed per unit area and time from the soles of the feet was decreased by a factor of seven when compared to the forearm and the amount absorbed through scrotal skin was increased by a factor of 40. They attributed some of this variation to stratum corneum thickness.

Bronaugh and coworkers (1983) showed that rat skin exhibits both site variability and a sex-related difference. They compared the permeability characteristics for several compounds through abdominal and dorsal skin from male and female Osborne-Mendel rats using in vitro diffusion cell techniques. In a short term (5 hr) experiment, they observed a tenfold increase in the percentage of applied dose traversing male abdominal skin as compared to male dorsal skin. Female abdominal skin also exhibited greater permeation than female dorsal skin, however, the increase was only slightly greater than twofold. They also measured rat skin thickness from frozen sections and associated the differences in observed permeability to the differences in stratum corneum and whole epidermis thickness. Male dorsal stratum corneum thickness was 35 microns whereas male abdominal stratum corneum measured only 14 microns. Female abdominal stratum corneum thickness was the same as that observed for males, however, the female dorsal stratum corneum was only 18 microns. Their observations for whole epidermis were similar. In contrast, Behl and coworkers (1984) determined that the in vitro permeability of full-thickness, male, hairless-mouse skin from dorsal and abdominal sites on the same animal were the same. However, the dorsal skin was less permeable than abdominal skin in experiments where the stratum corneum had been removed by stripping from the dorsal and abdominal skin samples. Recently, an additional demonstration of variability

in skin thickness was presented (Monteiro-Riviere et al. 1990). These workers also demonstrated an important influence of the method of preparation of the skin sample on those measurements. Using Sprague-Dawley rats (sex not specified), they determined the thickness of stratum corneum as about 5 microns from both the dorsal and abdominal sites using paraffin prepared sections. In contrast, their frozen section preparation results indicated the thickness of dorsal stratum corneum on the order of 12 microns as compared to abdominal stratum corneum of about 6 microns. The method of skin sample preparation (frozen vs. paraffin), resulted in a more than doubled measurement of the dorsal stratum corneum thickness.

The variability of observed skin permeation from different sites may not be solely a function of the number of cell layers or thickness of the stratum corneum. Quantitative analysis of human stratum corneum lipids has demonstrated variability in lipid composition at four sites with known differences in permeability, with the sphingolipid content exhibiting a direct relationship to permeability (Lampe et al. 1983).

The variation in skin permeability is evident in clinical medicine. Some manufacturers of transdermal nitroglycerin therapy have asserted that their optimally designed delivery systems can provide reliable, consistent delivery of medication, almost implying a zero-order input similar to an intravenous infusion. Curry and Aburawi (1985)

highlighted the problems which can be masked in relying upon only the mean data which was used to support the aforementioned assertions. They showed that after administration of 1, 2, or 4 doses of a prototype transdermal product, mean plasma nitroglycerin concentrations exhibited a profile similar to those obtained with an intravenous infusion. In another study using the same transdermal nitroglycerin delivery system, the individual plasma concentration curves for seven subjects revealed wildly fluctuating levels with several orders of magnitude between peaks and troughs. The extreme variability in plasma nitroglycerin concentrations can not be adequately explained by stratum corneum thickness and/or lipid composition, alone.

This variability of percutaneous nitroglycerin absorption may be due to variations in:

a. stratum corneum thickness and lipid composition
b. epidermis thickness and composition
c. the density of capillary loops
d. the depth of capillary penetration
e. the potential for capillary recruitment
f. the permeability of capillary wall
g. blood flow through capillary beds
h. the amount of arteriovenous shunting below dermal capillaries.

Loss of internalized xenobiotics or endogenous substances through the skin can be compared to the transfer

of substances from the circulatory system to the surrounding interstitium in any tissue or organ system. The rate of entry of a substance into the various tissues of the body depends upon the blood flow rate through the respective capillary beds and the permeability of the capillaries for the particular substance. There is wide variation of permeability characteristics of capillaries throughout the various organ systems in the body. However, permeability through the capillary wall is not assumed to be a predominant barrier for most substances except possibly in the brain. The rate at which the substance leaves the blood stream is dependent on its lipid solubility, molecular weight, molecular charge, and physical state of binding to macromolecules within the blood stream (Goldstein, Aronow and Kalman 1974).

3. Skin Permeability: Theory

A. Fick's law of diffusion

Passive diffusion is the principle mechanism for percutaneous movement for most compounds. However, the migration of substances through the skin is a complex process largely because of the structural diversity of the different layers. Schaefer and coworkers (1982) described a system of twelve functional compartments to model the path of a topically applied drug from the surface, through the skin, to

the blood system, and final excretion. The contribution of some compartments is minimal when compared to other compartments, enabling one to simplify the model to three main areas; the stratum corneum, the epidermis-dermis, and the blood (Schaefer, Zesch and Stuttgen 1982). The simplified model may be applicable to any tissue site in the body, where the capillaries deliver and remove substances.

The stratum corneum exhibits a reservoir property in addition to its barrier properties. The concept of the stratum corneum as a reservoir became evident from observations where the pharmacological effects of local steroid administration could be demonstrated in skin sites 15 days after steroid application (Vickers 1963). Hadgraft (1979) developed a theoretical approach to take advantage of this reservoir. He postulated that drugs could be tailored to either improve their storage in or to increase their rate of removal from the stratum corneum. Surber and coworkers (1990) took Hadgraft's theory to the laboratory and developed procedures to permit measurement of solute partition coefficients between the stratum corneum and a vehicle to enable improved predictions of percutaneous drug flux and rational optimization of topical formulations.

It is apparent that a substance dissolved in the stratum corneum matrix may remain there for days after topical application, until the normal process of shedding and cell renewal removes it or it diffuses inward. The sorptive

property, especially for water insoluble compounds, and lack of blood supply of the stratum corneum differentiates the skin from other tissues with respect to the amount of a substance which is accessible to the capillary, especially for percutaneous absorption. It is now widely recognized that the stratum corneum is a major rate-limiting barrier layer in the skin, as far as water-soluble compounds are concerned (Scheuplein and Bronaugh 1983).

Skin may be regarded as a laminate of uniform membranes, as a simplification, to enable measurement of constants that describe diffusion of substances through it (Schalla and Schaefer, 1987). Scheuplein and Blank (1971), in their discussion on the biophysics of skin permeability, described the application of the integrated form of Fick's diffusion law to the transfer of substances across the skin. As a first approximation, skin may be regarded as a composite membrane for the application of Fick's first law which may be expressed as:

$$J = (-D)(A)(dc/dl) \qquad \text{eq. (1)}$$

where: J = the net rate of diffusion (moles per unit of time)

A = the area of the plane of skin (cm^2)

dc/dl = the concentration gradient across the plane of skin ($moles/cm^3/cm$)

D = the diffusion coefficient (cm^2 per unit time).

The minus sign indicates that the direction of diffusion is down a concentration gradient. The diffusion coefficient may be thought of as proportional to the average speed with which a diffusing molecule can move in the surrounding medium. The Einstein relation,

$$\text{average displacement squared} = 2(D)(t) \qquad \text{eq. (2)}$$

where: t = the time elapsed since diffusion began (unit of time),

can be used to estimate the importance of distance in the diffusion process. The time required for a molecule with a D of 1×10^{-8} cm^2/second[1] would be 50 seconds for a distance of 10 microns (human stratum corneum thickness throughout most of the body) compared to over 20 hours for a distance of 400 microns (average stratum corneum thickness of palms and soles). Furthermore, the concentration within the membrane is dependent upon the hydrophobicity or lipophilicity of the compound for the membrane. This means that the concentration difference within the membrane is not the outside concentration on one side (C_1) minus the outside concentration on the other side (C_2) but is related by the partition coefficient, (K), which describes the preferential solubility

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[1] Throughout this dissertation, the dimensions of the permeability coefficient are expressed in the usual cm/hr. However, the dimensions of the diffusion coefficient are usually expressed in cm^2/sec.

of the compound for two different media (inside vs. the outside of the membrane). After rearrangement of equation (1) to include the partition coefficient between the membrane and the substance, the relationship can be stated as

$$J = (-D)(A)(K)(C_1-C_2)/(L_1-L_2) \qquad \text{eq. (3)}$$

where: K = the partition coefficient (dimensionless)

C_1 = the concentration on side one (moles/cm^3)

C_2 = the concentration on side two (moles/cm^3)

L_1-L_2 = the thickness of the membrane (cm)

and J, D and A are as defined for equation (1).

Additionally, since D, K, and (L_1-L_2) may be constants for a particular substance and a particular membrane, $(D)(K)/(L_1-L_2)$ may be expressed as a permeability coefficient (Kp). This allows for expression of the flux equation as

$$J = (Kp)(A)(C_1-C_2) \qquad \text{eq. (4)}$$

where: $Kp = (D)(K)/(L_1-L_2)$ (cm/sec)[2]

and J, A, C_1 and C_2 are as defined above.

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[2] In defining equation (4), the dimensions of Kp are in cm/sec, which is not the usual dimensions for expressing the permeability coefficient. As explained in footnote (1), Kp is usually expressed in cm/hr.

If everything which traversed the membrane was immediately removed or the concentration on the driving side was orders of magnitude higher than the receiving side, in effect setting C_2 to zero, then the total amount that has crossed through the membrane with respect to time may be obtained by solving equation (4). This makes possible the expression of an "effective" composite transcutaneous K_p (Kp_{eff}) as

$$Kp_{eff} = \frac{\text{amount transferred(0 to t)/A}}{\text{AUC}} \qquad \text{eq. (5)}$$

where: amount transferred(0 to t)/A = the total flux per unit area (moles/cm^2)

and AUC = the area under the concentration-time curve(0 to t) of side one of the membrane (moles-unit of time/cm^3).

The Kp_{eff} of a substance through skin can be viewed as a measure or index of transcutaneous permeation that is normalized for the skin surface area, A, and the diffusional driving force concentration, AUC. Utilization of equation (5) requires appreciation of several assumptions, including 1.) the skin is considered as a homogeneous layer, 2.) there is no interaction between the skin and the penetrating substance such as binding or metabolism, and 3.) the system is at steady-state.

B. Partitioning permeation into physiologic and anatomic components

Adamczyk and coworkers (1966) have demonstrated that the skin serves as an organ of clearance from the body, at least for gaseous compounds. The effective transcutaneous permeability coefficient multiplied by the unit area is a method for expressing clearance through skin. The dimensionality of this concept is in agreement with the traditional expression of clearance which is in volume per unit time.

White and coworkers (1983) have described the analogous variables and passive elements in different kinds of systems and expressed solute conductance as clearance. Application of the mathematics of electric circuits allows the expression of total clearance through a system of barriers in series as

$$\text{Clearance}(Cl)_{total} = 1/(1/Cl_1 + 1/Cl_2 + 1/Cl_n) \quad \text{eq. (6)}$$

The concept of serial additiveness of the different skin components has been described previously (Scheuplein and Blank 1971; Crank 1975). Scheuplein and Blank (1971) characterized permeability through skin as a diffusional resistance, where the reciprocal of the composite permeability coefficient is equal to the sum of the separate diffusional resistances of the stratum corneum, viable epidermis, and the papillary layer

of the dermis. They also considered capillary blood flow as a potential contributor to the overall apparent diffusional resistance of a substance traversing the skin and reaching the systemic circulation. However, they did point out that under normal blood flow conditions, the apparent diffusional resistance of perfusion was negligible compared to the diffusional resistance of the stratum corneum except possibly for permanent gases or small lipid-soluble molecules.

The apparent in vivo permeability of whole skin to chemicals migrating outwards from within the body is due to the relative contributions of capillary, epidermis-dermis, and stratum corneum permeabilities, and rate of capillary blood flow. The serial nature of these individual determinants of permeation leads to an expression of the effective composite transcutaneous permeability coefficient (Kp_{eff}) as

$$Kp_{eff} = \frac{1}{1/Kp_{sc} + 1/Kp_{ve} + 1/Kp_{cap} + 1/Q} \qquad \text{eq. (7)}$$

where: Kp_{sc} = the permeability coefficient of stratum corneum (cm/hr)

Kp_{ve} = the permeability coefficient of viable epidermis-dermis (cm/hr)

Kp_{cap} = the permeability coefficient of capillary wall (cm/hr)

Q = capillary blood flow ($cm^3/hr/cm^2$).

4. Existing methods for studying skin permeability and physiology

A. In vitro methods

The large body of experimental work which has contributed to our understanding of skin permeability has been performed in vitro, mainly focusing on inward diffusion. Most of this work has been carried out using diffusion cells, which are special glass chambers in which a sample of skin, varying from whole skin to separated skin layers such as stratum corneum, can be secured. The diffusion cell may be a two-chambered system where the identical solvent (usually water or saline) is used in the donor and receptor sides. However, for investigation of the influence of different solvents and to maintain the surface of the skin at ambient conditions, the one-chambered diffusion cell is more commonly chosen. The receptor chamber may be static as described by Franz (1975) or it may be a flow-through system as developed by Bronaugh (1985). The flow-through systems offer several advantages, including enhanced removal of diffused substances via continuously renewed receptor fluid, and more physiological provision of nutrients and removal of waste products in viable skin preparations. In both systems, a compound of interest is placed on the donor side and is collected on the opposite or receptor side.

There are several challenges to studying and interpreting skin permeation in an in vitro system, including difficulty controlling variable thickness of the skin sample used, influence of method of preparation of the skin sample prior to use, and accounting for the absence of influence of blood flow on the permeation characteristics of the compound under investigation. Nevertheless, the information gained from the in vitro work has yielded many insights into the inert membrane characteristics of skin. Franz (1975) attempted to validate the use of the in vitro model system by comparing his in vitro (human skin samples) derived permeation data for twelve identical organic compounds studied in vivo by Feldmann and Maibach (1970) using urinary collections of topically applied radiolabeled compounds. Franz was unable to find quantitative agreement between the in vitro and in vivo data. However, he showed a significant, $p < 0.01$, rank correlation between the two sets of data. Bronaugh (1982) also investigated the in vivo/in vitro comparative permeation of three of the compounds (benzoic acid, acetylsalicylic acid and urea) studied by Franz; however, his experiments were conducted using female Osborne-Mendel rats. Bronaugh was able to demonstrate good qualitative and quantitative agreement in rat skin permeability between the in vitro and in vivo methods.

Nevertheless, controversy exists whether the in vitro methods are reliable and accurate predictors of percutaneous

permeability in humans. Kligman (1983) proposed that the in vitro data are more credible based on technical grounds alone and he considered in vivo data to be suspect when there was disagreement with in vitro data. On the contrary, Wester and Maibach (1977) were of the opinion that in vivo determinations must be conducted as long as in vivo/in vitro discrepancies appear.

An alternative in vitro model, the isolated perfused porcine skin flap (IPPSF), for studying percutaneous absorption and cutaneous toxicology was described by Riviere and coworkers (1986). The IPPSF was created from the abdominal skin of weanling Yorkshire pigs and harvested along with its main artery and vein. The viable skin preparation could be maintained for 10-12 hours as an isolated organ system using a computer-controlled, temperature-regulated perfusion chamber (perfusion media consisted of a Krebs-Ringer bicarbonate buffer, glucose and bovine serum albumin). During percutaneous absorption experiments (Williams and Riviere 1989; Carver, Williams and Riviere 1989) the afferent arterial and efferent venous perfusate concentrations were analyzed and the absorption rate was calculated. Using a three-compartment pharmacokinetic model to simulate mass transfer from the surface to capillary perfusate in the IPPSF, they were able to demonstrate excellent correlation (R^2 = 0.88) between in vivo percutaneous absorption, obtained from 6-day excretion studies in pigs, and in vitro estimates of bioavailability.

B. In vivo methods

i. Inward permeation systems

One of the earliest methods of studying percutaneous absorption in vivo was described by Feldmann and Maibach (1974). They investigated skin absorption of 12 radiolabeled pesticides and herbicides applied to the ventral forearm of humans. Urine was collected for five days after skin application of the pesticides. The urinary recovery data was corrected for incomplete excretion with data obtained after intravenous dosing with the compounds. Their urinary recovery method showed that not only were these pesticides and herbicides absorbed into the skin, but penetrated through it and were carried away by the systemic circulation to other parts of the body. Disadvantages of this method include the necessity for ancillary intravenous dosing data to correct for incomplete urinary excretion, and possible differential metabolism resulting from various routes of administration.

Wester and Noonan (1978) compared the topical bioavailability of a potential anti-acne agent by two methods: 1.) cumulative excretion in urine and feces and 2.) the area under the plasma concentration time curve (AUC). The percentage of the dose absorbed through the skin was calculated as the ratio of AUC's following topical and intravenous administration. They showed that the AUC method

produced results similar to the cumulative excretion method for measuring percutaneous absorption. The AUC method later was used to study the bioavailability of topical application of nitroglycerin in Rhesus monkeys (Wester et al. 1983). The investigators administered radiolabeled drug and measured plasma nitroglycerin levels, total plasma radioactivity and total urinary radioactivity. Bioavailability by the topical route agreed with plasma AUC (total carbon-14) and total urinary excretion of the radioactive label. However, the bioavailability was about 20% greater then the estimate derived from plasma nitroglycerin data, a difference which the investigators postulated was due to a percutaneous first-pass metabolic effect.

An inconvenience with both the cumulative excretion method and the plasma AUC method is the necessity to administer an intravenous dose to calculate absolute bioavailability. In addition, the very low plasma concentrations of drug resulting from low topical bioavailability and dilution/excretion effects usually requires the use of radiolabeled compounds.

Rougier and coworkers (1983) described an in vivo method for studying percutaneous absorption by using the stratum corneum reservoir property. In their studies, which employed substances varying widely in structure and physicochemical properties, a linear relationship was demonstrated between total penetration at the end of 96 hours

and the amount within the stratum corneum at the end of a 30 minute application time. Their claim of noninvasiveness of their method may be argued by some, since evaluation of amount in the stratum corneum requires removal of this layer by tape stripping.

Reifenrath and coworkers (1984) evaluated different animal skin samples for comparability in permeability to that of man using skin grafting techniques. However, their assessment of permeability was performed essentially by the same methods as described previously, i.e., the recovery of radioactive excreta after separate topical and intravenous dosing.

A novel in vivo method for studying dynamic skin permeation was developed by Wojciechowski and coworkers (1987). Their model consists of a skin sandwich which is generated as a flap by grafting a split-thickness skin graft to the subcutaneous surface of the epigastric skin on the abdomen of athymic (nude) rats. The island skin flap is a piece of living skin isolated and maintained by an independent and defined blood supply. The uniqueness of this system is that local transdermal drug absorption can be unambiguously investigated before the drug enters the general systemic circulation.

In vivo methodology for studying the percutaneous penetration of toxic gases, excluding the inhalational route, was investigated by McDougal and coworkers (1985). They

devised a dermal vapor exposure system whereby rats breathed purified air while inside a chamber which contained chemicals in the vapor phase. Serial blood sampling enabled them to generate a blood concentration/time profile which was then used to calculate the flux through the skin and an effective composite permeability coefficient. They investigated several compounds, varying in molecular weight and partition coefficients, including isoflurane, the probe compound used in this project (McDougal et al. 1990).

Pharmacodynamic methods have been utilized to assess skin penetration of chemicals. These methods depend upon the ability of the topically applied chemical to reach a region within the skin to elicit a response which may be measured or observed. Stoughton and coworkers (1960) measured the vasodilation produced by the topical application of nicotinic acid and other nicotinates and assigned a "penetration index" as a basis of comparing the activity of the compounds. McKenzie and Stoughton (1962) used apparent vasoconstriction ("blanching" of skin color) as an index of absorption of various steroids. Guy and coworkers (1983) using laser Doppler velocimetry (LDV)[3] to measure the change in cutaneous blood

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[3] Laser Doppler velocimetry (LDV) and laser Doppler flowmetry (LDF) are used interchangeably in the literature to describe the measurement of blood flow with the laser Doppler instrument. This interchangeability may not be correct, since the instruments vary in signal-to-noise ratio and output parameters such as velocity, volume, and total blood flow. I could not find any reports comparing the several available LDV/LDF instruments. Throughout this dissertation, when the work of other investigators is cited, the terminology which

flow, demonstrated the dose-response of different concentrations of methyl nicotinate. The penetration of several concentrations of minoxidil solutions through scalp skin was studied by Wester and coworkers (1984). Using LDV to measure the change in blood flow, their data suggested a dose-response relationship. Ryatt and coworkers (1986), using LDV measurements, studied the accelerant properties of two agents (2-pyrrolidone and laurocapram) to increase the rate and extent of percutaneous penetration of hexyl nicotinate in human subjects. The local pharmacodynamic response (vasodilation) measurements demonstrated faster and increased absorption of hexyl nicotinate with the two agents as compared to vehicle alone.

ii. Outward permeation systems

Studies of the efflux of gases from the body has spawned several methods (Adamczyk, Boerboom and Kistemaker 1966; Hansen, Sonoda and McIlroy 1980; Jacobsen and Gothgen 1985; Delpy and Parker 1980; Littlejohn 1972; Stoelting and Eger 1969) for collection and quantification. The monitoring of transcutaneous oxygen and carbon dioxide to replace or augment invasive blood level measurements of those gases, was

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they used is reported. Laser Doppler measurements conducted as part of this project are referred to as laser Doppler flowmetry and are used to represent a "flow parameter".

the intent of most of the earlier work. Some of the collection devices rely on the inclusion of a heating element in the collection probe to heat the collection site skin surface in order to "arterialize" capillary blood flow and to maximize the delivery of gas to the skin. All of the aforementioned complex methodologies employ techniques for "sweeping away" the permeating gas or vapor by flushing the collecting chamber with an inert gas. In contrast, the simple and convenient transdermal vapor collection device (TVCD), which will be more fully described below, has no flow-through gases, no heating element, and creates a sink condition for the collection of permeating vapors.

Peck (1987) had originally invented a Dermal Substance Collection Device (DSCD) which had three essential components: (1) a substance binding reservoir, (2) a liquid transfer medium and (3) an occlusive cover. The DSCD, a band-aid like device, which when affixed to the epidermal surface, provides a conduit for migration of chemicals which are resident in the stratum corneum into the binding reservoir matrix. A more recent invention by Peck (1990), the Transdermal Vapor Collection Method and Apparatus, was specifically designed for substances not requiring a liquid medium conduit, i.e., vapors, between the skin surface and the device. The device has a large capacity for the substance collected but also has sufficiently low affinity to permit complete quantitative extraction. The active component of the vapor collection

device is dry activated carbon which is uniformly dispersed in a substrate of polytetrafluoroethylene (PTFE), from which it cannot be dislodged, and compressed into thin sheets. The activated carbon provides an extensive surface area for noncovalent binding of substances and serves as a binding reservoir, preventing the escape of collected vapor to the environment or migration back into the skin.

C. Selection of an animal model

There is as yet no single, generally accepted technique for determining the percutaneous penetration of xenobiotics. The foremost controversy is whether to use in vivo or in vitro models. Accepting the fact that, at least for certain substances, permeation through skin may be impacted by more than just the physical barrier properties of the stratum corneum, an in vivo model is a necessity. Wester and Maibach (1983) reviewed the various animal models used in percutaneous absorption studies and in view of the uncertain extrapolation of animal data to humans, advocated the use of humans whenever feasible. However, for understanding the contributions of skin physiology to permeation, the use of an animal model is practical.

The fuzzy rat was selected as the animal model for this research, predominantly because its skin is devoid of any fur and could be utilized for permeation studies with minimal

or no preparation such as clipping, shaving, or depilation. Bronaugh and Stewart (1986), using in vitro methodology, compared the permeability of fuzzy rat skin with the permeability of human and hairless mouse skin. Human skin was a better barrier than rodent skin except for water. Their observed permeability constant of water (0.0011 ± 0.0001 cm/hr, mean ± SEM) was identical for 200 micron dermatomed sections of human and fuzzy rat skin. With every compound they investigated, except for a fragrance ingredient (Compound 2), fuzzy rat skin was closer in permeability to human skin than was the skin of the hairless mouse. Furthermore, these rats were available to our laboratory at minimal expense and were utilized for investigating the permeability across skin of other compounds.

D. Skin blood flow

The measurement of blood flow at the capillary level is essential for evaluating the effect of skin blood flow on the transport of xenobiotics across the skin. The ideal method would be one that provided a continuous reading of the blood flow in a small, localized region and would not impart any changes of its own on the circulation by disturbing the normal state. The methods available include washout of radioactive indicators, thermal uptake with heated probes, various forms of plethysmography, implanted hydrogen

electrodes or radiation detectors, and trapping of labeled microspheres. The aforementioned methods have differing assets and limitations, with the main asset being the expression of blood flow in absolute values and the main limitation being their invasiveness.

Laser Doppler velocimetry (LDV), a recently introduced non-invasive technique for assessing microcirculation (Stern 1975), is the only method which shows the dynamic flow variations of blood in the tiny capillaries; however, the quantification is in relative terms, usually expressed in millivolts or arbitrary units. The LDV "reflects" blood flow using the Doppler principle applied to light energy (photons) in the same manner as sound energy is Doppler shifted. Light at 632.8 nm from a 2 mW helium-neon laser source is transmitted to a volume of skin through a quartz optical fiber. The light energy (photons) are back-scattered unshifted from stationary skin components and with shifted frequencies when reflected from erythrocytes moving in the blood vessels (Engelhart and Kristensen 1983). A second optical fiber collects the reflected light and a microprocessor converts the Doppler-shifted light to velocity and volume (vascular) parameters which multiplied give a flow parameter. The probe containing the incident light source and the collecting optical fiber is protected from natural lighting interference by the use of a light filter. The measurement area or depth is reported to be a hemisphere with a radius of about 1000

microns (Tenland 1982), however some investigators (Harry and Kenny 1984) have estimated a maximum penetration of up to 1500 microns. With the use of a 0.8 mm diameter probe, the surface area that is monitored is approximately 1 square mm. The output from the instrument, i.e. a flow parameter, can be defined as the product of the number of red blood cells and their mean velocity in the measuring volume of approximately one cubic mm. If capillary density is variable over millimeter distances, then such variations may change the volume component of blood flow and modify the measured parameter. The flow value that is measured by the LDV may also be influenced to some extent by factors such as pigmentation, hemoglobin content, vascular bed geometry, and epidermal thickness.

The measurement of blood flow by LDV has been linearly correlated with traditional methods and adequately validated in humans and animals by comparison with ^{133}Xe washout scintigraphy (Holloway and Watkins 1977; Engelhart and Kristensen 1983; Neufeld et al. 1988), standard plethysmographic techniques (Sundberg and Castren 1986; Johnson et al. 1984), hydrogen gas clearance technique (Kvietys, Shepherd and Granger 1985), [^{14}C]iodoantipyrine technique (Rundquist et al. 1985), radiolabeled-microsphere uptake (Kvietys, Shepherd and Granger 1985; Smits, Roman and Lombard 1986) and electromagnetic blood flow meter (Smits, Roman and Lombard 1986; Pershing et al. 1989).

Holloway and Watkins (1977) measured blood flow in the

forearm skin of normal subjects both by ^{133}xenon clearance and laser Doppler techniques. LDV measurements were made before and after xenon clearance flow measurements at the same site. Over a wide range (approximately 10 to 70 ml/min/100 gms) of blood flow values measured by xenon clearance, a good correlation ($R = 0.89$, $p < 0.001$) to laser Doppler measurements was observed.

Johnson and coworkers (1984) measured forearm blood flow in normal men by venous occlusion plethysmography and LDV using a variety of protocols. They observed correlation coefficients greater than 0.94 in all of their studies comparing LDV and plethysmographically measured blood flow.

Pershing and coworkers (1989) compared electromagnetic blood flow meter measurements to LDV in the skin sandwich flap model of Krueger and coworkers (1985). They calculated a correlation coefficient (R) = 0.96 over the range of blood flow values from 0 to 2.6 ml/min in the vessel directly supplying the skin sandwich flap.

The use of radioactive microspheres to study blood flow in various organs and tissue beds has become the most widely accepted method of many circulatory physiologists (Hales 1974; O'Neill, Haddy and Grega 1982). Kvietys and coworkers (1985) compared LDV and microsphere measured blood flow in the isolated cat jejunum. They observed a significant linear correlation ($R = 0.82$, $p < 0.0001$) between the two methods. Smits and coworkers (1986), in addition to comparison

with an electromagnetic flowmeter, also compared LDV to microsphere measured blood flow. They observed good correlations (R = 0.95 and 0.91) in the slower flow cremaster muscle and higher flow renal cortex. They alluded to limitation of the microsphere technique for measuring blood flow in regions as discrete as those sampled by LDV and the uneven distribution of microspheres in the renal cortex leading to overestimates of blood flow in some parts of the kidney and underestimates in others.

Laser Doppler flowmetry (LDF) was selected as the most appropriate methodology for the assessment of changes in cutaneous blood flow in this project. Its main advantages are simplicity of use, noninvasiveness, and reliability in detecting increases or decreases in flow rates. It is much more difficult to use for the absolute measurement of blood flow. LDF is a versatile technique for studying dynamic variations in microvascular blood flow during basal conditions or after various pharmacologic and physical provocations.

5. ISOFLURANE AS A MODEL COMPOUND FOR STUDYING SKIN PERMEATION

A. Physicochemical features of isoflurane

Most substances permeate skin as passive diffusion process (Scheuplein 1976). The three physical parameters which primarily reflect the rate of skin permeation are the

diffusion coefficient, D, the partition coefficient, P, and the thickness of the skin (Scheuplein 1978). The mathematical expression of D (cm^2/sec) can be thought of as a measure of the speed with which a diffusing molecule can move in the surrounding medium. The resistance to diffusion can be thought of as a frictional force. A physicochemical model of molecular diffusion derived by Stokes postulates that diffusional resistance is related to the spherical radius of the diffusing molecule (Barrow 1966). Since molecular radius is approximately proportional to the cube root of molecular weight, ordinarily, small molecular weight substances diffuse faster than those with large molecular weights. However, for chemicals of "low" mass (< 1000 daltons), molecular size is not the predominant factor for the penetrating ability of a substance through a membrane; the relative solubility of the diffusing chemical in lipid rich and hydrous regions of skin greatly affects its permeation. The high correlation between the oil(lipid)/water partition coefficient and the cutaneous permeability constant of a substance has not only supported this theory but has enabled predictable permeability from partition coefficient data (Collander 1937).

Isoflurane (1-chloro-2,2,2-trifluorethyl difluoromethyl ether) was selected as the model compound for the experiments which are the subject of this dissertation because it possesses many of the desirable features of a rapidly permeating compound. Isoflurane is one of a group of

volatile inhalational anesthetic agents that are halogenated ethers. The physical properties of isoflurane approach those of a suitable anesthetic agent, i.e., nonflammable and stable. Its physicochemical properties (Eger 1984) made it an ideal compound for use as a chemical marker for examining skin permeability. The molecular weight of isoflurane is low (184.5 daltons) and it boils at 48.5° C at 760 mm Hg. The water/gas, blood/gas and olive oil/gas partition coefficients, at 37° C, of isoflurane are 0.61, 1.43, and 90.8, respectively. The high fat soluble nature of isoflurane made it an ideal probe to test the rate limiting barrier hypothesis of the lipid-rich stratum corneum.

At room temperature, isoflurane is liquid but has a vapor pressure of about 250 mm Hg (water has a vapor pressure of about 17.5 mm Hg). To produce surgical anesthesia, it is traditionally administered through the use of precision vaporizers, specifically calibrated for isoflurane, from which flow delivered to the patient can be calculated to assure that safe concentrations are being delivered.

B. Clinical pharmacology of isoflurane

The pharmacology of isoflurane has been described in detail (Eger 1984), however, as with other molecular species capable of producing anesthesia, isoflurane's mechanism of action remains to be explained (Smith and Wollman 1985). The

minimum alveolar concentration (MAC) of isoflurane which produces anesthesia in man is about 1.2% in oxygen. In addition to its anesthetic properties, it has pharmacologic actions on the circulatory, respiratory and nervous systems. Stevens and coworkers (1971) investigated the cardiovascular effects of isoflurane and observed significantly less effect on the total circulation than other similar agents. However, they described a marked increase in skin blood flow which precluded significance due to variation in values. Seyde and Longnecker (1984), using microsphere techniques to study regional hemodynamics, did not observe a significant difference in cutaneous blood flow in non-anesthetized rats and those receiving isoflurane.

In man, isoflurane is virtually unmetabolized when compared to other similar agents, as only 0.2% of the administered dose appeared as urinary metabolites, while 95% of the dose administered was accounted for in exhaled air (Holaday et al. 1975). Hitt and coworkers (1974) observed approximately the same very low rate of isoflurane metabolite formation in Fischer-344 rats as in man. Isoflurane's resistance to biotransformation, in addition to its physicochemical characteristics described previously, were the influential factors in selecting it as the probe compound. The absence of metabolites simplified the assay and pharmacokinetic analysis.

The pharmacokinetics of isoflurane in man and pig,

after administration by inhalation, has been extensively studied (Carpenter et al. 1986; Yasuda et al. 1990; Yasuda et al. 1991a; and Yasuda et al. 1991b). The inhaled and exhaled isoflurane concentrations (analysis of vapor) were used in the aforementioned pharmacokinetic studies, in contrast with traditional pharmacokinetic methodology, i.e., measurement of drug concentrations over time in plasma/blood. The pharmacokinetic parameters were derived from the isoflurane "washin" (uptake) and "washout" (elimination) profiles which were generated from the ratio of alveolar isoflurane concentration to inspired isoflurane concentration over time curve and the ratio of the alveolar isoflurane concentration to the last alveolar isoflurane concentration during administration over time curve, respectively. The isoflurane uptake and elimination data fit multiexponential (multicompartment) disposition functions and a five-compartment mammillary (parallel rather than serially connected compartments) model was derived to reflect known physiologic compartments such as the lung, vessel-rich group, muscle group, an undefined fourth compartment, and fat group. The pharmacokinetic data in the pig was qualitatively similar to human data.

C. Method for administering isoflurane

Isoflurane, the selected probe compound, needed to be

administered in a highly controlled and convenient fashion. Although isoflurane is always given to humans by inhalation, this appeared to be problematic in rats due to the requirement for tracheal intubation and complete control of respiration. Moreover, the highly quantitative nature of the experiments to be detailed later required a refinement of the methodology used by the anesthesiologists to calculate the dose delivered and the body burden of an anesthetic. Yasuda and coworkers (1991b) have questioned the accuracy of the method they used for pharmacokinetic analysis which were based on the underlying assumption that the end-tidal respiratory concentrations accurately indicate the anesthetic partial pressures in arterial blood. They pointed out, that generally, there appeared to be good correlation between the two, however, the differences were larger during periods of initial administration and elimination. Furthermore, since my laboratory was not equipped to administer and monitor isoflurane by inhalation, an intravenous dosage form was desirable. Hence, the intravenous route of administration was considered to be an attractive alternative to the inhalation route.

Intravenous (IV) administration of volatile anesthetics had been previously reported. Eger and coworkers (1962) dissolved ether in saline and administered it intravenously. Krantz and coworkers (1962) developed an intravenous emulsion for delivering methoxyflurane

intravenously which was used on 6 patients requiring surgery with no apparent untoward effects. However, Cascorbi and others (1968), cautioned against the expanded use of methoxyflurane emulsion in man because thrombophlebitis occurred at the injection site. Intravenous infusion of halothane dissolved in Intralipid™ in dogs was described by Biber and coworkers (1984) and a favorable comparison to dosing via inhalation was demonstrated. Intravenous fat emulsion has been used as the vehicle for IV dosing of poorly aqueous soluble agents such as hexamethylmelamine (Wickes and Howell 1985; Ames and Kovach 1982) and diazepam (von Dardel et al. 1983). Wickes and Howell (1985) asserted that the sparingly aqueous soluble hexamethylmelamine was suspended in the fat emulsion, however, Ames and Kovach (1982) and von Dardel and coworkers (1983) believed that the drug is dissolved in the oleaginous phase of the emulsion.

The need for parenteral alimentation for patients led to the development of the intravenous therapeutic modality to reverse the clinical manifestations of Essential Fatty Acid Deficiency (EFAD). Additionally, the high caloric content of fat emulsions makes them an excellent nutritional replacement for patients who are metabolically stressed but need restricted fluid intake. Several pharmaceutical manufacturers provide a sterile, nonpyrogenic fat emulsion for intravenous administration (U.S. Department of Health and Human Services 1990). The fat source in the preparations is safflower and/or

soybean oil, which are mixtures of neutral triglycerides with the following major fatty acids; linoleic, oleic, palmitic, stearic, and linolenic. The emulsified fat particles radii of approximately 0.2 micron is similar to naturally occurring chylomicrons. The infused fat particles are thought to be cleared from the blood similar to the clearance of chylomicrons (Carlson and Hallberg 1963).

Straathof and coworkers (1984) observed a prolongation of the half-life of the lipophilic drug, phenytoin, in rats given a 45 hour infusion of fat emulsion seven hours after an intraperitoneal dose of phenytoin as compared to a 45 hour infusion of saline. In contrast, von Dardel and coworkers (1983) did not find a significant difference in the pharmacokinetics of diazepam in humans between the traditional dosage form and diazepam dissolved in fat emulsion after intravenous administration.

6. Selection of pharmacologic and physical modulators

A. Vasodilator modulator

The intention of these studies was to induce localized perturbations of skin physiology and structure in the region of the small, well defined skin area where isoflurane was collected. To preclude the variability which could be introduced by topical application, the pharmacologic

modulators were injected intradermally. Minoxidil was selected as the vasodilating modulator after review of the pharmacologic agents which produce vasodilation independent of inhibitory effects within the adrenergic nervous system. A convincing consideration for selecting minoxidil was based on reports of topical application of minoxidil producing a marked increase in cutaneous blood flow (Wester et al. 1984; Hirkaler and Rosenberger 1989). Hirkaler and Rosenberger (1989), using simultaneous laser Doppler velocimetry, assessed the change in cutaneous blood flow produced by topical minoxidil in Sprague-Dawley rats. They observed increased blood flow (up to 50% greater than vehicle) for all minoxidil concentrations (0.015, 0.15, 0.375 and 0.75 mg/kg) evaluated.

Initially, a 1% minoxidil solution (0.5 mg/50 μl) was considered to be an optimal concentration to produce vasodilation since minoxidil topical solution (2%) is used as a therapeutic modality to treat human male pattern baldness (alopecia androgenetica) of the vertex of the scalp with a recommended dose of one ml to be applied twice daily. However, in a pilot study (N = 2) using the 1% minoxidil solution, both rats died approximately 30 minutes after intradermal administration of the minoxidil. Therefore, a 0.33% minoxidil solution was used in subsequent experiments.

B. Vasoconstrictor modulator

Phenylephrine (5 μg/50 μl) was selected as the modulator for producing vasoconstriction, mainly because of its almost pure alpha activity (Berthelsen and Pettinger 1977) and the reported evidence of activity when administered by a variety of routes. The cutaneous vasoconstrictor response was observed after intravenous (Willete, Hieble and Sauermelch 1991), subcutaneous (Millay, Larrabee and Carpenter 1991), topical (Harpin and Rutter 1983), iontophoretic (Krueger et al. 1985; Lindblad, Ekenvall and Etzell 1989; Hornqvist, Back and Henriksson 1984; Ekenvall et al. 1988), and intradermal (Morley, Page and Paul 1983) administration. In addition, its pharmacologic action on skin blood flow has been quantified using laser Doppler methodology (Krueger et al. 1985).

C. Capillary permeability enhancing modulator

A variety of autocoids given intradermally will increase the permeability of capillaries and venules. It is now generally accepted that the slow reacting substance of anaphylaxis (SRS-A) plays an important role in allergic reactions. SRS-A is thought to be a mixture of leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and possibly other closely-related leukotriene derivatives (Inagaki et al. 1986). LTD_4 (200 ng/50 μl) was selected as the modulator to enhance

capillary permeability based on published reports of its effectiveness (Rinkema, Bemis and Fleisch 1984; Ueno et al. 1981; Peck, Piper and Williams 1981).

D. Stratum corneum disrupting techniques

i. Chemical

Substances that enhance the permeability of the stratum corneum may be called accelerants, sorption promoters, or penetration enhancers. The most widely used penetration enhancers are the aprotic solvents such as dimethyl sulfoxide (DMSO), dimethylformamide, and dimethylacetamide. When applied to the external surface, solvents partition into and interact with the stratum corneum, thereby decreasing the barrier to drug diffusion. The solvents may also modify the vehicle-to-skin drug partition coefficient.

The focus of the series of experiments examining stratum corneum function necessitated neutralization or disruption of its external physical barrier properties without perturbing underlying physiology (especially blood flow and capillary permeability). The aprotic solvents do little permanent damage to the stratum corneum whereas low molecular weight and more volatile organic solvents are usually harmful to the skin barrier. Mixed solvents, having both polar and non-polar characteristics, are the most deleterious because

they remove large quantities of lipid material (Scheuplein and Bronaugh 1983). A mixture of ether and ethanol in a ratio of 10 to 1 was selected as the chemical modulator to disrupt the stratum corneum based on the report of Scheuplein and Bronaugh (1983).

ii. Mechanical

Tape-stripping of the skin has been re-discovered as a well characterized method for investigating skin permeability excluding the barrier function of the stratum corneum (Scott and Dugard 1986; Bronaugh and Stewart 1985). Tape-stripping was selected as the methodology for mechanical stratum corneum disruption because of its simplicity and generally recognized acceptance as an effective methodology for removing that barrier.

SPECIFIC AIMS

The specific aims of this research were to develop and to study an in vivo skin permeation model that would be sensitive to perturbations of skin capillary physiology and stratum corneum. The specific goals to be achieved in evolution of the model were the following:

1. develop a quantitative delivery and assay system for the probe compound, isoflurane,

2. establish an in vivo model to measure transdermal flux,

and

3. examine the ability of the model to measure the effect of physicochemical perturbations on Kp_{eff} such as blood flow, capillary permeability, and stratum corneum changes.

MATERIALS AND METHODS

1. Isoflurane assay

All chemicals used were reagent grade unless otherwise noted. Concentrations of isoflurane in hexanes[4] intravenous fat emulsion, blood, and the amount of isoflurane collected by the TVCD were analyzed using gas-liquid chromatography. Hexanes (pesticide grade from Fisher Scientific, Silver Spring, MD) was the diluent used for all isoflurane analyses. The analyses were performed on a Perkin-Elmer (Rockville, MD) model Sigma 2B, gas chromatograph (GC) equipped with an electron capture (Ni^{63}) detector (ECD). The chromatographic separation of hexanes-isoflurane mixture was attained on a 6-ft x 1/8 in-i.d. stainless steel column packed with 0.1% SP-1000 fluid impregnated on 80-100 mesh Carbopack C (Supelco). The temperatures of the injector, column and detector were maintained at 200, 125, and 300° C, respectively. The carrier gas was ultra-high purity grade nitrogen (Air Products & Chemicals, Inc., Allentown, PA)

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[4] Pesticide grade hexanes was used as the diluent and extracting solvent for this project. This reagent is generally a mixture of several isomers of hexane (C_6H_{14}), predominantly n-hexane and methylcyclopentane (C_6H_{12}). It is specifically recommended as the reagent of choice for GC/ECD analysis of pesticides because it has virtually no interfering substances. Throughout this dissertation, the term "hexanes" will be used to indicate the solvent.

flowing at 50 ml/min. A Perkin-Elmer AS-300 auto-sampler was used for injection of 1 μl aliquots. The settings on the auto-sampler were as follows; nitrogen at 8 psi, compressed air at 46 psi, flush time of 20 seconds, injection time of 2 seconds, and 2 injections per sample vial. The output from the detector was channeled to a Perkin-Elmer Sigma 15 chromatography data station and the signal was expressed as peak area (counts).

A calibration curve was prepared by the addition of liquid isoflurane (Forane™, Anaquest, Madison, WI) to hexanes to make six standards (solutions A to F) ranging in concentration from 299.2 pcg/μl to 29.92 ng/μl. In addition, low concentration (448.8 pcg/μl) and high concentration (14.96 ng/μl) dilutions were prepared which were used as quality control checks of the standard curve. Calibration curve and quality control solutions were prepared fresh weekly prior to analysis of samples and stored at -20° C[(5)]. The dilutions were made using accurate volumetric glassware and solutions A to F had concentrations of 29920, 14960, 7480, 1496, 598.4, and 299.2 pcg/μl, respectively. Method validation and quality control (QC) during application of the assay method relied on the replicate analysis of QC samples which were randomly interspersed among samples from experiments.

[(5)] Storing isoflurane dissolved in hexanes in gas tight containers at -20° C was felt to be satisfactory to prevent loss due to vaporization. At that temperature, the vapor pressure of isoflurane is only 18.4 mm Hg, which is very similar to the vapor pressure of water (17.5 mm Hg) at room temperature.

A. Procedure for assaying isoflurane in blood

Blood samples were analyzed by a modification of the method of Miller and Gandolfi (1979). 1.5 ml of hexanes was accurately measured into 2 ml septum vials and weighed on an analytical balance (Mettler Instrument Corporation, Highstown, NJ). Blood samples were quickly added to the vials containing the hexanes and re-weighed to determine the weight and volume of the blood sample. The vials were placed on a rotator (Scientific Industries Inc., Bohemia, NY) for at least one hour after which approximately one ml of the hexanes layer was removed and placed in auto-sampler vials for loading in the auto-sampler and GC analysis. The concentrations in blood samples, expressed as μg/ml, were calculated from the calibration curve and normalized for the volume of blood using the specific gravity (=1.0535) of rat blood (Ringler and Dabich 1979).

The usual method for examining the extraction efficiency of a solvent is performed by spiking drug-free biological fluid with varying known concentrations of the drug and assaying for the amount recoverable. The difficulty of handling neat isoflurane precluded the spiking of blood samples. Therefore, the following reverse extraction procedure experiment was conducted to measure the extractibility of isoflurane into blood containing fat emulsion. A fuzzy rat was infused with fat emulsion (isoflurane-free) at a rate of

0.0125 ml/min through the external jugular vein. Blood samples (0.26 to 0.51 ml) were removed through the femoral artery at 2.5, 5, 15, 30, 60, 90, 92.5, 95, 100, 110, 120, and 130 minutes after the start of the infusion. The blood samples were added to pre-weighed vials containing 1.5 ml of the calibration standard curve solutions and processed as detailed above. To mimic experimental conditions as closely as possible, the blood samples were added to pre-selected concentrations as follows: 2.5 and 130 min samples to solution F (description of solution concentrations is above), 5 and 120 min samples to solution E, 15 and 110 min samples to solution D, 30 and 100 min samples to solution C, 60 and 95 min samples to solution B, and 90 and 92.5 min samples to solution A. 1.5 ml of the calibration standard curve solutions in identical vials served as control. The calculated concentrations from samples with blood were compared with samples with the control.

B. Procedure for assaying isoflurane in fat emulsion

Intravenous fat emulsion containing isoflurane was analyzed for isoflurane content by extraction with hexanes. Twenty μl of the emulsion was added to 4.0 ml of hexanes, vortexed for 60 seconds, then placed on rotator for at least one hour. 100.0 μl was removed and added to a new vial containing 4.0 ml of hexanes. The vial was vortexed for 60

seconds and approximately one ml was placed in an auto-sampler vial for analysis. The concentration of isoflurane in fat emulsion was calculated from the standard curve with appropriate corrections for the dilutions.

C. Characterization of isoflurane in TVCD

The adsorption and desorption characteristics of the charcoal/PTFE component of the TVCD (detailed description of TVCD construction is below) with respect to isoflurane were evaluated using the methodology which follows. Circular discs were cut from the charcoal/PTFE sheets using a #6 punch dye (1.1 cm diameter). The disks were placed inside the cap of a 4 ml septum vial (with the charcoal surface facing into the vial) in which 50 μl of isoflurane/fat emulsion had been added. A vial with no charcoal/PTFE disk in the cap served as the control. The vials were incubated at 35° C for 90 minutes inside the oven of the GC after which they were placed in a freezer (-20° C) for 120 minutes. After removal from the freezer, the disks were placed under vacuum (created by placing them one inch below the tip of a glass dropper pipette connected to vacuum line) for 0, 30, 60, 180, and 840 minutes, after which they were extracted in 4.0 ml of hexanes. 4.0 ml of hexanes was added to the original vial after the disks were removed. The vials were placed on a shaker for 60 minutes after which 100 μl was removed and added to 4.0 ml of hexanes.

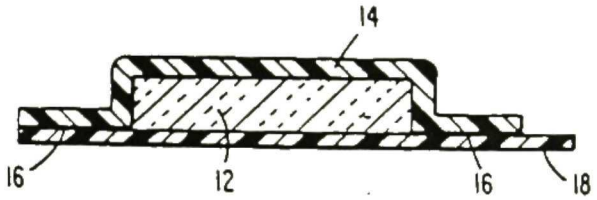
After 10 minutes of shaking, samples were placed in auto-sampler vials for GC analysis. The amount on the disk and the amount remaining in the original vial were calculated from the standard curve.

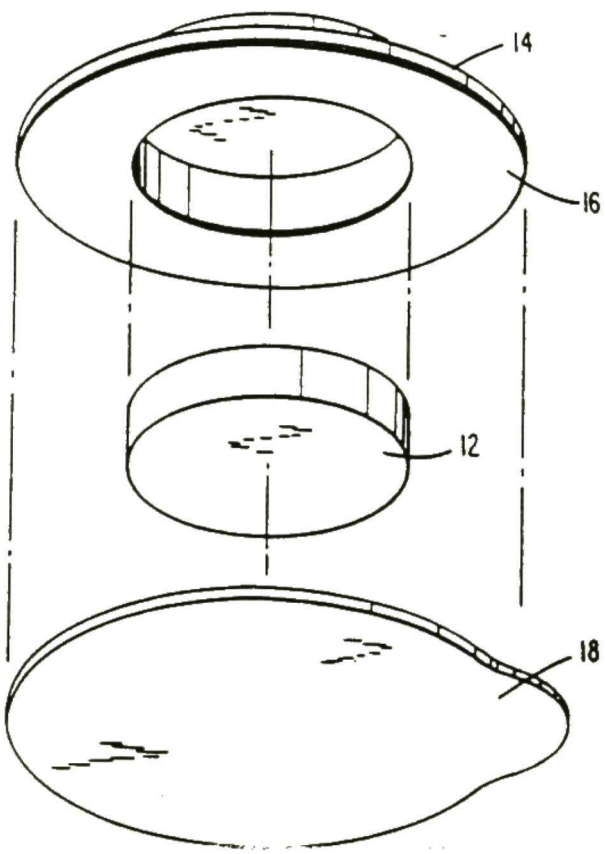
2. Isoflurane collection device

The isoflurane transdermal vapor collection device (TVCD) is an embodiment of the Transdermal Vapor Collection Method and Apparatus invented by Peck (1990) shown in figure 3. Sheets of polytetrafluoroethylene (PTFE) in which activated charcoal had been impregnated (P/N #F10027, W. L. Gore & Associates, Inc., Elkton, MD) were custom manufactured to this laboratory's specifications and included an active and an inactive surface. The active surface comprised the charcoal/ PTFE layer which was laminated to an impervious FEP™ film (the inactive surface). Hand-made TVCD's were constructed by cutting 1.1 cm diameter disks (using #6 punch dye) from the sheets, triple washing the disks in hexanes, then drying under a stream of nitrogen and placing them over the center hole of Double-stick disks™ (No. 2181, Medical Products Division, 3M, St. Paul, Minn.) over a sheet of aluminum foil. A sheet of custom made adhesive foil (Adhesives Research, Glenrock, PA) was placed over the outer surface of the disk and the individual circular TVCD's, along with the impermeable foil on the bottom, were punched out using a 2.54-cm diameter die.

FIGURE 3. SCHEMATIC REPRESENTATION OF TRANSDERMAL VAPOR COLLECTION DEVICE

The drawing at the top is an enlarged cross-sectional view of the TVCD and the drawing on the bottom is an exploded perspective view of the device. The numbers identify components of the device which are more fully explained in the text. The charcoal/PTFE (binding reservoir) disk, adhesive foil, Double-stick disks™ and aluminum foil cover are numbers 12, 14, 16 and 18, respectively. (Modified from Peck 1990)


14
16
12
16
18


14
16
12
18

The TVCD's were usually made on the day of use but if made in advance, they were enclosed in heat-sealed, airtight plastic bags (Kapak Corp., St. Louis Park, Minn.) and stored at 4° C until used. At the time of experimental use, the protective aluminum foil cover was removed, exposing the adhesive tape and charcoal/PTFE surfaces for topical application. At the end of experiments, the TVCD's were removed at set time points, usually at the end of isoflurane infusion, from the skin surface and the charcoal/PTFE component was peeled away from the adhesive foil and immediately placed in 1.0 ml of hexanes for extraction.

3. Animal model

"Fuzzy" rats (WFfz) were obtained courtesy of Dr. James Cooper, DVM, AAMRL, Wright Patterson AFB, Ohio. These rats were derived from a breeding pair originating in the laboratory of Dr. F. G. Ferguson, Pennsylvania State University, Laboratory Animal Resources, University Park, PA. Fuzzy rats (Ferguson, Irving and Stedham 1979) are characterized by hypotrichosis (relative to normal rats: fewer and smaller hair follicles, smaller hair shafts and a reduction in medullated hair shafts). The rats were allowed at least one week to acclimatize to USUHS animal facilities before use and were kept in isolation from other animals due to a suspected infection. They received laboratory chow

(Ralston-Purina, St. Louis, MO) and tap water ad lib., and were maintained on a 12 hour light/dark cycle. At the time of use in the experiments, the rats were between 60 to 90 days old.

To avoid the possible introduction of bias in the series of experiments examining the effects of various TVCD site perturbations, the experiments were performed in a random assignment derived from a table of random numbers (Daniel 1978). On the day of the experiment, rats were dosed with 0.3 ml mixture of acepromazine/ketamine (0.3 mg of acepromazine maleate, Aveco Co., Inc., Fort Dodge, IA and 2.7 mg of ketamine hydrochloride, Ketalar™, Parke Davis, Morris Plains, NJ) intramuscularly. A 12-18 inch length of polyethylene tubing (PE-50, Clay Adams) was surgically implanted in the left femoral artery and a similar implant was performed on the right jugular vein. In some experiments Evans blue dye was injected through the vein just prior to administering pharmacologic modulators and starting isoflurane infusion. Blood samples (approximately 0.2 ml) were drawn in a tuberculin syringe from the artery, after flushing of the line, at set time points (usually 17) up to 240 minutes. After drawing the blood sample, the line was refilled with heparinized saline (100 U/ml).

The repetitive blood sampling (hypovolemia) was not expected to alter the hemodynamic status of the rats. Ploucha and Fink (1986) showed that a four ml (about 20% of blood

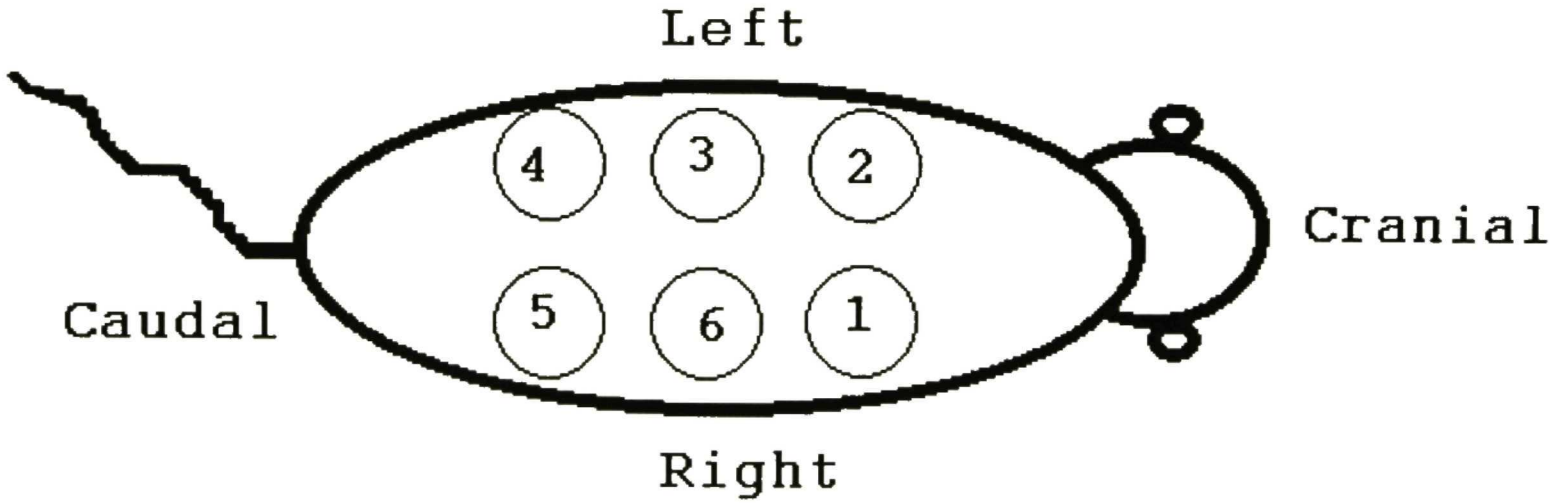
volume) hemorrhage reduced mean arterial pressure and cardiac output 25 and 43%, respectively, in male Sprague-Dawley rats weighing slightly more than 300 g. The rats used in these experiments were about 350 g and only about 15% of the blood volume was expected to be removed. In addition, the rats were infused with fat emulsion containing isoflurane, which replaced about 1/3 of the volume lost. Furthermore, experiments in our laboratory had shown that there was no significant change in the hematocrit of the rats when subjected to a similar blood sampling paradigm.

Isoflurane is predominantly (over 90%) eliminated through the lungs as unchanged drug. To reduce the potential of contamination from exhaled isoflurane circulating within the laboratory, a scavenger system was devised to collect and remove the exhaled isoflurane from the experimental area. One end of a three foot long piece of polyethylene tubing (i.d. about 0.5 cm) was pushed through a hole in the closed end of a cylinder-shaped plastic syringe holder (approximate dimensions were 4 inches long by 1.5 inch diameter at open end). The other end of the tubing was attached to the laboratory's central vacuum line. The open end of the plastic cylinder was placed approximately one inch from the mouth and nose of the rats and a light vacuum was maintained to remove the exhaled isoflurane.

Five sites (Fig. 4) were selected for placement of the TVCD's on the back (unless otherwise noted) of the rats with

FIGURE 4. DIAGRAM OF PLACEMENT OF TVCD'S ON RAT DORSUM

The schematic diagram depicts the placement of the TVCD's on the rat dorsum. The same numbering sequence (1 - 5) was used in all the experiments (unless otherwise noted) and identifies the location of the TVCD's. Cutaneous blood flow, using LDF, was measured at site 6.


Left
4
3
2
Cranial
Caudal
5
6
1
Right

avoidance of any area with pronounced scratches. The same five sites (1 - 5) were used for each experiment and a sixth site (6) was the site for placement of the LDF probe. The TVCD's were placed with great care being taken to assure good adherence around the whole device. If an animal exhibited signs of loss of anesthetic effect during the preparation, another 0.15 ml of acepromazine/ketamine was administered intramuscularly. The animals were euthanized with an intravenous injection of T-61 Euthanasia solution (Hoechst Roussel Agri-Vet Company, Somerville, NJ) at the conclusion of the experiment.

4. Administration of intravenous isoflurane

The intravenous formulation of isoflurane was prepared by mixing 2 ml of isoflurane with 22 ml of intravenous fat emulsion (Liposyn™ 20%, Abbott Labs., Chicago, IL) gravimetrically on an analytical balance to assure accuracy. Using the specific gravity of isoflurane, concentrations were expressed as milligrams of isoflurane per milliliter of mixture. The formulation was stored under refrigeration for a maximum of 14 days.

Isoflurane in intravenous fat emulsion was administered through the jugular vein site as a constant infusion (unless otherwise noted) with a syringe pump (Model 355, Sage Instruments, Cambridge, MA), which was calibrated

to deliver a precise and reproducible amount of the formulation. The infusion rate was set at 0.0125 ml/min for a duration of 90 minutes. The approximate dose of isoflurane administered was 140 mg in a volume of about 1.2 ml.

5. Measurement of cutaneous blood flow

The cutaneous blood flow was assessed using a laser Doppler perfusion monitor (Medex Periflux PF3, Perimed, Stockholm, Sweden). Calibration of the instrument was performed weekly and consisted of establishing a zero perfusion reading and adjustment of the reading to a standard which was supplied by the instrument manufacturer. The zero was established by placing the LDF probe inside the probe holder which had been fixed to a piece of white cardboard. Since there was no movement under the incident laser light, the backscattered light contained no Doppler-shifted components resulting in zero perfusion units. Calibration to a standard was established using the PF 100 Motility Standard. The standard consisted of a small bottle containing about 2.5 ml of a colloidal suspension of latex particles. The Brownian motion of those particles was used as the motility standard. At standard temperature, (22° C) the Motility Standard should have produced a 250 perfusion unit reading on the instrument. The LDF manufacturer provided for the adjustment of the instrument both for the zero and the 250 PU levels by

adjusting the associated potentiometer. However, all calibrations with the LDF never required any adjustment as all white surface measurements produced a zero reading and all of the measurements of the Motility Standard where within ± 10% of the standard motility of 250 PU. When applied to the skin surface, a small amount of transmission gel was applied to the probe head at the point where the laser light was emitted. The probe head was attached to the application site by placing it in a probe holder which was fixed to the skin with double stick adhesive. The output from the LDF (a perfusion unit measurement every 2 seconds) was channeled directly into a Zenith personal computer through an RS-232 interface and the data was collected as a long, continuous column. Prior to administration of the pharmacologic modulators, a 5 to 10 minute baseline measurement of cutaneous blood flow was obtained. The site on the skin of the LDF probe was marked prior to removing it to administer the modulators so as to return it as accurately as feasible to the site were the baseline measurements were taken.

6. Measurement of capillary permeability

An attempt was made to measure capillary damage and extravasation using the Evans blue dye leakage technique. However, since quantitative and reliable measurements could not be obtained, the observations are not be included in the

Results section.

7. Modulators

A. Minoxidil

Minoxidil (Loniten™, The Upjohn Company, Kalamazoo, MI) was only available in 10 mg tablets. However, taking advantage of the solubility and characteristic ultraviolet (UV) spectra of minoxidil, the active ingredient was analytically extracted and used to make a solution for intradermal injection. One tablet was placed in a test tube and 1 ml of absolute ethanol added. The tablet was crushed with a glass stirring rod, the test tube vortexed, then centrifuged at 500 rpm for 30 minutes. The ethanol layer was carefully decanted, leaving a pledget of white powder at the bottom of the tube. The addition of ethanol, mixing and decanting was repeated two times and the ethanol supernatants were combined and accurately diluted to 5.0 ml with ethanol. Fifty μl was further diluted to 5.0 ml in ethanol and the absorbance read in a scanning UV spectrophotometer (Cary 118, Varian, Sugarland, Texas). Using the extinction coefficient for minoxidil (11,790 at UV max [ethanol] 285 nm [Windholz et al. 1983]) the amount extracted from the tablets was calculated.

Solutions of minoxidil in normal saline, which also

served as the control in a series of experiments, were made by extracting the minoxidil from the tablets as detailed above and then allowing the ethanol to evaporate at room temperature. The powder that remained was dissolved in saline to make a 0.33% solution of minoxidil.

Fifty μl of the minoxidil solution (167 μg) were injected intradermally using a 250 μl Hamilton syringe with a 26G needle. The injection technique was performed so as to place the injected volume within the dermis by entering the skin almost parallel to the skin surface with the bevel of the needle facing up.

B. Phenylephrine

Five μg of phenylephrine (Sigma Chemical) in 50 μl of normal saline solution was injected intradermally as described above. Changes in cutaneous blood flow were assessed as described above.

C. Leukotriene D_4

LTD_4 was obtained through the generosity of Upjohn Diagnostics (The Upjohn Company, Kalamazoo, MI) in a concentration of 50 μg/0.5 ml in methanol. LTD_4 was stored at -20° C until use. The stock solution was diluted to 200 ng/50 μl by diluting 40 μl to 1000 μl with normal saline just prior

to use. The LTD_4 was administered intradermally as described above. Changes in cutaneous blood flow were assessed as described above.

D. Ether-ethanol

Chemical alteration of the stratum corneum was effected by treating the skin site used for collection of isoflurane with a mixture of ether and ethanol. Ten ml of ethyl ether were mixed with 1 ml of ethanol USP and approximately 1 ml (until noticeable saturation) of the mixture was applied to the cotton pledget in a Hill Top Chamber™[6] (Hill Top Research, Inc., Cincinnati, OH). On the afternoon previous to the day on which isoflurane was to be administered, fuzzy rats were lightly anesthetized as described above to permit placement of the Hill Top Chambers containing the ether-ethanol. To ensure that the Hill Top Chambers remained firmly in place, the rats were loosely wrapped circumferentially with Vetrap™ (3M) and returned to the animal facility over night. The duration of the ether-ethanol application was approximately 16-20 hours after which the Hill Top Chambers were removed and the experiment for collecting the isoflurane through the altered skin sites was

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[6] The Hill Top Chamber is a specially constructed band-aid like occlusive device. It is comprised of a square (about 3 cm by 3 cm) piece of adhesive tape in the middle of which is a round plastic well holding a 0.95 cm^2 absorbent pad.

conducted as detailed above.

The effect of the ether-ethanol application to the skin of the fuzzy rats was assessed by visual (microscopic) examination. Several sections of normal (untreated area) and treated skin samples were excised from one rat each in the ether-ethanol and tape stripping groups after the termination of the experiment (after the animal was euthanized) and prepared on microscopic slides by the USUHS Pathology Service. Figure 5 is a low power (X 40) micrograph of a representative normal skin sample from the mid-lower back of the rat. The normal skin structures (stratum corneum, epidermis, dermis, sebaceous glands) were clearly delineated and readily visible. Some of the outer layers of the stratum corneum appeared to have separated from the adjoining skin surface, however, this may be due to an artifact of the sample preparation. Figure 6 is a high power (X 550) magnification of the aforementioned slide. The multi-laminate stratum corneum was clearly visible and appeared to be the same thickness as the underlying epidermal layer. Several cells of the epidermal layer (near the stratum corneum junction) appeared flattened and are progressing through the transformation of the lower epidermal layers into the stratum corneum.

The difference in gross (microscopic) appearance of fuzzy rat skin and histology textbook's description of human skin is readily apparent. Human skin is usually shown with numerous "valleys and ridges" between the epidermal-dermal

FIGURE 5. PHOTOMICROGRAPH (LOW MAGNIFICATION) OF NORMAL DORSAL FUZZY RAT SKIN

This micrograph (hematoxylin and eosin [H & E] stain, X 40) illustrates the typical histological appearance of normal skin from the caudal dorsum of a male fuzzy rat. The darkest (outermost layer, rightwards in the micrograph) is the stratum corneum, below which is the viable epidermis. The innermost layer is the dermis.

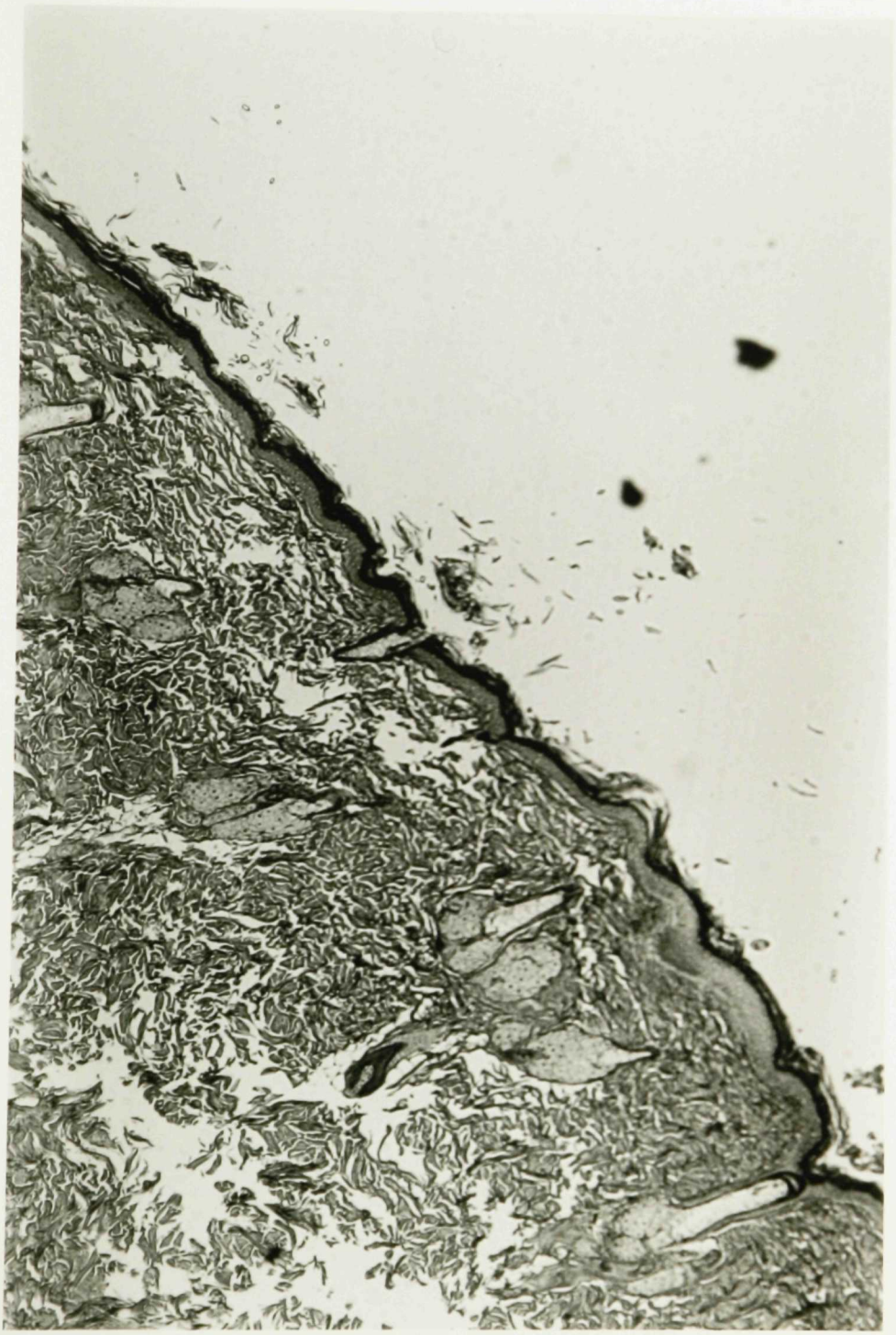
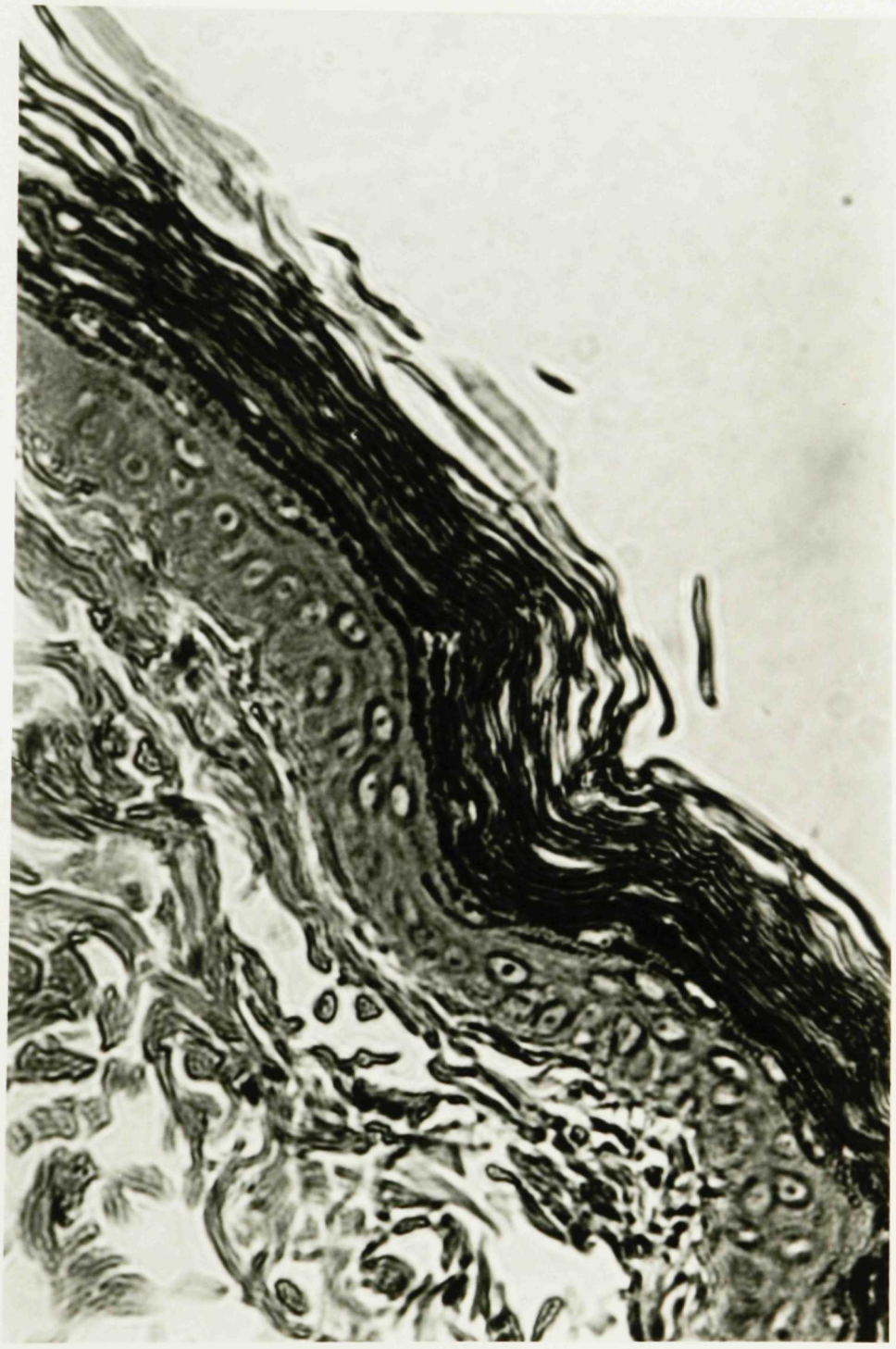


FIGURE 6. PHOTOMICROGRAPH (HIGH MAGNIFICATION) OF NORMAL DORSAL FUZZY RAT SKIN

This micrograph (H & E, X 550), a magnification of a section from figure 5, illustrates the typical histological appearance of the outermost skin layers, including the stratum corneum (the outermost laminated layer, rightwards in the micrograph), viable epidermis (cellular layer adjacent to the stratum corneum), and a small part of the dermis (innermost, left lower corner of micrograph).



junction, which are the epidermal pegs and dermal papillae. The micrographs of fuzzy rat skin appeared to be devoid of such anatomic structures and the epidermal-dermal junction appeared as a fairly smooth, flattened layer.

E. Tape stripping

Fuzzy rats were anesthetized and arterial and venous cannulas surgically implanted as described previously. Prior to administration of the isoflurane, the six sites on the back of the rat were subjected to stratum corneum removal by the tape-stripping technique. Double-stick scotch tape was cut into segments slightly larger than 1 cm and stuck on to the end of the plunger of an one ml tuberculin syringe (approximate area of 0.8 cm^2). The plunger was pressed lightly to the skin surface and removed, a new piece of tape was used and the process repeated for a total of ten stripping applications. Pilot studies indicated that after the tenth tape stripping, the skin had a glistening appearance, suggesting complete removal of all the layers of the stratum corneum. After all the sites had been stripped, baseline LDF measurements were obtained and the rest of the experiment was conducted as outlined above. The effect of tape stripping of the stratum corneum was assessed by visual (microscopic) examination of several skin samples as was done with the ether-ethanol treatment group.

8. Data analysis

The response of the Ni^{63} electron capture detector is linear only over a very narrow range (one order of magnitude) with detector response decreasing with increasing concentrations (McNair 1974). The isoflurane concentrations from this project were anticipated to range over two orders of magnitude. Therefore, following the recommendation of McNair (1974) for demonstrating the linear range of detector response, the calibration curve data (peak areas and nominal concentrations) were converted to base 10 logarithmic form prior to regression analysis. Log transformation is an acceptable technique for linearizing a power function of the form: $Y = (b)(X^m)$ (Chatterjee and Price 1977). Linear regression of the log-transformed assay data (of the form: $y' = \log(b) + (m)(x')$, where $y' = \log Y$ and $x' = \log X$) were performed on either a Zenith or IBM personal computer/MS-DOS system using MKMODEL (Holford/Elsevier-Biosoft, Cambridge, UK), an extended least squares non-linear regression program.

Model-independent and compartment modeling methods were used for analyzing the isoflurane-blood concentration data. Model-independent methods are a less complex approach based purely on mathematical description of blood or plasma profiles of drug or metabolites and calculation of useful pharmacokinetic values without invoking a particular model. The area under the blood concentration of isoflurane versus

time curve (AUC) was estimated using a PC/MS-DOS adaptation of MOMENT, a program developed in our laboratory, which uses the trapezoidal rule to calculate the AUC. The clearance of isoflurane from the blood was obtained by dividing the dose by the AUC.

MKMODEL was used to estimate pharmacokinetic parameters by non-linear regression analysis of the experimental data using a two-compartment model with zero order input. MKMODEL produced an estimate of the optimal weighting factor in addition to the pharmacokinetic parameters. However, if the estimate of the weighting factor was outside the range of 1.5 to 2.5, the data were re-analyzed with a constant weighting factor of 2. The estimates of clearance obtained by the model-independent and compartmental methods were compared using student's t-test with a $p < 0.05$ indicating a significant difference (Gibaldi and Perrier 1975). Elimination half-life was calculated by dividing 0.693 by the parameter estimate (obtained from MKMODEL) of the terminal elimination rate constant.

The Kp_{eff} was calculated by dividing the amount of isoflurane collected in the TVCD per unit area by the AUC and converting to cm/hr, the usual unit for expressing permeability coefficients (Blank, Scheuplein and MacFarlane 1967). The mean Kp_{eff}'s were compared by ANOVA and Duncan's Multiple Range Test or Bonferroni's Test (alpha = 0.05) was used to correct for multiple comparisons (SAS Institute 1988).

Extreme observations of Kp_{eff} values, found to be outliers when tested using Dixon's test at the 1% level of significance, were not included in the final comparison of experimental perturbations (Snedecor and Cochran 1980).

At the present time, there is no universally accepted and standardized methodology for the analysis of blood flow measurements using LDF technology. Since the collection of isoflurane in the TVCD was for a duration of 90 minutes (an average flux over time), the associated, continuous LDF measurements were also averaged over the 90 minute (approximately 2700 observations) interval. The LDF output was converted from the sizable string of data to useful columns using a custom Fortran program written in our laboratory. The baseline and post-treatment values were compared using student's t-test with a $p < 0.05$ indicating a significant difference.

RESULTS

1. Isoflurane assay

A. Validation

The novel chromatographic system was effective in the analysis of isoflurane in hexanes. The retention time for isoflurane was approximately 1.43 minutes. Although a few unknown peaks of low amplitude appeared in chromatograms from the spiked hexanes, the isoflurane peak was well separated and was not affected by those unknown peaks. Figure 7 shows a representative standard curve and demonstrates excellent linearity in the range 299.2 - 29,920 pcg in an injected volume of one μl. The equation of the curve was Y = 0.741X-1.9376[7] with a log likelihood of 194[8]. To assess the precision of this analytical procedure, reproducibilities for both intra-assay (within-day) and inter-assay (between-day) variations were determined (Table 1) using standard

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[7] The equation of this line as a power function was $Y = 0.0115X^{0.7411}$ and is shown as the inset graph in Figure 7.

[8] The log likelihood indicates how well the parameter values in the model describe the data. The correlation coefficient or coefficient of determination are useful measures for goodness-of-fit in ordinary least squares regression. Weighted least squares and extended least squares regressions requires a more complex measure such as the log likelihood. In the comparison of parameter estimation models, the larger log likelihood value is optimal.

FIGURE 7. REPRESENTATIVE CALIBRATION STANDARD CURVE

GC/ECD response (peak area in arbitrary units) was plotted as a function of isoflurane concentration on logarithmic scale. Each diamond represents 10 observations. Extended least squares regression analysis of log-transformed observations was done using MKMODEL and the equation of the line was Y = 0.741X-1.9376. The inset graph is a plot of the same data on linear scale.

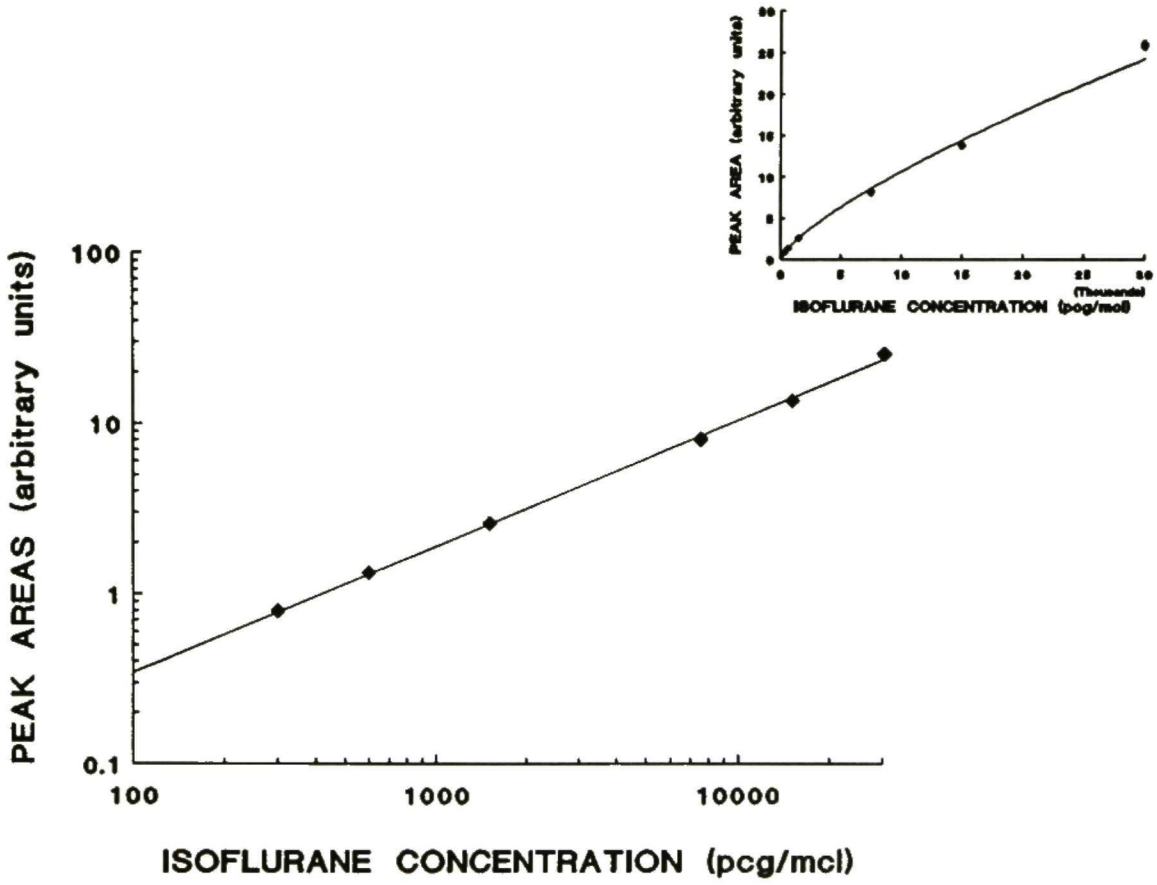

PEAK AREAS (arbitrary units)
100
10
1
0.1
100
1000
10000
ISOFLURANE CONCENTRATION (pcg/mcl)
PEAK AREA (arbitrary units)
ISOFLURANE CONCENTRATION (pcg/mcl)

TABLE 1.

PRECISION AND ACCURACY IN THE DETERMINATION OF ISOFLURANE IN HEXANES

Isoflurane was diluted in varying concentrations in pesticide grade hexanes ranging from 299.2 to 29920 pcg/μl. The table shows the mean (± SEM) concentrations, % CV and % relative error determined after analysis by GC/ECD. Standard curve analyses giving rise to these variability estimates were analyzed by MKMODEL, a non-linear extended least squares curve fitting computer program (Holford, 1987) as described in Materials and Methods. The number of replicates is indicated in the table (N).

Nominal Concentration (pcg/μl)	Concentration Determined (mean ± SEM) (pcg/μl)	CV (%)	Relative error (%)
Intra-assay variation (N = 10)			
299.2	298.7 ± 1.1	1.2	< 1
598.4	600.8 ± 1.5	0.8	< 1
1496	1491 ± 5	1.1	< 1
7480	7081 ± 27	1.2	-5
14960	14264 ± 35	0.8	-5
29920	33152 ± 105	1	11
Inter-assay variation (N = 33)			
448.8	474.7 ± 2.8	3.4	5.8
14960	13082 ± 103	4.5	-12.6

concentrations made up on one day and stored at -20° C until used. The coefficients of variation (CV) for six different concentrations in the within-day study varied between 0.8 and 1.2%; those in the between-day study ranged from 3.4 to 4.5%. The accuracy of the measurements was concurrently evaluated by comparing the nominal concentrations of isoflurane with their estimated concentrations. The relative errors in the within-day study ranged from -5 to 11%.

Five sets of calibration curve data were analyzed in a comparison of MKMODEL and SAS (a more widely recognized statistical analysis software program), and the results are presented in Table 2. The small differences in parameter estimates are probably accounted for by the different variance models used by the programs. SAS uses the principle of least squares to produce estimates of the slope and intercept and assumes that the error associated with each observation is the same. MKMODEL, using extended least squares estimation, has the ability to estimate the parameters of an error model from the data and therefore uses the information in each observation with an appropriate weight. Student's t-test indicated no significant difference ($p > 0.05$) in the mean estimates of the slope and intercept from the two methods. The mean correlation coefficient of the regression analysis using SAS was 0.997. MKMODEL did not provide the correlation coefficient as a measure of goodness-of-fit, but reported a mean log likelihood value of 49.3.

TABLE 2.

COMPARISON OF CALIBRATION EQUATIONS FROM MKMODEL AND SAS

Regression analysis of log-transformed peak area (Y) versus log-transformed standard isoflurane concentrations (X) were done using MKMODEL (Holford, 1987) and SAS. Five sets of calibration curve data were compared. The equations are shown in the power function form (not log-transformed) for easier comparison. Mean (± SEM) values are reported. The mean estimates of the slope and the intercept were not significantly different ($p > 0.05$).

Method	Equation	CV of slope (%)
MKMODEL	$Y = 0.00167(\pm 0.00027)X^{0.933(\pm 0.011)}$	2.69
SAS	$Y = 0.00179(\pm 0.00027)X^{0.927(\pm 0.011)}$	2.61

B. Assay of isoflurane in blood and fat emulsion

The appearance of chromatograms obtained from blank fuzzy rat blood and blood containing isoflurane was identical to those of hexanes without blood and there was no evidence of any endogenous substance causing interference at the retention time of isoflurane. The unknown peaks which were present did not interfere with the separation and quantification of isoflurane.

The extraction recovery of isoflurane from blood was assessed by comparing the concentration of isoflurane after the addition of blood and without blood (Table 3). The extraction recovery ranged from 97.7 to 99.3% of the control samples for isoflurane concentrations of 0.44 to 46.99 μg/ml. The lower limit of quantification (sensitivity of the assay) was estimated to be about 1 μg/ml.

The extraction of isoflurane from the mixture with fat emulsion was comparable to that from blood. There were no substances within the fat emulsion matrix which when extracted in hexanes produced any interfering peaks near the retention time of isoflurane. Hence, calibration curves for quantification of isoflurane in blood or fat emulsion were derived from analysis of standards made up in hexanes alone.

C. Assay of isoflurane in TVCD

Representative chromatograms of blank and isoflurane-

TABLE 3.

EXTRACTION OF ISOFLURANE BY FUZZY RAT BLOOD

Arterial blood from a fuzzy rat receiving isoflurane-free fat emulsion was added to a vial containing one of the calibration standard solutions (in duplicate). Calibration standard solutions, placed in identical vials, served as controls. Isoflurane concentrations were determined by gas-chromatography.

Standard solution	Control[a] samples (μg/ml)	Blood samples (μg/ml)	Per cent[#] of control
F	0.44	0.43	
F	0.44	0.44	98.9
E	0.87	0.86	
E	0.86	0.84	98.3
D	2.13	2.08	
D	2.14	2.09	97.7
C	9.73	9.58	
C	9.69	9.68	99.2
B	19.45	19.57	
B	19.47	19.07	99.3
A	46.28	45.75	
A	46.99	45.48	97.8

[a] hexanes only

[#] calculated from the average of the replicates

containing collection devices are shown in figure 8. Even though there are several unknown peaks near the retention time of isoflurane, the isoflurane peak is separated, sharp and quantifiable. In an experiment to be described later (Characterization of Collection Devices), it was shown that there was total extraction of isoflurane from the charcoal/PTFE component of the TVCD's. Hence, as with the quantification of isoflurane in blood and fat emulsion, quantification of the amount of isoflurane recovered from TVCD's was derived from analysis of standards made up in hexanes alone.

2. Intravenous isoflurane formulation

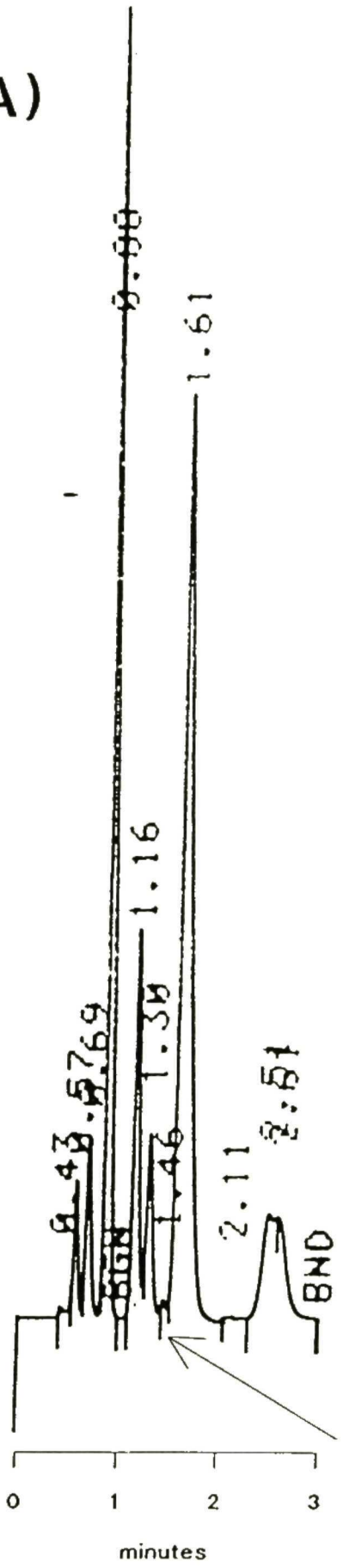
A. Physicochemical characteristics

Visual inspection of the formulation did not reveal any apparent incompatibility between isoflurane and Liposyn™ as there was no observable disturbance in the homogeneity of the emulsion. Analysis of isoflurane extracted in hexanes from isoflurane-enriched Liposyn™ showed quantitative recovery of drug (96%, N = 6, C.V. = 8.5%). The stability of isoflurane in Liposyn™ over a twenty-one day period is shown in Table 4. At the end of the study period (21 days), the determined isoflurane concentration was 97.7% of the nominal concentration of 119.6 mg/ml.

FIGURE 8. REPRESENTATIVE CHROMATOGRAMS OF EXTRACTION OF ISOFLURANE FROM COLLECTION DEVICES

Chromatograms of extracts of (A) blank collection device and (B) collection device removed from the back of a rat where 200 ng of leukotriene D_4 had been injected at the skin site of the TVCD prior to the administration of intravenous isoflurane. The devices were taken through the extraction procedure and analyzed by gas chromatography as described in Methods. The isoflurane peak area in (B) was 1.471 arbitrary units which was determined to be 1.16 μg of isoflurane. The arrow in (B) indicates the isoflurane peak and in (A), the anticipated peak appearance time of isoflurane.

(A)


1.61
1.16
1.30
1.46
2.11
2.51
BND
0
1
2
3
minutes

(B)

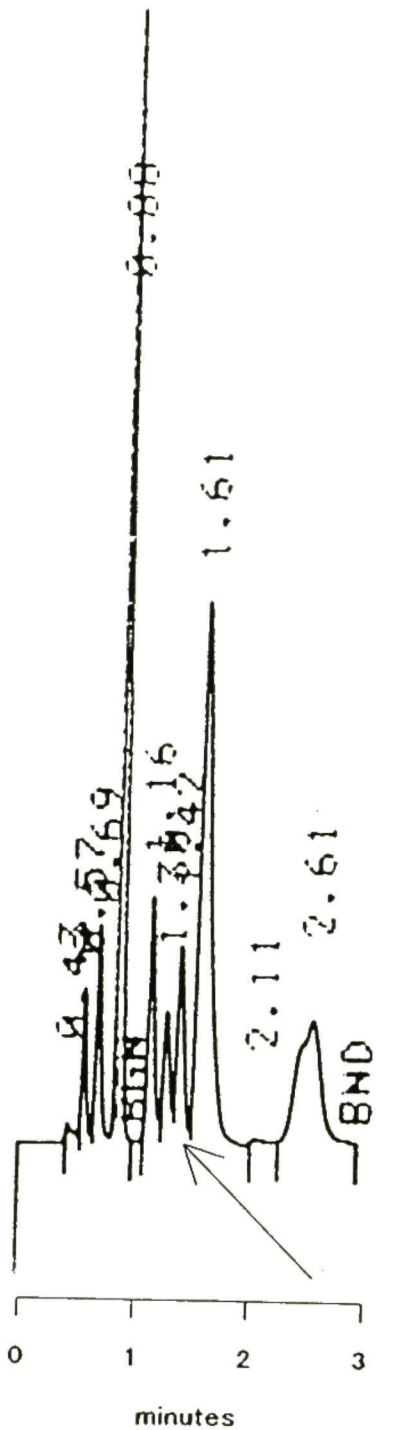

1.61
2.11
2.61
BND
0
1
2
3
minutes

TABLE 4.

STABILITY AND RECOVERY OF ISOFLURANE FROM FAT EMULSION

The concentration of isoflurane was determined from samples of isoflurane-Liposyn™ (fat emulsion) mixture stored over a twenty-one day period. Samples of the mixture were extracted in hexanes and analyzed for isoflurane by gas-chromatography. The determined concentrations were compared to the nominal concentration of 119.6 mg/ml.

Reference date	Concentration determined (mg/ml) (mean ± SEM, N = 3)	Per cent of nominal
Day 1	121.4 ± 4	101.5
Day 2	121.2 ± 2.8	101.3
Day 15	116.6 ± 0.4	97.5
Day 21	116.8 ± 0.4	97.7

B. In vivo characteristics

The initial in vivo experiments demonstrated that the usual, simple administration technique for pharmacokinetic studies, i.e. single, bolus intravenous dose, would not be feasible to characterize the pharmacokinetics of intravenously administered isoflurane. This was shown by the inability to recover measurable quantities of isoflurane in blood or TVCD's after a single, bolus dose, which was restricted to a maximum of 0.2 ml (24 mg of isoflurane) volume so as not to kill the animal.

In subsequent experiments, rats were dosed with either multiple bolus doses only, multiple bolus doses and constant infusion or constant infusion only. In order to examine the pharmacokinetics of intravenously administered isoflurane-fat emulsion mixture, a dose-ranging study was conducted. Six male fuzzy rats, with a mean weight of 281 grams (range 231 to 329 grams), were administered doses (multiple bolus dosing was used in 2 rats and a combination of multiple bolus and infusion was used in the other rats) of isoflurane ranging from 116.7 to 1857.2 mg/kg of rat body weight. The area under the isoflurane blood concentration time curve (AUC) increased linearly with dose (range 847 to 14,727 μg-min/ml) and the mean (± SEM) model-independent derived systemic clearance of isoflurane from the blood was 166 ± 13 ml/min/kg. Even though there was an apparent trend for decreasing clearance with increasing dose, the parameter estimate of the slope of the

regression (clearance vs. dose) line was not significantly different from zero ($p = 0.4597$).

The isoflurane-enriched fat emulsion mixture was used as the dosing formulation in over 50 animal experiments with no observable harmful effects on the animals. The amounts of isoflurane administered ranged from 15.2 to 525.6 mg (49.8 to 1857.2 mg/kg). When the isoflurane-fat emulsion mixture was given after the acepromazine/ketamine anesthetic had worn off, it was observed to produce an apparent stage II+ (loss of consciousness, excitement minimal or marked, breathing irregular) anesthesia in the rats.

3. Characterization of collection devices

The absorptive capacity of the charcoal/PTFE component of the TVCD was determined by the following in vitro procedure which is more fully described in Materials and Methods. Septum vials containing 5,915 ± 76 (mean ± SEM,) μg of isoflurane in 50 μl of isoflurane-fat emulsion mixture[9], which had charcoal/PTFE disks on the inside of the cap (except for control) were incubated at 35° C for 90 minutes. Hexanes were added to the vials and the amounts of isoflurane remaining (not adsorbed to charcoal/PTFE disks or lost) were analyzed. This "up-loading" procedure resulted in a loss of 1,695 μg

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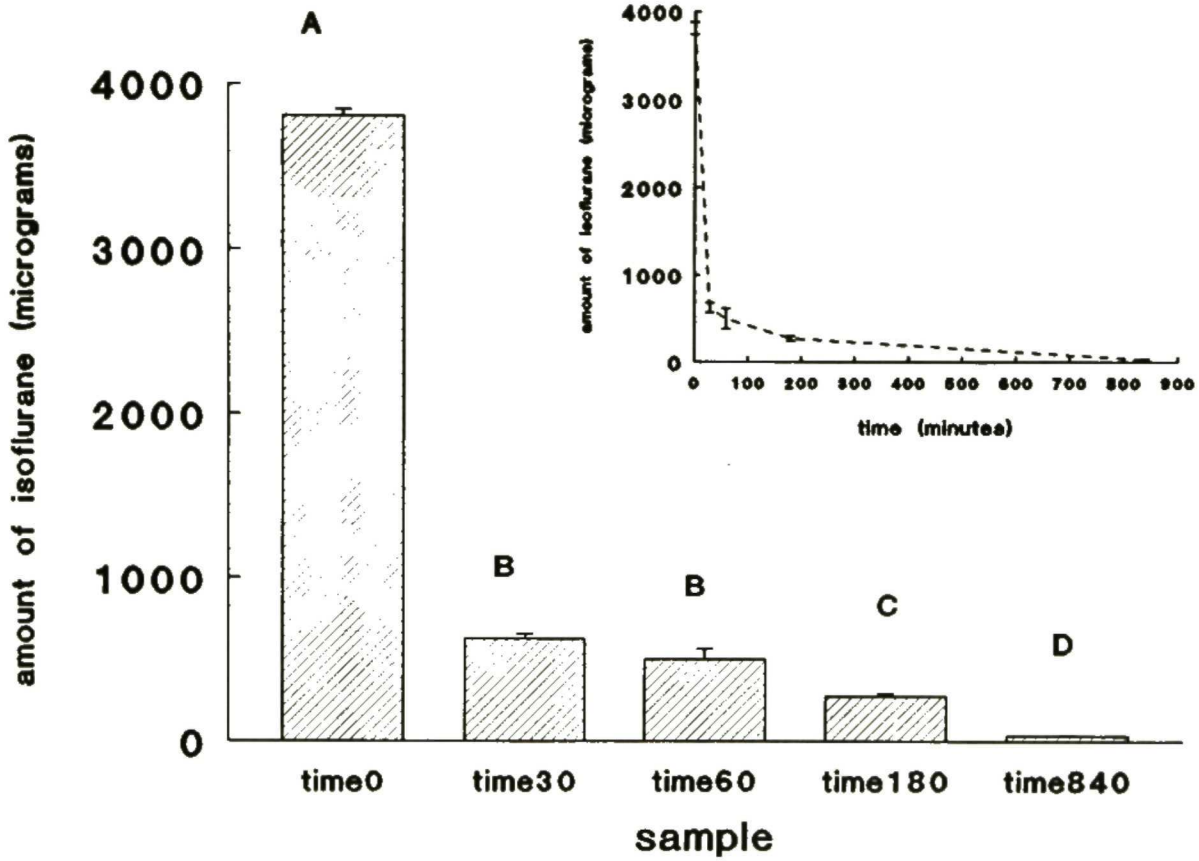
[9] The amount of isoflurane in 50 μl of the isoflurane-fat emulsion mixture was determined as described in Materials and Methods.

(approximately 30%) of the starting amount of isoflurane as the amount in the control vials was 4,220 ± 136 (mean ± SEM, N = 3) μg. This loss of isoflurane may be attributable to leakage from the vial or adsorption to septum or plastic vial cap. Over 4,000 μg of isoflurane were available for adsorption to the disks, of which 90% was adsorbed, as the mean (± SEM) amount recovered in the original vials was 427 ± 12 (N = 15) μg.

The charcoal/PTFE disks from the above experiment were removed and exposed to room temperature air to desorb, the complete procedure more fully described in Materials and Methods. The desorption (in air) of isoflurane from the charcoal/PTFE disks occurred very rapidly initially but then progressed at a slower rate (Fig. 9). The mean (± SEM) amount of isoflurane recovered from the disks at zero minutes was 3,810 ± 39 μg, which when added to the mean (± SEM) amount which was not adsorbed (413 ± 29 μg) in those vials, is almost identical to the total amount (4,220 ± 136 μg) available for up-loading in the control vials. After only 30 minutes of exposing the disks in air, the bulk of the isoflurane had desorbed, as only 625 ± 33 μg were extractable from the disks. However, even after 840 minutes of air exposure, there was a very small (34 ± 4 μg) amount of isoflurane extractable from the disk. ANOVA with Bonferroni's correction for multiple comparisons indicated only the 30 and 60 minute samples not to be significantly different from each other or the other samples.

FIGURE 9. AMOUNT OF ISOFLURANE DESORBED FROM CHARCOAL/PTFE DISKS

The graph shows the amount of isoflurane extracted from the charcoal/PTFE disks which had been allowed to desorb in open air at room temperature for specific time intervals. Time0, time30, time60, time180, and time840 are 0, 30, 60, 180, and 840 minutes of desorption time, respectively. All of the disks had adsorbed approximately 4,000 μg of isoflurane vapor during a 90 minute incubation at 35° C. After the desorption time, the disks were extracted in hexanes and the amount of isoflurane was analyzed by gas chromatography. The height of the bars represent the mean amount (from three replicates) of isoflurane recovered from the disks. The error bars represent the standard error of the mean. Bars with the same letter on top were not significantly different from each other and bars with different letters were statistically different. The inset graph is a plot of the same data showing the rapid (see text) biexponential rate of desorption over the 840 minute study period.


A
B
B
C
D
4000
3000
2000
1000
0
amount of isoflurane (micrograms)
time0
time30
time60
time180
time840
sample
amount of isoflurane (micrograms)
time (minutes)
0
100
200
300
400
500
600
700
800
900

The desorption data were fit to a bi-exponential decay curve function of the form

$$Y = Aexp^{(-B)(t)} + Cexp^{(-D)(t)}$$

where: Y = the amount of isoflurane on the disk (μg)

A, C = coefficients

B, D = rate constants of desorption

t = time (minutes)

($B_{half\text{-}life}$, $D_{half\text{-}life}$ = ln2/B, ln2/D)

using LSNLR, a least squares non-linear regression algorithm developed in our laboratory. The results of the regression are shown below:

$$Y = 3{,}150exp^{-0.14t} + 660.7exp^{-0.0048t}$$

R^2 = 0.9984, SEE = 64.6 μg

$B_{half\text{-}life}$ (rapid phase) = 4.95 min

$D_{half\text{-}life}$ (slow phase) = 144.4 min

where R^2 is the coefficient of determination and SEE is the standard error of the estimate. Thus, the desorption process is characterized by a dominant rapid phase (approximately 82% lost with a half-life of about 5 min) accompanied by a lesser, slower phase (approximately 18% lost with a half-life of about 145 min).

The construction of the TVCD's in aluminum foil was effective in preventing contamination of the charcoal/PTFE

disks from environmental sources other than the exposed contact surface. Specially constructed TVCD's (the charcoal/PTFE disk sealed on both sides with adhesive aluminum foil tape) placed on the dorsal skin (N = 3) of a rat given isoflurane had no detectable amount recovered, whereas the TVCD's exposed to the skin (N = 3) surface had a mean (± SEM) amount of isoflurane of 3.7 ± 0.2 μg. In this experiment, the exhaled isoflurane was not removed using the "scavenger" vacuum apparatus described previously. Three charcoal/PTFE disks which were placed approximately four inches from the face of the rat picked up 7.7 ± 0.8 μg of isoflurane, demonstrating the necessity for removing the exhaled isoflurane from the experimental area.

4. Animal model

A. In vivo permeation of isoflurane through normal skin

Five male rats with a mean weight of 281 grams (range of 200 to 364 grams) were used to characterize the in vivo permeability of isoflurane through unperturbed skin and to evaluate the hypothesis that the flux is linearly related to the driving concentration in skin blood, (i.e. a test of consistency with Fick's law of diffusion). The rats were given doses (by bolus and infusion in two rats and constant infusion in the others) of isoflurane ranging from 136.1 to 525.6 mg which resulted in isoflurane-blood concentration area under

the curve (AUC) values ranging from 1,345 to 15812 μg-min/ml (Fig. 10). The TVCD's (N = 26) varied in size, ranging in area from 0.5 to 1.78 cm^2 and 3 to 12 were placed on the back of each rat similar to the diagram of figure 4. Seventeen of the TVCD's were removed immediately at the end of the isoflurane dosing period, one was removed 60 minutes post-dosing and the rest were removed 120 minutes post-dosing. The amount of isoflurane recovered from the TVCD's ranged from 0.35 to 16.44 $\mu g/cm^2$.

In order to test the applicability of Fick's Law, the data as a whole were analyzed by linear regression with the model of the form:

$$J = \beta_0 + \beta_1(AUC) + \beta_2(site) + \epsilon$$

where: J = the amount of isoflurane extracted from each TVCD during the collection period ($\mu g/cm^2$)

AUC = the area under the blood concentration-time curve from the start of isoflurane dosing to TVCD removal

β_0 = the intercept of the regression line

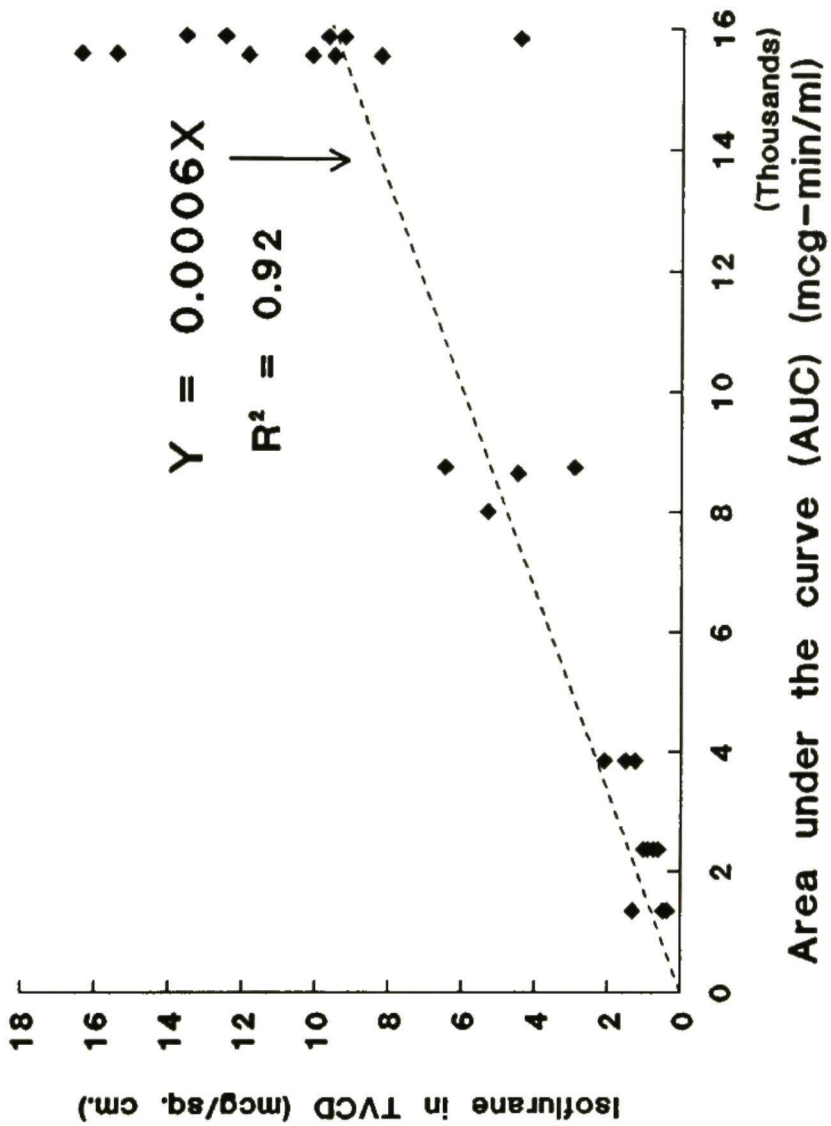
β_1 and β_2 are the slopes of the regression line

ϵ is the residual error of the multiple regression.

The parameter estimates of β_0 and β_2 were not significantly different from zero and, hence, the model was reduced by the elimination of the term including site and forcing the

FIGURE 10. RELATIONSHIP BETWEEN ISOFLURANE RECOVERED FROM TVCD AND ISOFLURANE IN BLOOD AUC

Graphical presentation of the relationship between the area under the isoflurane-blood concentration time curve and the corresponding amount of isoflurane recovered in the TVCD. Data were derived from experiments using five fuzzy rats. Each diamond represents one TVCD. The dashed line represents the regression line with the intercept forced through zero.


Y = 0.0006X
R² = 0.92
Isoflurane in TVCD (mcg/sq. cm.)
Area under the curve (AUC) (mcg-min/ml)
(Thousands)
0
2
4
6
8
10
12
14
16
18

intercept through zero. This was analogous to the integrated from of Fick's first law of diffusion,

$$J = (Kp)(A)(AUC) \qquad \text{eq. (8)}$$

where: Kp = the permeability coefficient (cm/hr)

A = the skin contact area of the active charcoal portion of the TVCD (cm^2)

J and AUC are as above (with J in μg and AUC in μg-hr/ml).

The coefficient of determination (R^2), which served as an indicator of goodness-of-fit of the linear model was 0.92. The estimate of Kp, 0.036 cm/hr, was significantly different from zero ($p > 0.0001$).

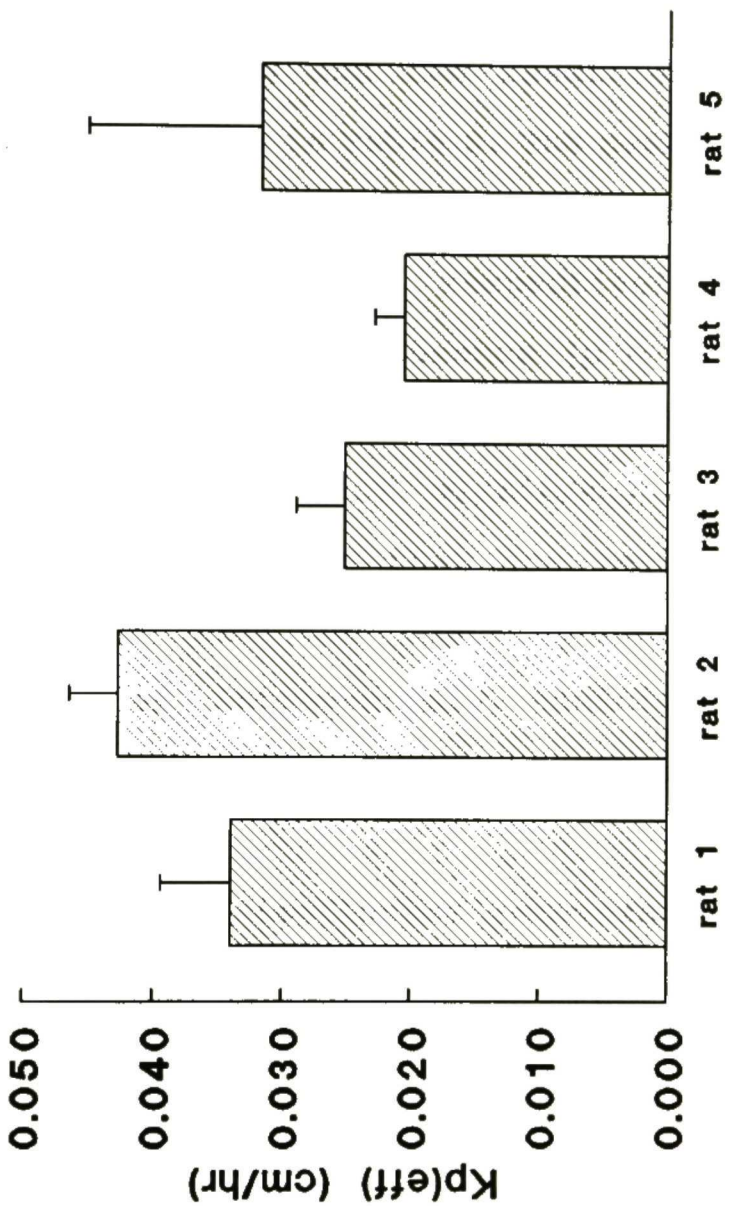
The individual Kp_{eff}'s were calculated by site and animal using equation (8) and the mean (± SEM) Kp_{eff}'s by animal are shown in figure 11. The grand mean (± SEM) Kp_{eff} of 0.0347 ± 0.0028 cm/hr (range 0.0152 to 0.0636 cm/hr, CV = 42%) was nearly identical to the permeability coefficient estimated by the regression approach.

B. Control studies

Five male fuzzy rats with a mean weight of 367 grams (range was 340 to 414.5 grams) were used as control animals to observe the influence of intradermal injections and

FIGURE 11. EFFECTIVE PERMEABILITY COEFFICIENTS IN NORMAL FUZZY RATS

The Kp_{eff}'s were calculated using equation (8) at each site on five fuzzy rats and the mean Kp_{eff} of each rat is represented by the height of the bars (the error bars represent the standard error of the mean). There were 4, 12, 3, 4, and 3 TVCD's on rats 1, 2, 3, 4, and 5, respectively.


0.050
0.040
0.030
0.020
0.010
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

introduction of a 50 μl fluid volume on permeation. Fifty μl of normal saline solution were injected intradermally on the back at six sites similar to the ones diagramed in Figure 4 as described in Materials and Methods. Isoflurane was administered by constant intravenous infusion in doses ranging from 141.4 to 145.1 mg and the resulting pharmacokinetic parameters are presented in Table 5. The peak isoflurane-blood concentrations and AUC values ranged from 14.8 to 22.9 μg/ml and 1306 to 2228 μg-min/ml, respectively. There was no statistically significant difference ($p = 0.84$) between the means of the model-independent (227.8 ± 28.1 ml/min/kg) or model-dependent (230 ± 29.1 ml/min/kg) derived systemic clearance values indicating that the zero-order input, 2-compartment model fit the distribution and elimination characteristics of intravenously administered isoflurane acceptably well. The mean (± SEM) elimination half-life was 29.8 ± 6.2 minutes.

The amount of isoflurane recovered from the TVCD's ranged from 0.17 to 1.83 $\mu g/cm^2$ which resulted in individual Kp_{eff} values from each site on each rat ranging from 0.0053 to 0.0589 cm/hr. The mean (± SEM) Kp_{eff} of each rat is shown in figure 12 and the grand mean (± SEM) Kp_{eff} was 0.028 ± 0.0027 cm/hr (CV = 48%).

Skin blood flow was measured using LDF. Baseline measurements were obtained just prior to the intradermal injection of the saline solution and placement of the TVCD's. After replacement of the LDF probe to the baseline measurement

TABLE 5.

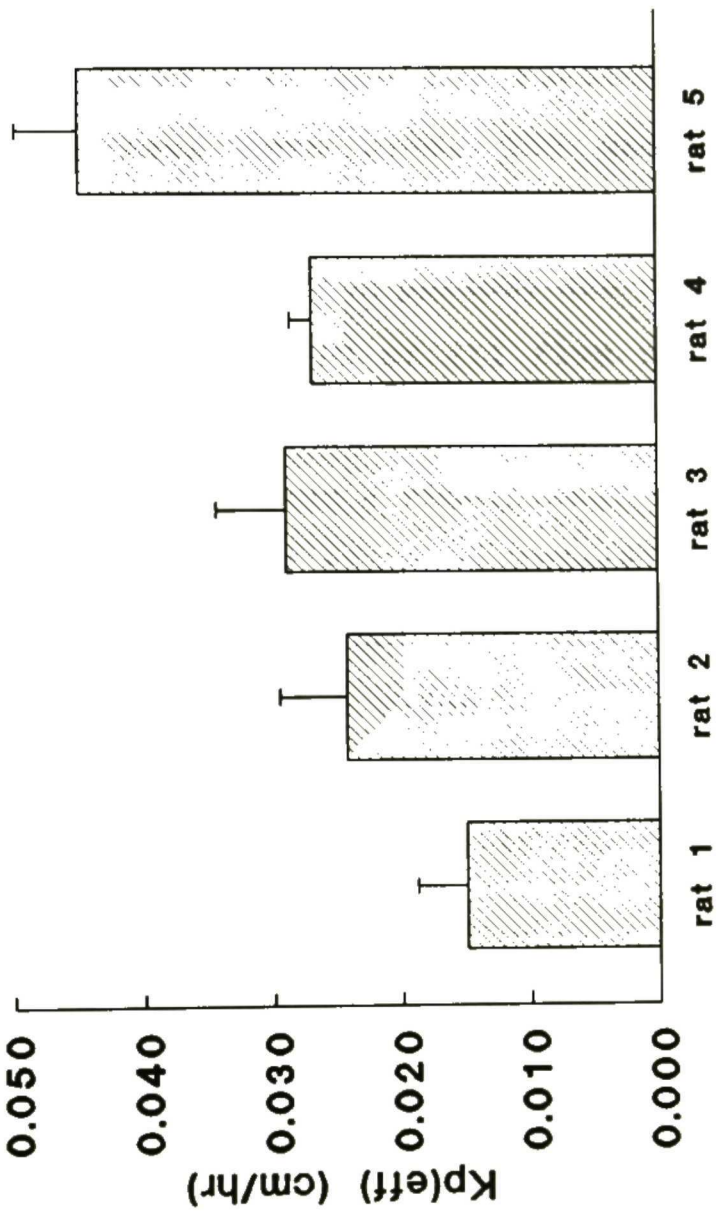
PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS (CONTROL GROUP)

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	22.9	2228	157.1	155.6	36.3
2	14.8	1306	326.8	333.5	19.5
3	20.7	1819	220.7	215.3	27.5
4	17.2	1585	235.4	209.7	15.7
5	22	2061	198.8	236	50.2
MEAN	19.5	1800	227.8	230	29.8
SEM	1.5	165	28.1	29.1	6.2
% CV	17	20	28	28	46

FIGURE 12. EFFECTIVE PERMEABILITY COEFFICIENTS IN "CONTROL" RATS

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The TVCD sites had intradermal injections of 50 μl of normal saline prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean.


0.050
0.040
0.030
0.020
0.010
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

site, skin blood flow was continuously monitored until the time of removal of the TVCD's (about 90 minutes). The LDF observations of the pre-(baseline) and post-intradermal injection periods were averaged individually for each rat. Rats 1 to 3 had received intravenous injection of Evans blue dye which interfered with the LDF readings and only their baseline values have been used. The grand mean (± SEM) perfusion units (PU) of all the rats (except rats 1 to 3 in the post-intradermal injection period) are shown in figure 13. The mean (± SEM) baseline PU of all five rats was 14 ± 1.34 and ranged from 11.4 to 19.1 PU. Intradermal injection of the saline solution did not have a significant effect on relative blood flow as the mean of the post-injection period of rats 4 and 5 was 15.6 PU and was not statistically different ($p = 0.61$) from the mean baseline value.

C. Effects of modulators on isoflurane permeation through skin

i. Phenylephrine

Five male rats with a mean weight of 330 grams (range was 320 to 340 grams) were used in the experiments investigating the effect of localized vasoconstriction (produced by phenylephrine) on the outward permeation of isoflurane. Isoflurane was administered by continuous intravenous infusion in doses ranging from 138.9 to 142.6 mg and the resulting pharmacokinetic parameters are presented in

FIGURE 13. SKIN BLOOD FLOW IN "CONTROL" RATS

Skin blood flow (expressed in perfusion units) was measured using LDF. Baseline measurements, over an approximate seven minute period (about 210 observations), were obtained just prior to the intradermal injection of 50 μl of 0.9% sodium chloride solution in each of five rats. The post-injection period, measured at the baseline measurement site, consisted of 90 minutes of continuous monitoring (about 2700 observations) in each rat. The height of the bars represent the mean PU of all rats and the error bars represent the standard error of the mean. The mean baseline and post-injection values have N's of 5 and 2, respectively, and were not significantly different.

(Rats 1 to 3 were excluded from the post-injection period because of Evans blue dye interference with the LDF measurements.)

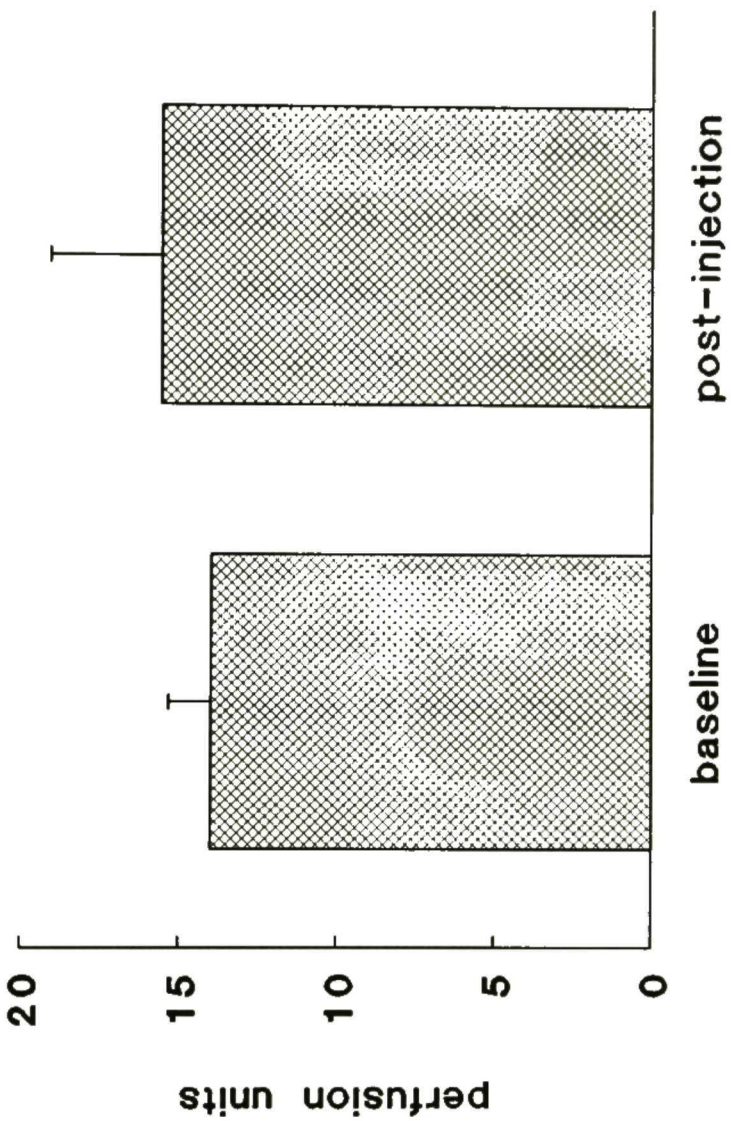

perfusion units
20
15
10
5
0
baseline
post-injection

Table 6. The peak isoflurane-blood concentrations and AUC values ranged from 15.2 to 40.6 μg/ml and 1359 to 3645 μg-min/ml, respectively. There was no statistically significant difference ($p = 0.08$) between the means of the model-independent (219 ± 33.9 ml/min/kg) and model-dependent (251 ± 36.9 ml/min/kg) estimates for systemic clearance of isoflurane and the estimates were similar to those derived in the "control" experiments. The mean (± SEM) elimination half-life was 54.3 ± 5.9 minutes which was not significantly different than half-life in the "control" group.

The amount of isoflurane recovered from the TVCD's ranged from 0.4 to 1.63 $\mu g/cm^2$ which resulted in individual Kp_{eff} values from each site on each rat ranging from 0.0079 to 0.073 cm/hr. The mean (± SEM) Kp_{eff} of each rat is shown in figure 14 and the grand mean (± SEM) Kp_{eff} was 0.0336 ± 0.0037 cm/hr (CV = 55%).

Relative skin blood flow measurements, using LDF, are shown figure 15. The mean baseline perfusion units of the five rats in the phenylephrine treatment group was very similar to the baseline measurements of the "control" group. The mean (± SEM) baseline blood flow of all five rats was 12.6 ± 1.4 PU and ranged from 10.5 to 17.8 PU. The mean (± SEM) blood flow of four rats (rat 1 was excluded because it had received an intravenous injection of Evans blue dye) during the post-intradermal injection period was 6.1 ± 0.6 PU and was significantly different ($p = 0.013$) from the baseline period. Visual examination of the individual perfusion unit over time

TABLE 6.

PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS AFTER LOCAL APPLICATION OF PHENYLEPHRINE

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	17.5	1737	241.5	309	56.3
2	26.4	2402	180.6	227.2	50.6
3	40.6	3645	113.4	116.7	52.1
4	19	1743	244.3	291.4	37.9
5	15.2	1359	315.3	310.7	74.5
MEAN	23.7	2177	219	251	54.3
SEM	4.6	404	33.9	36.9	5.9
% CV	43	41	35	33	24

FIGURE 14. EFFECTIVE PERMEABILITY COEFFICIENTS IN FUZZY RATS AFTER LOCAL APPLICATION OF PHENYLEPHRINE

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The TVCD sites had intradermal injections of 50 μl of phenylephrine (5 μg) solution prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean.

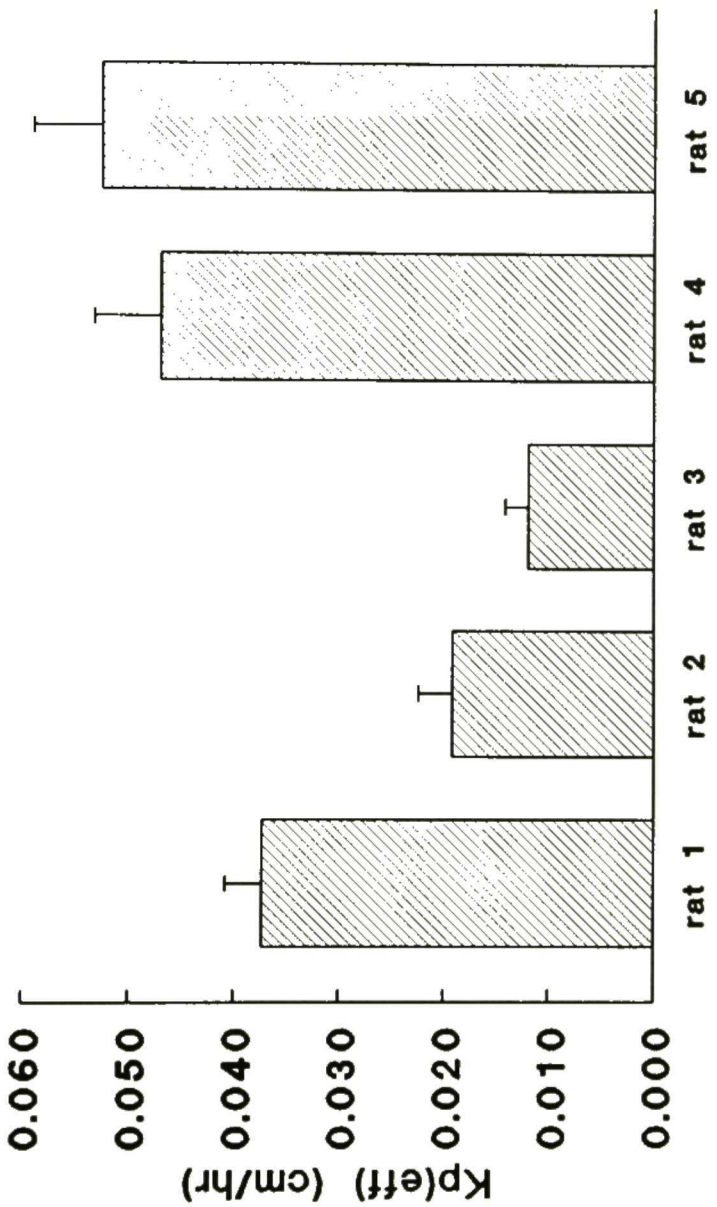
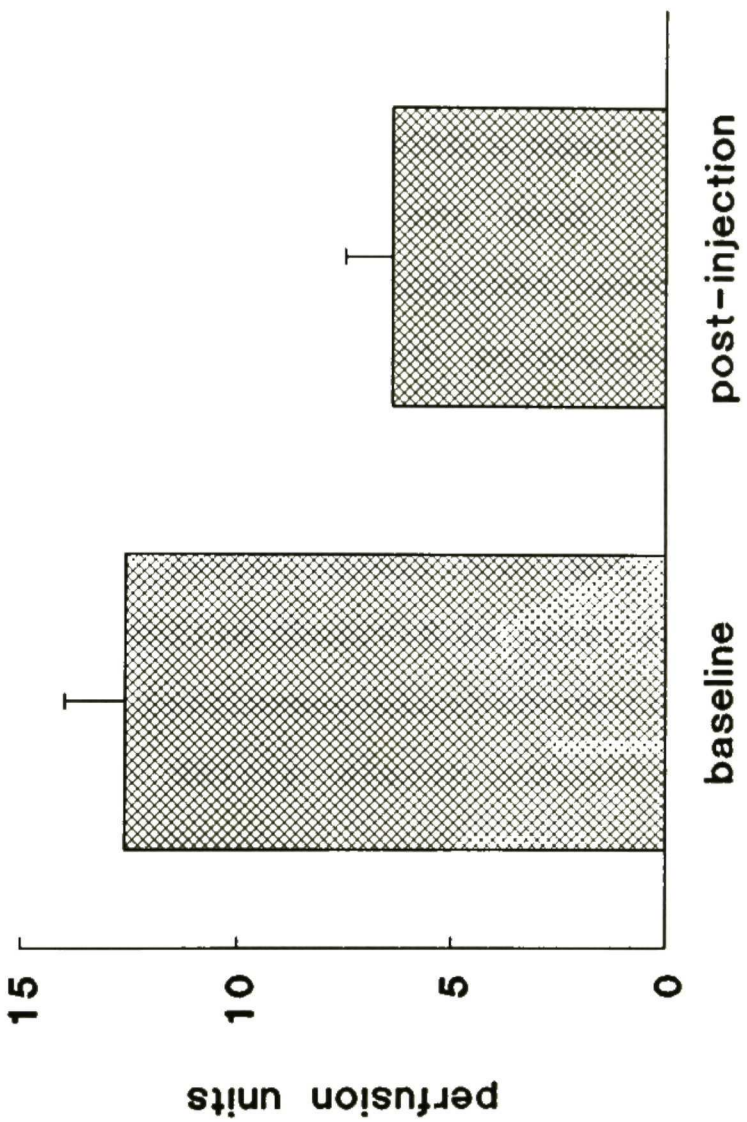

0.060
0.050
0.040
0.030
0.020
0.010
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

FIGURE 15. SKIN BLOOD FLOW IN FUZZY RATS BEFORE AND AFTER LOCAL APPLICATION OF PHENYLEPHRINE

Skin blood flow (expressed in perfusion units) was measured using LDF. Baseline measurements, over an approximate seven minute period (about 210 observations), were obtained just prior to the intradermal injection of 50 μl of phenylephrine (5 μg) solution in each of five rats. The post-injection period, measured at the baseline measurement site, consisted of 90 minutes of continuous monitoring (about 2700 observations) in each rat. The height of the bars represent the mean PU of all rats and the error bars represent the standard error of the mean. The mean baseline and post-injection values have N's of 5 and 4, respectively, and were significantly different ($p = 0.013$).

(Rat 1 was excluded from the post-injection period because of Evans blue dye interference with the LDF measurements.)


perfusion units
15
10
5
0
baseline
post-injection

profiles indicated a relatively short duration of phenylephrine vasoconstriction, as in all four rats there was an increasing trend toward baseline levels.

ii. Minoxidil

Five male fuzzy rats with a mean weight of 357 grams (range 306 to 403 grams) were used in the experiments investigating the effect of localized vasodilation (produced by minoxidil) on the outward permeation of isoflurane. Isoflurane was administered by continuous intravenous infusion in doses ranging from 138.1 to 149.2 mg. The 30-minute blood sample of rat 5 (45.5 μg/ml) did not fit the expected pharmacokinetic profile observed in other experiments, (i.e., rapid increase in isoflurane-blood concentration in the first 15 minutes, then fairly steady concentrations until the infusion was terminated) and was excluded from the calculations for clearance and half-life and was not considered to be the representative Cmax. The resulting pharmacokinetic parameters are presented in Table 7. The peak isoflurane-blood concentrations and AUC values ranged from 18.9 to 24.7 μg/ml and 1493 to 2723 μg-min/ml, respectively. There was no statistically significant difference ($p = 0.09$) between the means of the model-independent (223.3 ± 19.1 ml/min/kg) and model-dependent (255.8 ± 32.1 ml/min/kg) estimates for systemic clearance of isoflurane and the estimates were similar to those derived in previous

TABLE 7.

PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS AFTER LOCAL APPLICATION OF MINOXIDIL

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	19.3	1493	294.6	372.2	61.9
2	19.1	1775	195	223	63.6
3	23.9	2028	222.5	275.2	53.3
4	18.9	1841	218	216	44.7
5#	24.7	2723	186.5	192.8	44.7
MEAN	21.2	1972	223.3	255.8	53.6
SEM	1.3	206.5	19.1	32.1	4
% CV	14	23	19	28	17

The isoflurane-blood concentration of the 30-minute sample (45.5 μg/ml) did not fit the expected pharmacokinetic profile. Therefore, the 30-minute sample was excluded from the pharmacokinetic calculations of Cl_{M-I}, Cl_{FIT}, and half-life and was not considered as the Cmax observation.

experiments. The mean (± SEM) elimination half-life was 53.6 ± 4 minutes and was also similar to that in previous experiments.

The effect of minoxidil in increasing the amount of isoflurane permeating through the skin was clearly apparent. The amount of isoflurane recovered from the TVCD's ranged from 0.83 to 8.98 $\mu g/cm^2$ which resulted in Kp_{eff} values ranging from 0.0228 to 0.3464 cm/hr. Three Kp_{eff}'s appeared to be extreme observations, were significant as outliers by Dixon's test, and were excluded from additional data analysis. The individual Kp_{eff} values from each site on each rat ranged from 0.0228 to 0.0984 cm/hr and the mean (± SEM) Kp_{eff} of each rat is shown in figure 16. The grand mean (± SEM) Kp_{eff} was 0.0528 ± 0.0037 cm/hr (CV = 33%).

Relative skin blood flow measurements, using LDF, are shown in figure 17. The mean baseline perfusion units of the five rats in the minoxidil treatment group was similar to baseline measurements of previous experiments. The mean (± SEM) baseline blood flow of all five rats was 10.6 ± 1.4 PU and ranged from 7.5 to 15.3 PU. The mean (± SEM) blood flow of three rats (rats 2 and 4 were excluded because they had received an intravenous injection of Evans blue dye) during the post-intradermal injection period was 18.6 ± 0.7 PU and was significantly different ($p = 0.0072$) from the baseline period. Visual inspection of the individual perfusion unit over time profiles indicated that the full putative vasodilatory effect of minoxidil had not been attained at the

FIGURE 16. EFFECTIVE PERMEABILITY COEFFICIENTS AFTER LOCAL APPLICATION OF MINOXIDIL

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The TVCD sites had intradermal injections of 50 μl of minoxidil (167 μg) solution prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean. Rats 2, 3, and 4 had an N = 4, while rats 1 and 5 had an N = 5.

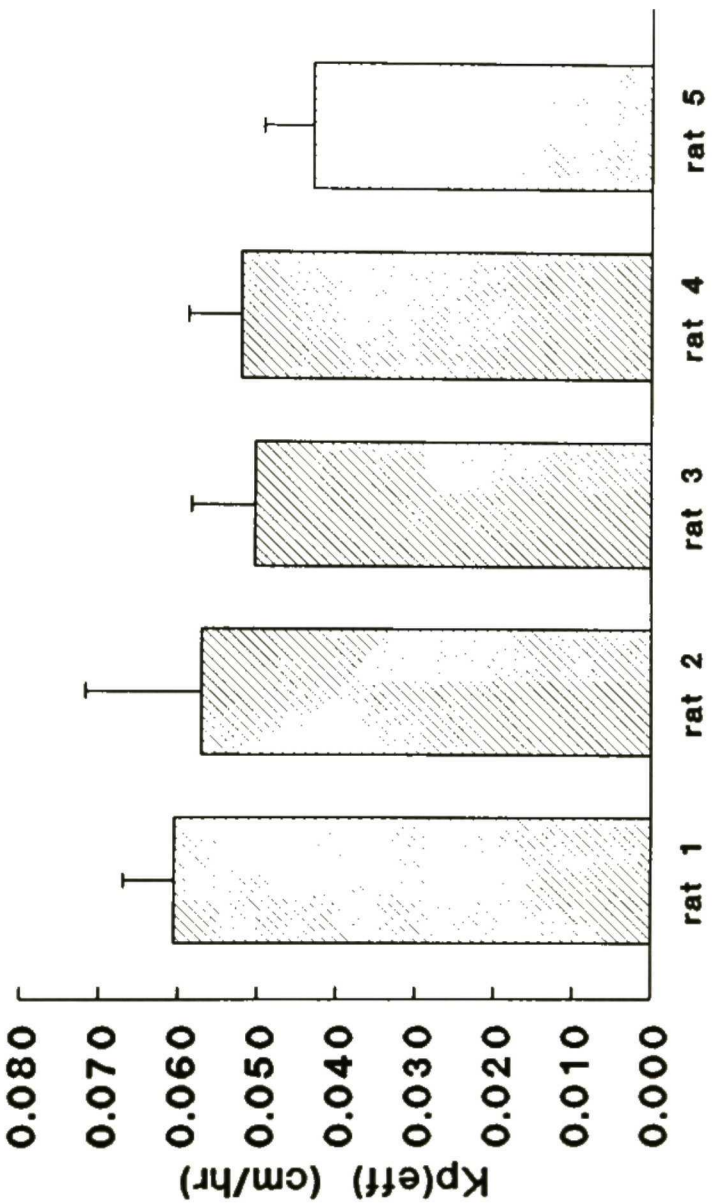
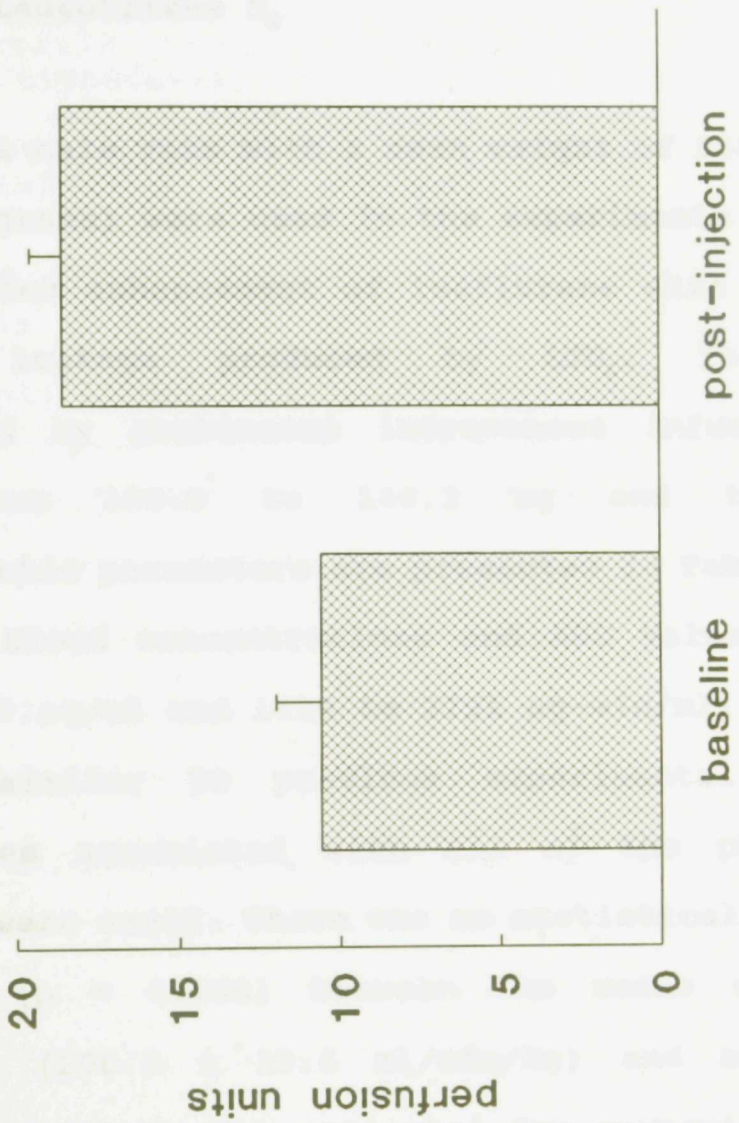

0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

FIGURE 17. SKIN BLOOD FLOW BEFORE AND AFTER LOCAL APPLICATION OF MINOXIDIL SOLUTION

Skin blood flow (expressed in perfusion units) was measured using LDF. Baseline measurements, over an approximate seven minute period (about 210 observations), were obtained just prior to the intradermal injection of 50 μl of minoxidil (167 μg) solution in each of five rats. The post-injection period, measured at the baseline measurement site, consisted of 90 minutes of continuous monitoring (about 2700 observations) in each rat. The height of the bars represent the mean PU of all rats and the error bars represent the standard error of the mean. The mean baseline and post-injection values have N's of 5 and 3, respectively, and were significantly different ($p = 0.0072$).

(Rats 2 and 4 were excluded from the post-injection period because of Evans blue dye interference with the LDF measurements.)


20
15
10
5
0
perfusion units
baseline
post-injection

end of the 90 minute monitoring period as there was an apparent increasing trend of the perfusion units in all three rats.

iii. Leukotriene D_4

Five male rats with a mean weight of 348 grams (range 323 to 381 grams) were used in the experiments investigating the postulated enhancement of isoflurane skin permeation by capillary leakage produced by LTD_4. Isoflurane was administered by continuous intravenous infusion in doses ranging from 138.9 to 149.2 mg and the resulting pharmacokinetic parameters are presented in Table 8. The peak isoflurane blood concentrations and AUC values ranged from 15.5 to 28.7 μg/ml and 1436 to 2721 μg-min/ml, respectively, and were similar to previous experimental groups. The variabilities associated with all of the pharmacokinetic parameters were small. There was no statistically significant difference ($p = 0.143$) between the means of the model-independent (206.1 ± 19.6 ml/min/kg) and model-dependent (211.5 ± 21.4 ml/min/kg) estimates for systemic clearance of isoflurane. The mean (± SEM) elimination half-life was 44.8 ± 3.5 minutes.

The postulated capillary permeability enhancing effect of LTD_4 produced a noticeable increase in the amount of isoflurane permeating through the skin. The amount of isoflurane recovered from the TVCD's ranged from 0.12 to 17.52

TABLE 8.

PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS AFTER LOCAL APPLICATION OF LEUKOTRIENE D_4

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	24.3	2186	197.8	197.5	48.1
2	20.9	1716	212.3	215.5	40.3
3	21.8	2195	186.5	186.5	36.1
4	15.5	1436	276	291	43
5	28.7	2721	157.9	167	56.3
MEAN	22.2	2051	206.1	211.5	44.8
SEM	2.2	221	19.6	21.4	3.5
% CV	22	24	21	23	17

$\mu g/cm^2$ which resulted in Kp_{eff} values ranging from 0.0031 to 0.5548 cm/hr. One of the Kp_{eff}'s appeared to be an extreme observation, was significant as an outlier by Dixon's test, and was excluded from additional data analysis. The individual Kp_{eff} values from each site on each rat ranged from 0.0031 to 0.1279 cm/hr and the mean (± SEM) Kp_{eff} of each rat is shown in figure 18. The grand mean (± SEM) Kp_{eff} was 0.0554 ± 0.0058 cm/hr (CV = 51%) which was similar to the Kp_{eff} observed in the minoxidil group.

Relative skin blood flow measurements, using LDF, are shown in figure 19. The LDF measurements of rat 3 were lost due to a communication malfunction between the LDF instrument and the computer. The mean baseline perfusion units of the four rats in the LTD_4 group was similar to baseline measurements of previous experiments. The mean (± SEM) baseline blood flow of the four rats was 12.3 ± 1.9 PU and ranged from 7.8 to 16 PU. The mean (± SEM) blood flow of three rats (rats 2 was excluded because it had received an intravenous injection of Evans blue dye) during the post-intradermal injection period was 6.1 ± 0.8 PU and was significantly different ($p = 0.0032$) from the baseline period.

iv. Ether-ethanol

Five male rats with a mean weight of 366 grams (range of 333 to 406.5 grams) were used in the experiments investigating the effect of chemical disruption of the stratum

FIGURE 18. EFFECTIVE PERMEABILITY COEFFICIENTS AFTER LOCAL APPLICATION OF LEUKOTRIENE D_4

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The TVCD sites had intradermal injections of 50 μl of leukotriene D_4 (200 ng) solution prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean. Rat 3 had N = 4 and the other rats had N = 5.

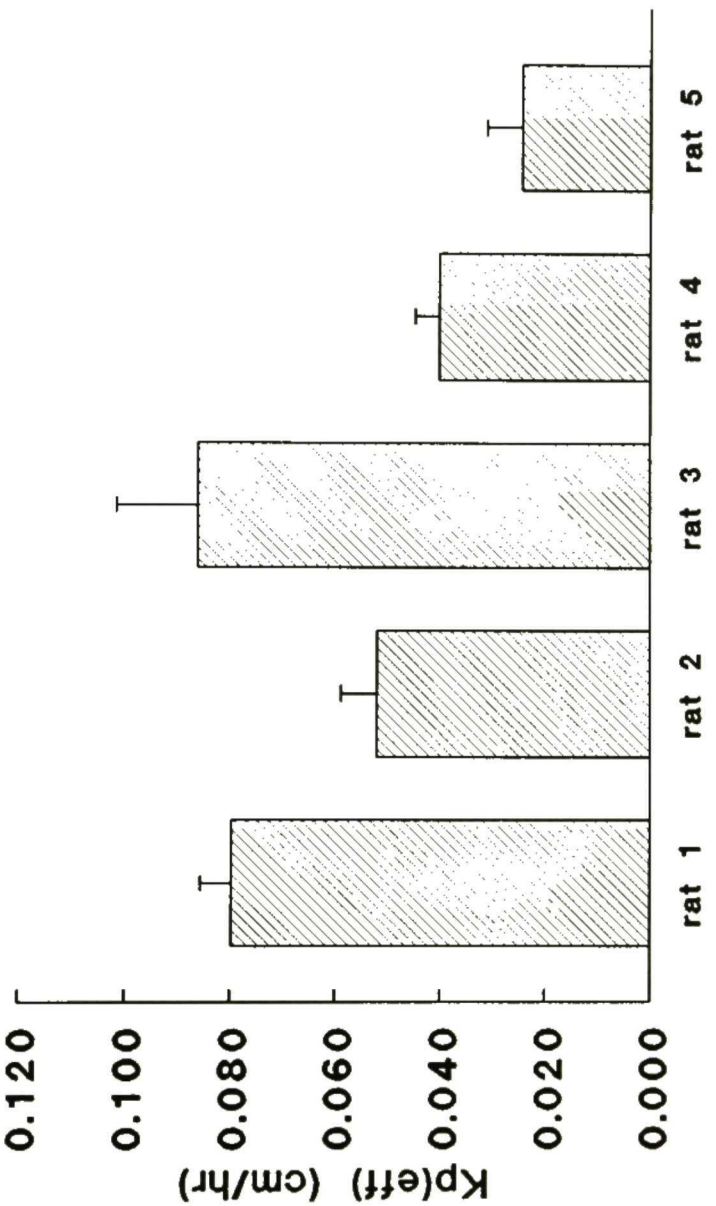
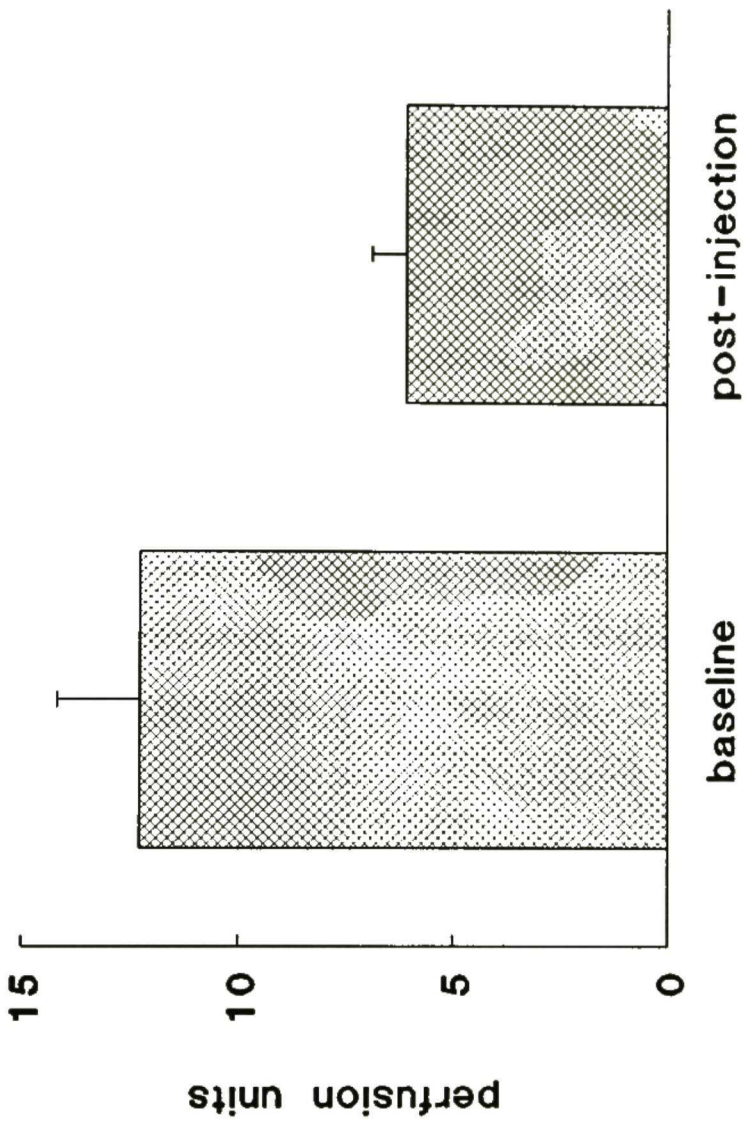

0.120
0.100
0.080
0.060
0.040
0.020
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

FIGURE 19. SKIN BLOOD FLOW BEFORE AND AFTER LOCAL APPLICATION OF LEUKOTRIENE D_4

Skin blood flow (expressed in perfusion units) was measured using LDF. Baseline measurements, over an approximate seven minute period (about 210 observations), were obtained just prior to the intradermal injection of 50 μl of leukotriene D_4 (200 ng) solution in each of five rats. The post-injection period, measured at the baseline measurement site, consisted of 90 minutes of continuous monitoring (about 2700 observations) in each rat. The height of the bars represent the mean PU of all rats and the error bars represent the standard error of the mean. The mean baseline and post-injection values have N's of 4 and 3, respectively, and were significantly different ($p = 0.0032$).

(Rat 2 was excluded from the post-injection period because of Evans blue dye interference with the LDF measurements.)


perfusion units
15
10
5
0
baseline
post-injection

corneum by ether-ethanol on the outward permeation of isoflurane through the skin. The approximate 16 hour pre-treatment of the skin with ether-ethanol solution had a markedly noticeable effect on the skin. The skin sites directly under the cotton pad of the Hill Top Chambers appeared shriveled and dehydrated. A thin layer of the skin, most likely the stratum corneum, was very loosely attached to the underlying layer and appeared to be very easily removable with a pair of forceps. Several of the ethanol-ether treated sites were excised at the termination of the experiment, prepared on microscope slides, and visually examined for gross changes in the anatomic structure of the skin. Figures 20 and 21, which are representative micrographs, are low power views of ether-ethanol treated LDF site and ether-ethanol treated site 5 respectively, from the same rat. In Figure 20, the whole epidermal layer is separated, but still within the visual field of the microscope lens, from the underlying dermis, however, in Figure 21, the epidermal layer is conspicuously missing. The total separation of the epidermal layer from the underlying dermis may be in response to the dehydrating and/or delipidization by the ether-ethanol solution or from the additional exacerbation of slide preparation.

After placement of the TVCD's over the sites which had local application of ether-ethanol solution, isoflurane was administered by continuous intravenous infusion in doses ranging from 132.5 to 149.2 mg. The resulting pharmacokinetic

FIGURE 20. PHOTOMICROGRAPH OF SKIN FROM LDF SITE AFTER LOCAL APPLICATION OF ETHER-ETHANOL SOLUTION

This micrograph (H & E, X 40) illustrates the appearance of the skin from the LDF site (for a description of location see Figure 4) on the dorsum of a male fuzzy rat. The site had undergone a 16 hour local application of an ether-ethanol solution as described in Materials and Methods. Compare this micrograph with Figure 5. The epidermal layer (middle of micrograph) has visibly separated from the underlying dermis.

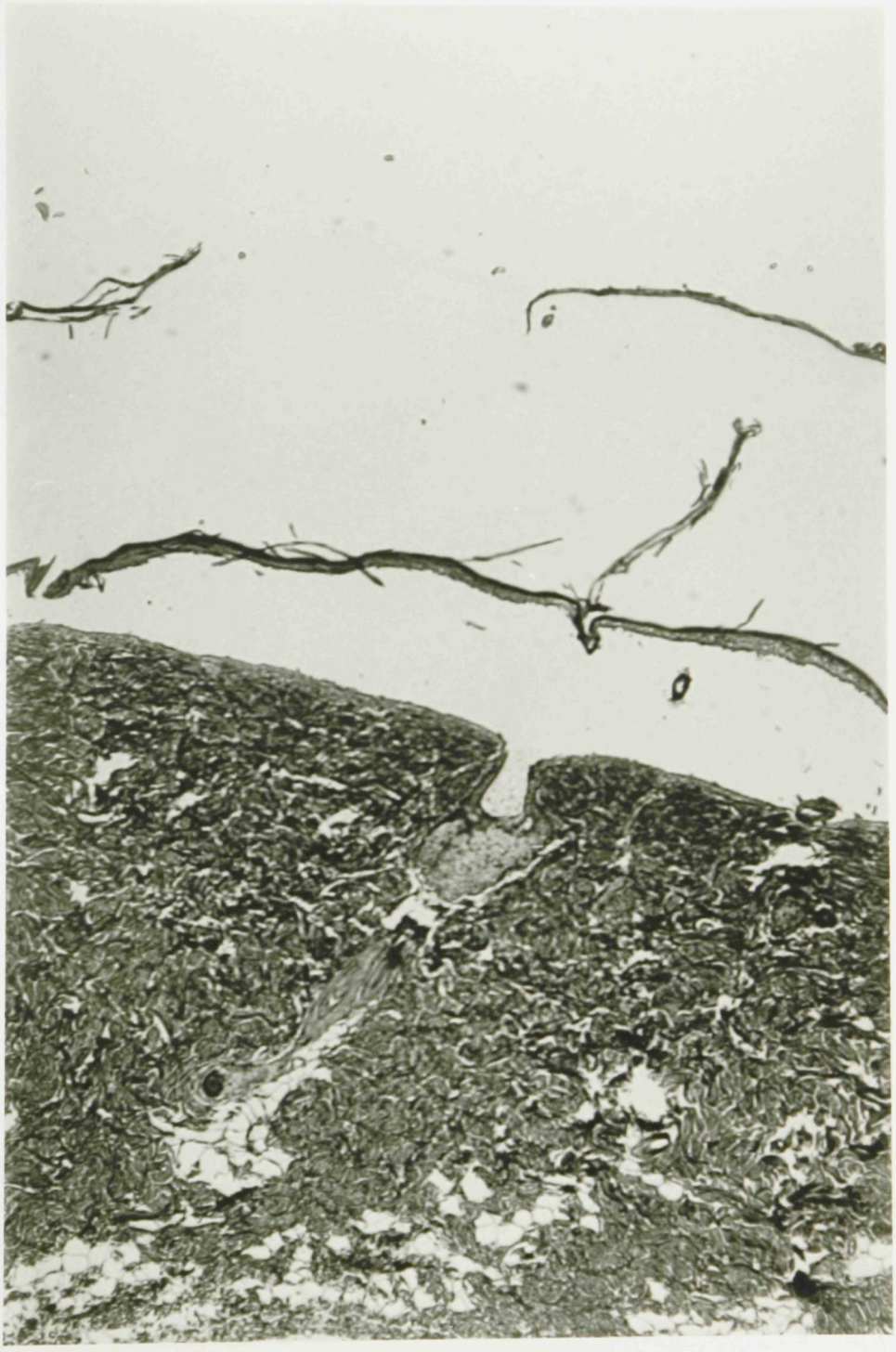
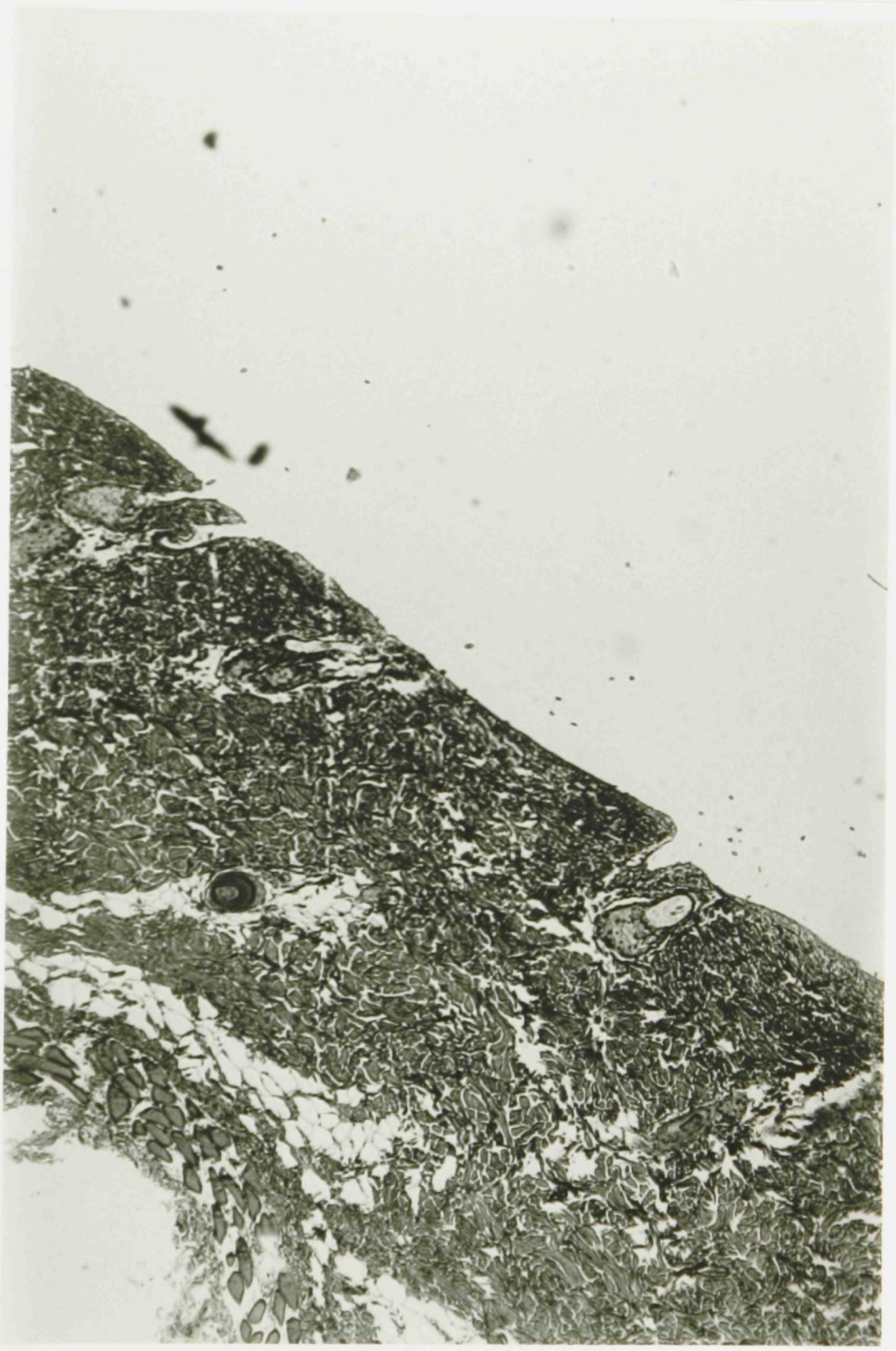


FIGURE 21. PHOTOMICROGRAPH OF SKIN FROM SITE 5 AFTER LOCAL APPLICATION OF ETHER-ETHANOL SOLUTION

This micrograph (H & E, X 40) illustrates the appearance of the skin from site 5 (see Figure 4 for a description of site location) on the dorsum of a male fuzzy rat. The site had undergone a 16 hour local application of an ether-ethanol solution as described in Materials and Methods. Compare this micrograph with Figure 21. The epidermal layer is totally missing in this micrograph.



parameters are presented in Table 9. The peak isoflurane-blood concentrations and AUC values ranged from 12.8 to 27.3 μg/ml and 1094 to 2547 μg-min/ml, respectively. These values were slightly lower when compared to previous experimental groups, however, the variability (coefficient of variation of about 30%) within this group was similar to the other groups. There was no statistically significant difference ($p = 0.39$) between the means of the model-independent (255.9 ml/min/kg) and model-dependent (267 ml/min/kg) derived estimates for systemic clearance of isoflurane and the estimates were similar to those observed with previous experimental groups. The mean (± SEM) elimination half-life was 43.7 ± 6.1 minutes and was also similar to that in previous experiments.

Topical ether-ethanol application to the skin had a very discernible effect on disrupting the barrier function of the stratum corneum as evidenced by the increased permeation of isoflurane. The amount of isoflurane recovered from the TVCD's ranged from 0.54 to 3.68 $\mu g/cm^2$ which resulted in Kp_{eff} values ranging from 0.0228 to 0.1721 cm/hr. The mean (± SEM) Kp_{eff} of each rat is shown in figure 22 and the grand mean (± SEM) Kp_{eff} was 0.0656 ± 0.0082 cm/hr (CV = 62%).

Skin blood flow was measured using LDF. There were no baseline (pre-treatment) measurements taken in this experiment because of the 16 hour pre-treatment time. Rats 3 and 4 had received an intravenous injection of Evans blue dye and their LDF measurements have been excluded. The mean (± SEM) skin blood flow of the three rats during the post-intradermal

TABLE 9.

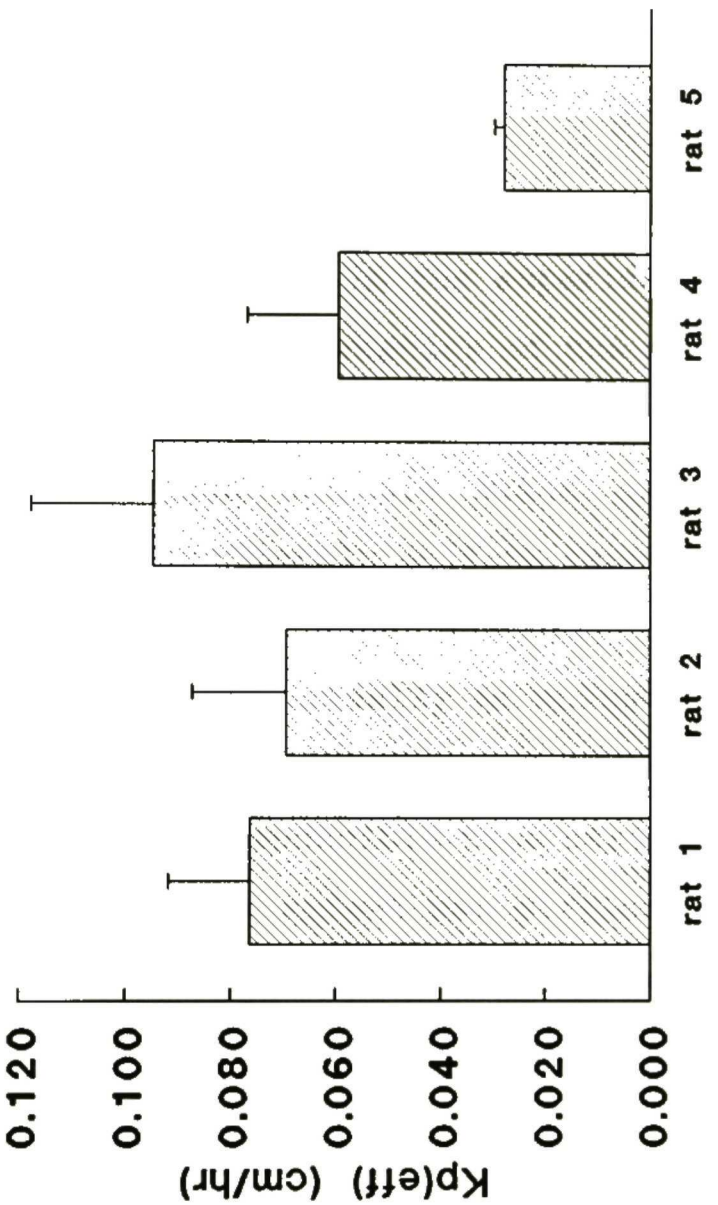
PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS AFTER CHEMICAL DISRUPTION OF THE STRATUM CORNEUM BY ETHER-ETHANOL SOLUTION

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	16.1	1525	275	300	53.3
2	12.8	1094	331.5	312.9	42
3	15.2	1466	272.9	275.5	26.7
4	15.3	1450	256.1	304.2	60.8
5	27.3	2547	144	142.6	35.5
MEAN	17.3	1616	255.9	267	43.7
SEM	2.6	245	30.7	31.7	6.1
% CV	33	34	27	27	31

FIGURE 22. EFFECTIVE PERMEABILITY COEFFICIENTS AFTER DISRUPTION OF STRATUM CORNEUM WITH ETHER-ETHANOL SOLUTION

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The TVCD sites had ether-ethanol solution applied approximately 16 hours prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean. Each rat had N = 5.


0.120
0.100
0.080
0.060
0.040
0.020
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

injection period was 17.9 ± 3.4 PU. Visual examination of the individual perfusion unit over time profiles indicated very little fluctuation in blood flow over the 90 minute monitoring interval.

v. Tape stripping

Five male rats with a mean weight of 349 grams (range 329 to 375 grams) were used in the experiments investigating the effect of mechanical disruption of the stratum corneum by tape stripping on the outward permeation of isoflurane through the skin. Even though all six sites on every rat were subjected to ten approximately uniform tape strippings, there were differences which were visually discernible. The differences ranged from virtually no change in appearance to glistening of the skin surface. It was apparent that the ability to perform a reproducible tape stripping of each skin site to exactly the same extent was difficult.

Several of the tape stripped sites were excised at the termination of the experiment, prepared on microscope slides, and visually examined for gross changes in the anatomic structure of the skin. Figures 23 and 24, which are representative samples, are low and high power views of tape stripped skin from site 4 (see figure 4 for description of site location), respectively. The stratum corneum laminates have been virtually removed (Fig. 23), except for possibly 1 or 2 remaining laminates, however, the rest of the skin does

FIGURE 23. PHOTOMICROGRAPH (LOW MAGNIFICATION) OF SKIN FROM SITE 4 AFTER TAPE STRIPPING

This micrograph (H & E, X 40) illustrates the appearance of the skin from tape stripped site 4 (see Figure 4 for description of site location) on the dorsum of a fuzzy rat. The tape stripping procedure was accomplished by ten applications and removals of scotch tape (new piece each time) to the skin site. The slide was prepared with two pieces of skin from the same site with the outer layers facing each other. The stratum corneum layer is virtually removed when compared with the micrograph in figure 5.

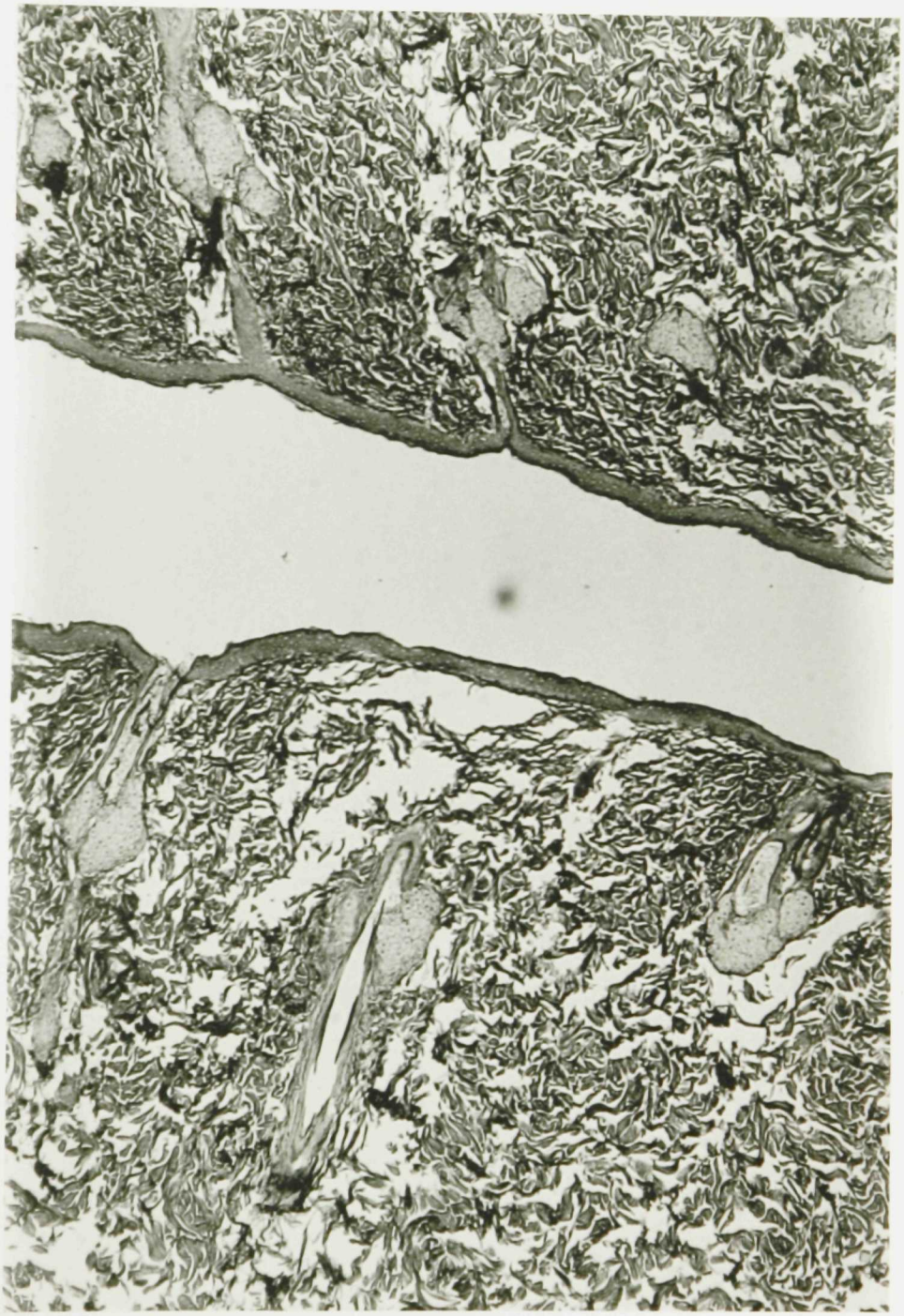
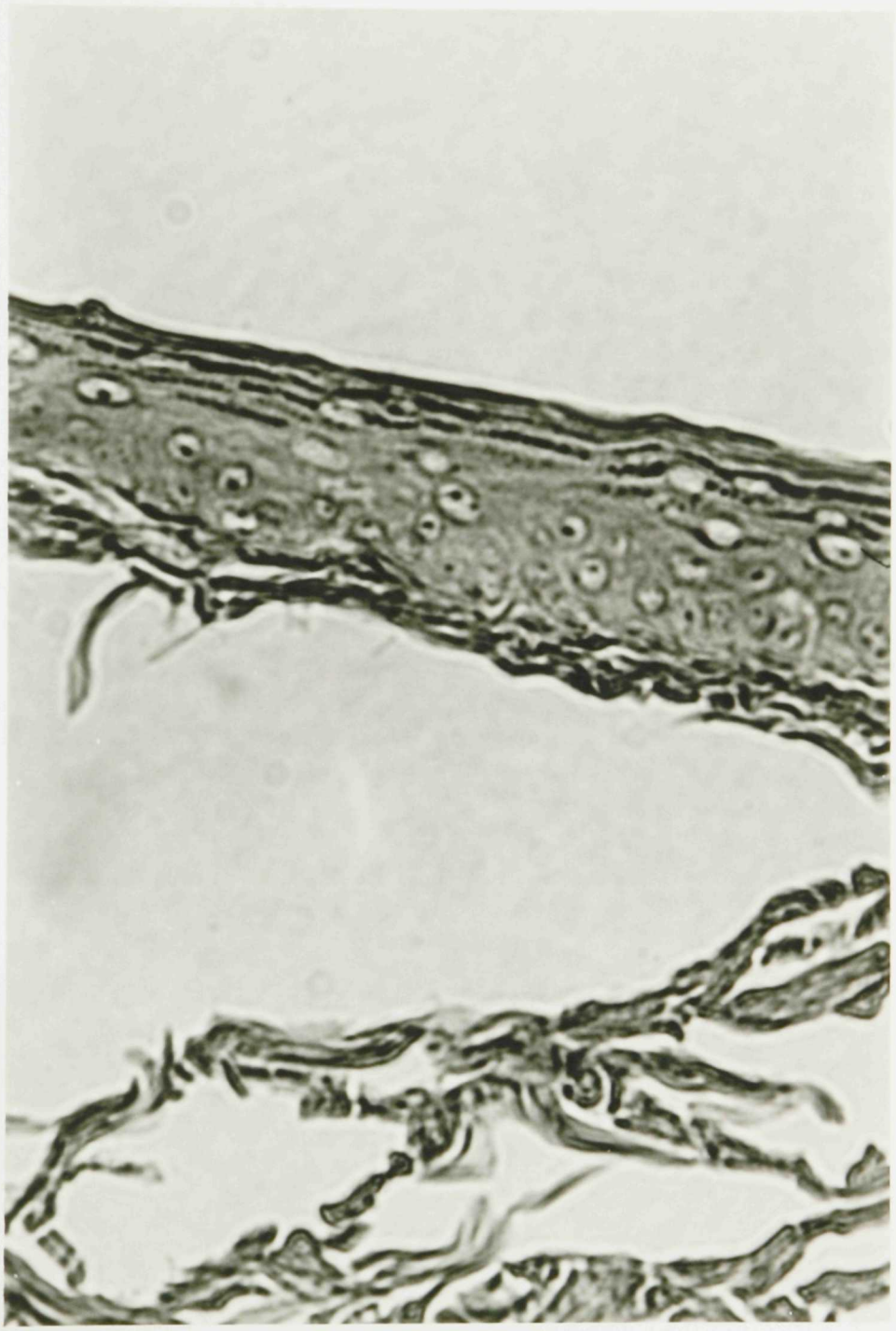


FIGURE 24. PHOTOMICROGRAPH (HIGH MAGNIFICATION) OF SKIN FROM SITE 4 AFTER TAPE STRIPPING

This micrograph (H & E, X 550), a magnification of a section from Figure 23, illustrates the histological appearance of the outermost skin layers after the site was tape stripped. Tape stripping was accomplished by ten applications and removals of scotch tape (new piece each time) to the skin site. Only a few layers of stratum corneum (uppermost layer of skin) are visible (compare to figure 6) and the epidermal layer appears to have separated from the underlying dermis.



not appear different from normal skin. Only a few of the laminates of the stratum corneum are visible in figure 24 as compared to the micrograph in figure 6. The separation of the epidermal layer from the underlying dermis was not observed in all of the tape-stripped skin microscope slides and it may be a slide preparation artifact.

After placement of the TVCD's over the sites which had been tape stripped, isoflurane was administered by continuous intravenous infusion in doses ranging from 138.9 to 149.6 mg. The resulting pharmacokinetic parameters are presented in Table 10. The peak isoflurane-blood concentration and AUC values ranged from 17.5 to 24.8 μg/ml and 1520 to 2295 μg-min/ml, respectively, and were similar to the results in previous experiments. There was markedly less variability in the pharmacokinetic parameters with this group of rats. There was no statistically significant difference ($p = 0.33$) between the means of the model-independent (230.5 ml/min/kg) and model-dependent (240.9 ml/min/kg) derived estimates for systemic clearance of isoflurane and the estimates were similar to those of previous experiments as was the mean (± SEM) elimination half-life of 45.9 ± 7.5 minutes.

Mechanical disruption of the stratum corneum by tape stripping had the most pronounced effect on the permeation of isoflurane through the skin. The amount of isoflurane recovered from the TVCD's ranged from 0.92 to 5.79 $\mu g/cm^2$ which resulted in Kp_{eff} values ranging from 0.0379 to 0.2435

TABLE 10.

PHARMACOKINETIC PARAMETERS OF ISOFLURANE IN FUZZY RATS AFTER MECHANICAL DISRUPTION OF THE STRATUM CORNEUM BY TAPE STRIPPING

Rats were administered intravenous isoflurane by continuous infusion and serial blood samples were analyzed for isoflurane content by gas chromatography. The Cmax value was obtained from visual inspection of the isoflurane-blood concentration over time profile and AUC was calculated to the last sampling time by the trapezoidal method. Cl_{M-I} and Cl_{FIT} are the model-independent and model-dependent derived estimates for clearance, respectively. Half-life was calculated from the terminal elimination rate constant.

RAT No.	Cmax (μg/ml)	AUC (μg-min/ml)	Cl_{M-I} (ml/min/kg)	Cl_{FIT} (ml/min/kg)	HALF-LIFE (minutes)
1	17.5	1636	245	243.6	57.3
2	18.1	1520	267.6	312	48.8
3	18.6	1699	235.7	229.1	32.8
4	23.4	1956	230.4	246.8	65.4
5	24.8	2295	174	172.8#	25.4#
MEAN	20.5	1821	230.5	240.9	45.9
SEM	1.5	138	15.5	22.2	7.5
% CV	16	17	15	21	36

The estimate of the weighting parameter in the curve fitting analyses of other rat data usually was 2, however, the estimate for rat 5 was approximately 0.1 which resulted in a very poor fit of the data. The pharmacokinetic parameter estimates for rat 5 were obtained by fixing the weighting parameter at a value of 2 and not allowing it to "float" (i.e., to be estimated) in the extended least squares regression analysis.

cm/hr. The mean (± SEM) Kp_{eff} of each rat is shown in figure 25 and the grand mean (± SEM) Kp_{eff} was 0.1017 ± 0.0097 (CV = 48%).

Relative skin blood flow measurements, using LDF, are shown in figure 26. The mean (± SEM) baseline blood flow of the five rats in the tape stripping group was 12 ± 1.7 PU and ranged from 9.4 to 18.6 PU. The mean baseline blood flow was very similar to that observed in previous experiments. The mean (± SEM) blood flow of three rats (rats 3 and 4 were excluded because they had received an intravenous injection of Evans blue dye) during the 90 minute post-tape stripping period was 10.3 ± 0.5 PU and was not significantly different ($p = 0.22$) from the baseline period. Visual examination of the individual perfusion unit over time profiles indicated very little fluctuation over the 90 minute monitoring interval.

D. Summary of LDF measurements of skin blood flow

The means of baseline blood flow of the individual experimental groups (saline control, phenylephrine, minoxidil, LTD_4, and tape stripping) were not significantly different from each other and all of the means were combined as an one overall baseline in the comparison of the individual modulators' effect on skin blood flow. The overall mean (± SEM) baseline blood flow was 12.3 ± 0.7 perfusion units (N = 24), which was the average of the baseline (prior to perturbing the skin) LDF measurements from the aforementioned

FIGURE 25. EFFECTIVE PERMEABILITY COEFFICIENTS AFTER DISRUPTION OF STRATUM CORNEUM BY TAPE STRIPPING

Isoflurane was extracted from TVCD's placed at five locations on the backs of fuzzy rats which had received a continuous intravenous infusion of isoflurane. The isoflurane on the TVCD's was extracted in hexanes and analyzed by gas chromatography. The skin of the TVCD sites had been tape stripped, which was accomplished by ten applications and removals of scotch tape (new piece each time), prior to placement of the TVCD's. The mean Kp_{eff} of each rat is represented by the height of the bars and the error bars represent the standard error of the mean. Each rat had N = 5.

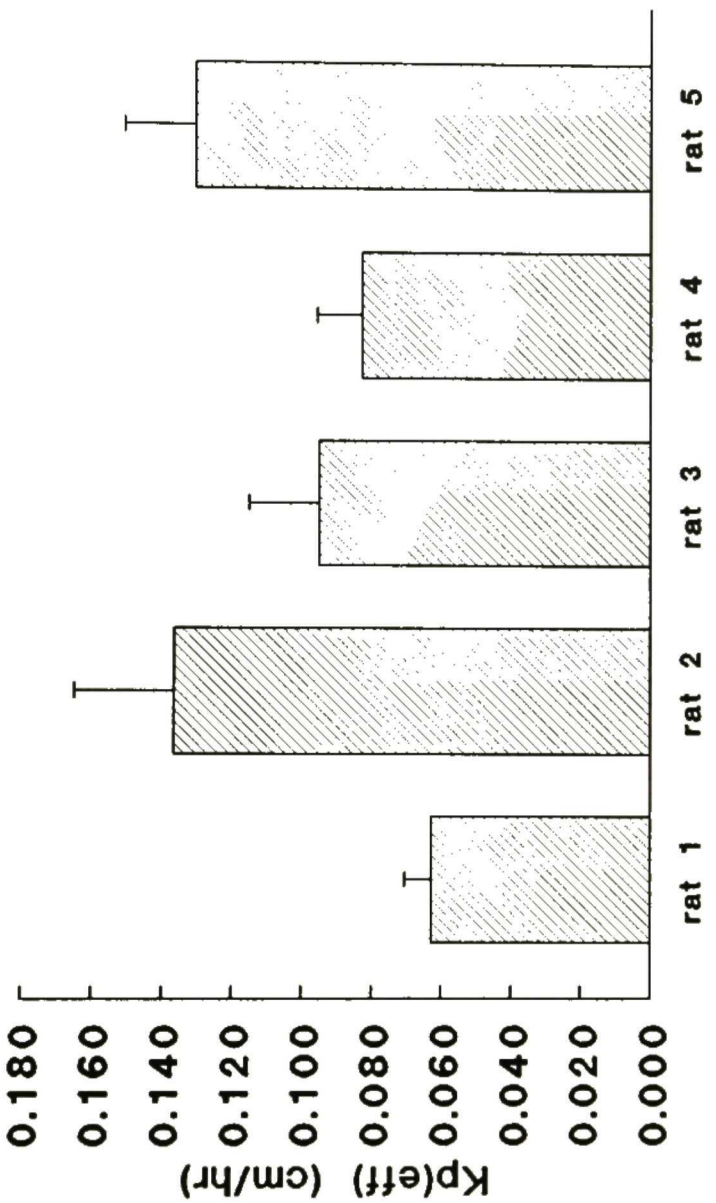
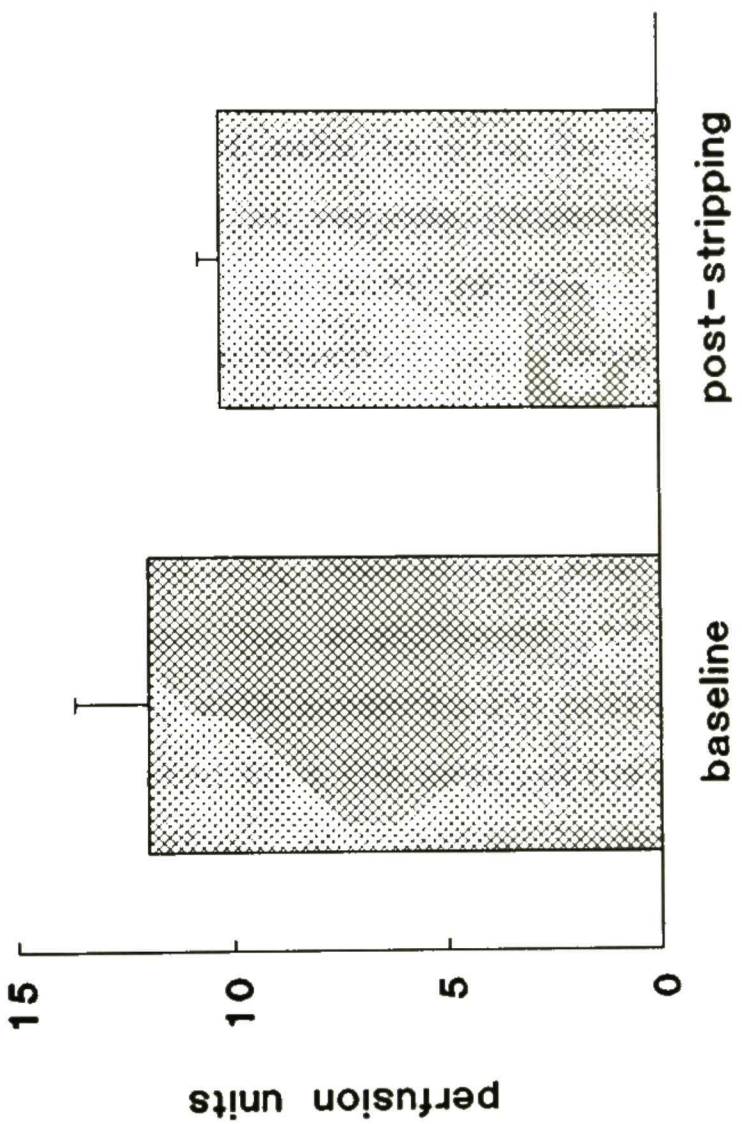

0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
Kp(eff) (cm/hr)
rat 1
rat 2
rat 3
rat 4
rat 5

FIGURE 26. SKIN BLOOD FLOW BEFORE AND AFTER TAPE STRIPPING OF SKIN SITES

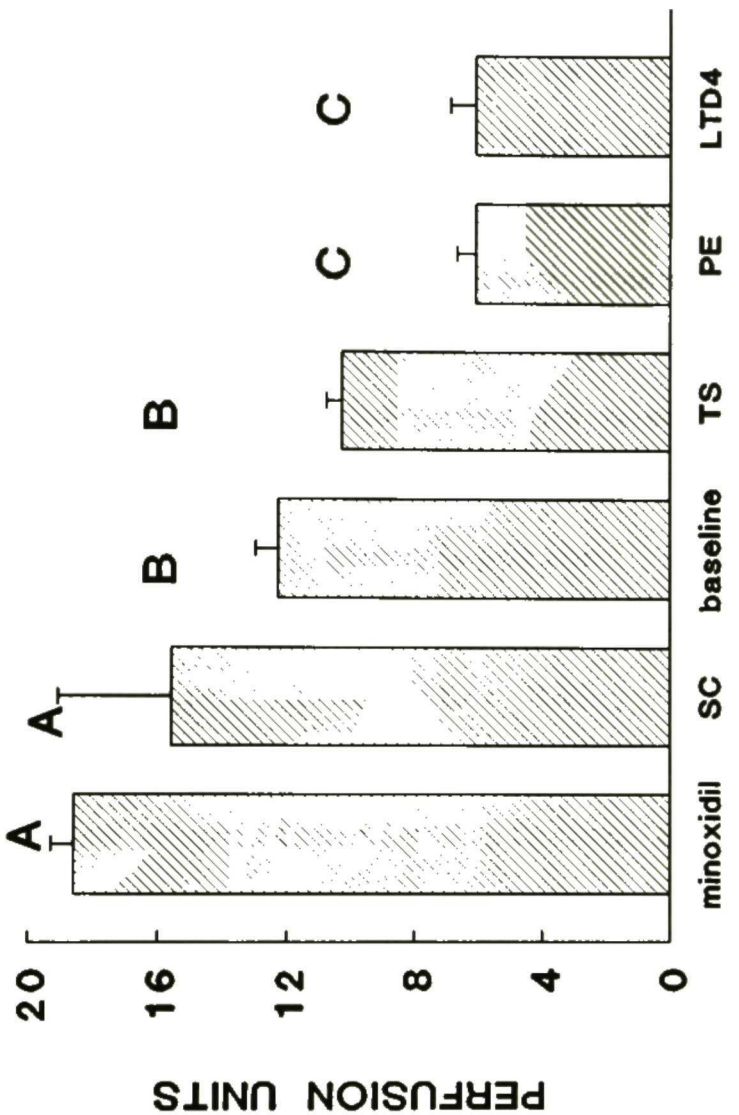
Skin blood flow (expressed in perfusion units) was measured using LDF. Baseline measurements, over an approximate seven minute period (about 210 observations), were obtained just prior to tape stripping of the skin sites, including the LDF site, in each of five rats. The post-tape stripping period, measured at the baseline measurement site, consisted of 90 minutes of continuous monitoring (about 2700 observations) in each rat. The height of the bars represent the mean PU of all rats and the error bars represent the standard error of the mean. The mean baseline and post-stripping values have N's of 5 and 3, respectively, and were not significantly different ($p = 0.22$).


perfusion units
15
10
5
0
baseline
post-stripping

experimental groups. The sound reproducibility (CV = 26%) of the baseline measurement provided supporting evidence that the LDF probe was placed at about the same location in all the rats and the blood flow, at least as measured with LDF, on the backs of fuzzy rats used in this research, was fairly uniform. The variation at that site was minimal even though the observations were from rats ranging in weight from 306 to 414.5 grams which were at various (uncontrollable) levels of anesthesia. The means (± SEM) of the baseline and post-"treatment" LDF measurements are shown in figure 27. Application of minoxidil resulted in a significant increase in perfusion units, as compared to baseline, while application of phenylephrine and LTD_4 resulted in significant decreases. Tape stripping had no significant effect on the LDF measurements. ANOVA, with Bonferroni's correction for multiple comparisons, indicated that the mean perfusion units following intradermal injection of saline (control group) was significantly increased from the overall mean baseline. However, the reliability of this result may be questionable since LDF measurements of only two rats were included in the post-intradermal injection period of the control group. Furthermore, in comparing the post-injection blood flow of each rat in the control group to its own baseline, the increase was not significant.

FIGURE 27. SKIN BLOOD FLOW PRE- AND POST-SKIN PERTURBATIONS

Skin blood flow was measured using LDF and expressed in perfusion units. Baseline measurements (an approximate 7 minute [210 observations] monitoring period) were obtained prior to performing the various experimental perturbations. The post-skin perturbation period was 90 minutes (2700 observations). The baseline measurement is the average of all the rats (N = 24). The minoxidil, SC (saline control), TS (tape stripping), PE (phenylephrine),and LTD_4 groups had N's of 3, 2, 3, 4, and 3, respectively. The mean perfusion units are represented by the height of the bars and the error bars represent the standard error of the mean. The letters (A, B, or C) above the bars indicate the statistical comparisons, i.e., the mean perfusion units of experimental groups with the same letter were not significantly different.

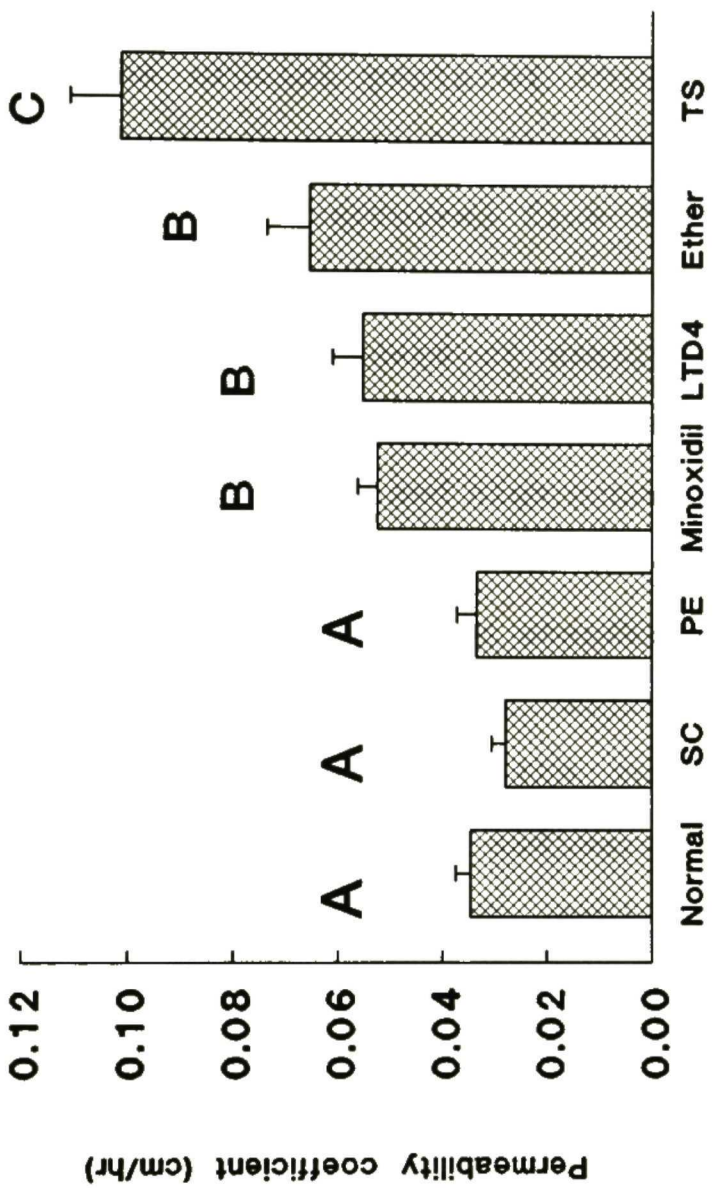

PERFUSION UNITS
20
16
12
8
4
0
A
A
B
B
C
C
minoxidil
SC
baseline
TS
PE
LTD4

E. Summary of effective permeability coefficient data

The group mean effective permeability coefficients from normal and perturbed skin experiments are shown graphically in figure 28. ANOVA indicated that there were significant differences between the mean Kp_{eff} observed under the various experimental conditions but there were no significant differences between the TVCD sites. Using Bonferroni's correction for multiple comparisons, the mean Kp_{eff} as a result of tape stripping of the stratum corneum was significantly increased as compared to normal skin and other experimental conditions. The mean Kp_{eff}'s of normal skin, saline control, and phenylephrine experiments were statistically similar but were significantly lower as compared to the other groups. Minoxidil, LTD_4, and ether-ethanol perturbations resulted in mean Kp_{eff}'s which were statistically similar but were significantly different as compared to the other groups.

FIGURE 28. EFFECTIVE PERMEABILITY COEFFICIENTS AFTER VARIOUS EXPERIMENTAL CONDITIONS

The mean Kp_{eff}'s of the various experimental conditions are represented by the height of the bars. Normal = unperturbed skin, SC = intradermal saline "control" solution, PE = intradermal phenylephrine solution, Minoxidil = intradermal minoxidil solution, LTD_4 = intradermal leukotriene D_4 solution, Ether = topical ether-ethanol application and TS = tape stripped skin. SC, PE, Ether, and TS included 25 observations each and Normal, Minoxidil, and LTD_4 had N's of 26, 22 and 24, respectively. The error bars represent the standard error of the mean. The letters (A, B, or C) above the bars indicate the statistical comparisons, i.e., the mean Kp_{eff}'s of groups with the same letter were not significantly different.


Permeability coefficient (cm/hr)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A
A
A
B
B
B
C
Normal
SC
PE
Minoxidil
LTD4
Ether
TS

DISCUSSION

Isoflurane assay

One of the crucial parts of this project was the development of a reliable assay for quantifying the amount of isoflurane in rat blood, recovered from TVCD's, and in the fat-emulsion mixture. A single calibration equation enabled quantitation over the two orders of magnitude range of the calibration curve. The technique of using two linear calibration equations (for low and high concentration samples) was evaluated but found not to provide as accurate estimates (of the back-calculated calibration standards) as the single log transformed calibration equation. Regression analysis of the calibration curve data using the relatively recently developed extended least-squares software, MKMODEL (which enables variance model parameter estimation), compared favorably with the well recognized and accepted least-squares SAS program. The advantages of using MKMODEL were its "user-friendliness", speed, and ability to estimate the weighting power which would provide the best fit of the experimental data. The results of the validation study indicated that the technique for isoflurane analysis yielded reproducible and accurate measurements of isoflurane concentration in pesticide

grade hexanes.

The recovery of isoflurane from blood, using a novel reverse extraction procedure, was essentially 100% for the range of concentrations examined. This finding agrees favorably with the report of McDougal and coworkers (1990), where they reported 100% extraction efficiency of isoflurane from blood.

Intravenous isoflurane

The formulation of isoflurane in fat emulsion made possible the intravenous administration of this anesthetic agent, heretofore only administered by inhalation. The short term safety of administering isoflurane intravenously was substantiated by the ability of the animals to tolerate the drug by this route during the course of the experiments. However, no morphologic or histologic examinations of blood vessels or other organs were conducted as to the complete safety of intravenously administered isoflurane. Administering isoflurane by intravenous infusion afforded a means of carefully titrating the dose and accurately quantifying the total dose delivered.

Scheuplein and Bronaugh (1983) described a period of lag time before a diffusing substance appears in appreciable quantity on the exit side of skin. To rapidly circumvent the transient (non-steady state) characteristics of skin permeability, constant blood concentrations of isoflurane

needed to be achieved very quickly. The information necessary to calculate an intravenous isoflurane infusion rate was derived from a dose-ranging experiment which was used to obtain a gross estimate for systemic clearance of 60 ml/min. Using the following equation,

$$C_{ss} = R_i/Cl$$

where: C_{ss} = the steady-state concentration in blood (μg/ml)

R_i = the rate of infusion (μg/min)

Cl = the clearance from blood (ml/min),

a theoretical C_{ss} of about 25 μg/ml was targeted with an approximate infusion rate of 1500 μg/min. The ability to successfully apply traditional pharmacokinetic concepts, such as the aforementioned formula, for this novel route of isoflurane administration is shown by the mean (± SEM) peak isoflurane concentration of 20.8 ± 1 μg/ml (N = 30), which was 80% of the targeted steady state concentration. Furthermore, visual observation of the shapes of the isoflurane-blood concentration profiles indicated that near peak concentrations were achieved rapidly (usually in less than 15 minutes) and maintained, with very little oscillations, until the infusion was terminated.

Pharmacokinetic parameter estimates of isoflurane have not been reported for the rat, even for inhalational

administration. However, comparison of estimates reported in humans and pigs can be made. Yasuda and coworkers (1991a) reported a mean (± SD) estimate for the pulmonary clearance of isoflurane in humans of 3.62 ± 0.41 L/min which they derived from the volume of the central compartment (V_1) times the elimination rate constant from the central compartment (k_{10}). In another report (Yasuda et al. 1991b), they indicated that the total body clearance of isoflurane in humans was 4 ± 0.5 L/min. In pigs, Yasuda and coworkers (1990) reported mean V_1 of 0.76 L and mean k_{10} of 1.34 min^{-1} which, when calculated as above, results in a clearance of 1.02 L/min. Normalizing the above clearance data by weight yields an approximate clearance of 50 ml/min/kg which is about 1/4 of the model-dependent derived systemic clearance observed in this project (227 ± 10 ml/min/kg, N = 30 [includes only the rats which received isoflurane by continuous intravenous infusion]). The disparity in the systemic clearance estimates may largely be influenced by difference in the control of pulmonary ventilation. Pulmonary ventilation (respiratory rate and tidal volume) is a major controlling factor in the systemic clearance of isoflurane since it is almost exclusively eliminated through the lung. Pulmonary ventilation in Yasuda's experiments was highly controlled (subjects and animals were intubated and on respirators), whereas in this project the animals were permitted to breathe on their own. Additionally, as pointed out previously, Yasuda and coworkers

(1991b) remarked about the uncertainty of their estimates for V_1 and the time constant for lung (the bulk of the central compartment) which may have contributed to erroneous estimates for some of their parameters. However, the multiexponential model derived rate constants (converted to elimination half-life) of the muscle group (the third compartment in their five compartment model) by Carpenter and coworkers (1986) and Yasuda and coworkers (1991a), of 53 and 55 minutes, respectively, compared very favorably with 45 ± 3 minute (N = 30) estimate of elimination half-life in this project. This favorable comparison may be due to chance or it may indicate that the elimination compartment (in the 2-compartment, zero-order input, pharmacokinetic model used in this project) represented elimination from a compartment similar to the third compartment of Yasuda and Carpenter. The estimate of elimination half-life of isoflurane was not heavily influenced by experimental procedure (i.e., control of pulmonary ventilation), but was mostly dependent on the partitioning of isoflurane within the various tissues in the body.

Characterization of collection device

The Freundlich or the Langmuir isotherm equations are usually used to characterize activated charcoal adsorption studies. Those two models relate the ratio of compound adsorbed per unit mass of charcoal to the free concentration

of the same compound at equilibrium. In this study, the goal was not to determine the maximum amount of isoflurane which could be adsorbed, since it was presumed that the charcoal/PTFE had a high capacity for isoflurane, but to ascertain that the desorption (by extraction in hexanes) was nearly complete. The high capacity is evident from the results of the in vitro on/off-loading studies which showed that nearly 4,000 μg of isoflurane could be adsorbed to the charcoal/PTFE active component of the TVCD. Open-air isoflurane desorption studies showed that the affinity of isoflurane for charcoal/PTFE was relatively low. Analysis of those studies indicated that the desorption process can be interpreted to be a two phase process, with half-lives of 5 and 144 minutes, respectively. The rapid and dominant initial half-life confirmed the necessity of rapid removal of the charcoal/PTFE disks from the TVCD and placing them in hexanes immediately. The 100% recovery of isoflurane demonstrated the complete extraction efficiency of the hexanes procedure.

Laser Doppler flowmeter measurements of skin blood flow

The results obtained in the various studies using LDF to measure skin blood flow clearly illustrate the strengths and advantages inherent in this non-invasive method over other non-dynamic measurement methods. However, the potential interference of Evans blue dye with LDF was not prospectively

recognized. The close proximity of the $lambda_{max}$ (610 nm) of Evans blue to the helium-neon laser light (632.8 nm) of LDF resulted in altered LDF response (in some cases less than 0.2 perfusion units which equates to no blood flow) in those rats which had been injected intravenously with Evans blue dye. Pilot studies in humans of skin blood flow in the forearm, palm and finger using LDF demonstrated that the methodology can qualitatively differentiate variations in skin blood flow from sites known to have differing blood flow.

The lack of universally accepted and standardized methodology for the analysis of blood flow measurements using LDF necessitated the development of a procedure which would be suitable for this project. Most investigators have used the voltage output from laser Doppler instruments and expressed flow as voltage or as arbitrary units (averaging the values from a short duration of observation, usually 1 - 3 minutes). Sundberg and Castren (1986) observed stable baseline LDF measurements after about 3 minutes. After review of LDF data from pilot experiments in this project, it was apparent that short periods of observation (less than 3 minutes) were highly variable and a longer (approximately 7 minutes) period was used to obtain an estimate for baseline (pre-perturbation) blood flow. The reproducibility of mean (± SEM) baseline blood flow of 12.3 ± 0.7 PU demonstrates that the procedure for estimating baseline blood flow was acceptable.

The relative increase in skin blood flow in the

intradermal saline (control) injection group was not significant when compared to its own baseline flow measurement, however, when compared to the overall baseline (from all the groups), it was significant. The moderate hyperemia may have been due to the trauma of injection similar to the observations of others (Holloway and Watkins 1977; Bisgaard, Kristensen and Sondergaard 1982; Millay, Larrabee and Carpenter 1991).

Amantea and coworkers (1983) attempted to quantify the degree of vasoconstriction produced by corticosteroids applied to the skin (the skin blanching assay) using laser Doppler velocimetry, but were unsuccessful. One can only conjecture that their failure resulted from the corticosteroid blanching action occurring at a depth within the skin which LDF could not quantify. In the experiments detailed previously, intradermal injection of phenylephrine resulted in a 50% reduction from baseline LDF measurements. However, the vasoconstrictor effect appeared to diminish in the latter half of the monitoring period, as the LDF measurements began returning toward baseline levels after about 45 minutes. The short vasoconstrictor action of phenylephrine was reported by Millay and coworkers (1991), where they observed cutaneous blood flow returning to near baseline levels approximately 30 minutes after subcutaneous injection of phenylephrine. Krueger and coworkers (1985) reported skin blood flow decreases to 15-20% of baseline which lasted 30-60 minutes after iontophoresis

of phenylephrine. After tape stripping of the skin, there was an observed, though not statistically significant, reduction in skin blood flow.

LTD_4 reduced skin blood flow to an even greater extent than phenylephrine and its action was maintained for the duration of the experiment. This observation compares favorably with the report of M. J. Peck and coworkers (1981), where they observed a 45% reduction in blood flow with a dose of 100 ng of LTD_4 in guinea-pig skin but is in contrast to the increase in flow observed in humans by Bisgaard (1987). The specific mechanism of action of LTD_4 on the microcirculation has not been completely elucidated and the contrasting observations may be due to species differences or different doses used by the investigators.

Initially (in a pilot experiment), minoxidil was used as a 1% solution which resulted in a substantial increase in skin blood flow, as measured by LDF. However, both animals died approximately 40 minutes after the intradermal injection. The two-thirds reduction of applied minoxidil concentration generated marked increase in skin blood flow relative to baseline. Almost identical results were reported by Hirkaler and Rosenberger (1989). Their topical application of minoxidil (at a dose of 0.15 mg/kg) on the abdominal skin of male Sprague-Dawley rats increased cutaneous blood flow by more than 80%. Wester and coworkers (1984), using laser Doppler methodology, were able to detect a significant increase in

blood flow on the skin of the scalp of humans with a 5% minoxidil solution but not with 1% and 3% solutions. In contrast, De Boer and coworkers (1988), also using laser Doppler methodology, did not observe increased blood flow on the human forearm even with a 5% minoxidil solution. An explanation for the contrasting observations may be related to the different sites used or a variation in absorption of minoxidil from dissimilar vehicles. The scalp, an area of relatively high skin blood flow, may have more of a reserve capacity for an increase but the forearm, an area of relatively low skin blood flow, may not have the capacity for a significant increase. The mechanism of action for the increased skin blood flow produced by the topical application of minoxidil is problematic in view of McCall and coworkers (1983) report that minoxidil is not active in vitro but must be metabolized by hepatic sulfotransferase to the active molecule, minoxidil N-O sulfate. Hamamoto and Mori (1989) have shown that sulfation of minoxidil occurs in rat keratinocytes which must occur very rapidly in view of the almost immediate increase in skin blood flow elicited by locally applied minoxidil. However, the observed cutaneous vasodilation could also be due to return of sulfated minoxidil to skin after distribution to and from the liver via the systemic circulation.

It is important to stress that the estimates of cutaneous blood flow (expressed as perfusion units) obtained

using LDF in this project were not absolute measurements of this parameter and were instrument- and laboratory-specific. However, in this project, the same instrument and calibration techniques were employed for all rats, making the comparison of relative blood flow across different modulators valid. Thus, this data should only be relied on to provide estimates of relative differences in blood flow between different modulators and not as absolute point estimates.

Evaluation of the in vivo animal model

The in vivo animal model used in this project performed very suitably for investigating the various physiologic and anatomic contributors affecting skin permeability. The fuzzy rat is an excellent animal species for skin permeation studies because it requires no prior preparation (to remove fur) of the skin surface. The ability to focus on a small, well defined area for permeability measurements, using the TVCD, enabled examination of the role of capillary permeability and localized skin blood flow in addition to the physical barrier properties of stratum corneum. The physicochemical properties of isoflurane and its resistance to biotransformation were dominant factors in its suitability as a marker compound for examining skin permeability. The development of the intravenous route for isoflurane administration was a technical achievement to

facilitate precise measurements of dose delivered and blood concentrations. Most importantly, it provided a highly controllable administration method to achieve steady-state blood concentrations to serve as the constant driving force for the outward flux of isoflurane through the skin. Administration of vasoactive substances by the intradermal route to alter skin physiology functioned reasonably well to maintain the desired effect in a very localized area. The dynamic measurement of relative skin blood flow, using LDF, was essential to associate the physiologic changes from the action of the vasoactive substances with the changes in the amount of skin permeation of the marker compound.

Isoflurane permeation and effect of modulators

Results from the study of isoflurane outward permeation through normal, un-perturbed skin, paralleled with McDougal and coworkers' (1990) inward permeation studies, are unequivocal proof for the bi-directionality of skin permeation. The effective permeability coefficient is a useful parameter which can be compared with estimated permeabilities from other studies because it is concentration independent and can easily be scaled for the exposed skin surface area. McDougal and coworkers (1990), after exposing living male Fischer-344 rats (whose fur was closely clipped) to 50,000 ppm isoflurane vapor, observed mean steady-state isoflurane blood

concentrations of about 1.8 μg/ml. Using a physiologically based pharmacokinetic model, which included a skin compartment, they calculated an estimate for isoflurane permeability constant of 0.025 ± 0.004 (mean ± SD) cm/hr. The mean (± SEM) Kp_{eff} of 0.0347 ± 0.0028 cm/hr observed in this project is in close agreement with McDougal's report. The rapid penetration of permanent gases (gases which remain in the vapor phase at standard temperature and pressure) through skin is well recognized (Scheuplein and Blank 1971) with permeability constants of 0.67, 0.21, 0.25 and 0.24 cm/hr for helium, argon, nitrogen and carbon dioxide, respectively. The observed Kp_{eff} of isoflurane is only one order of magnitude lower then the aforementioned permanent gases which most probably is accounted for by its highly lipid soluble nature and not by its molecular weight and size.

The mean (± SEM) Kp_{eff} of rats whose TVCD sites were injected with 0.9% sodium chloride solution was 0.028 ± 0.0027 cm/hr. This value was 80% of the Kp_{eff} observed in normal, unperturbed skin but in close agreement with McDougal and coworkers' (1990) observation. It might be postulated that adding a 50 μl volume of fluid between the TVCD and the skin capillary would serve as an additive barrier to diffusion especially for the lipophilic isoflurane.

The apparent 50% reduction from baseline LDF measurements, resulting from the intradermal injection of phenylephrine, did not translate to a comparable reduction in

the amount of isoflurane outward permeation. The mean (± SEM) Kp_{eff} of 0.0336 ± 0.0037 cm/hr was nearly identical to that observed in normal, unperturbed skin and was higher, but not significantly, than that of the sodium chloride solution (control) vehicle. Krueger and coworkers (1985) could only demonstrate a significant delay in the absorption of [^{14}C]benzoic acid across the skin after phenylephrine iontophoresis when compared to iontophoresis of vehicle and the total amount absorbed was the same in both cases.

Minoxidil resulted in a clearly demonstrable increase in the amount of isoflurane permeating through the skin. The mean (± SEM) Kp_{eff} of 0.0528 ± 0.0037 cm/hr was 152% and 188% increased over the mean Kp_{eff} of normal and saline control experiments, respectively. Ordinarily, skin permeation studies exhibit a high degree of variability with coefficients of variation greater than 50% not uncommon. The minoxidil induced vasodilation reduced the variability associated with between-rat and between-site differences remarkably with a CV of only 33%. Increasing the permeation of a substance through skin by localized heat-induced vasodilation is universally used in transcutaneous oxygen and carbon dioxide monitoring. However, in those instances the skin is heated under the monitoring electrode to the point of a first degree burn which raises skin blood flow 10-50 times above basal levels. To my knowledge, not a single study of increased transport of a substance through skin using a chemically induced vasodilation

has been published.

The discrepancy between apparent vasoconstriction having no effect and apparent vasodilation having an effect on the permeation of isoflurane needs further explanation. It may be hypothesized that AVA's which are subtended by the TVCD distort the estimation of the Kp_{eff}. The predominance of AVA's in the rat are found in its tail but there may be a sufficient amount on the dorsum. It is well recognized that capillaries are the predominant blood vessels across which blood-to-tissue-to-blood transfer occurs. However, a highly lipophilic substance such as isoflurane may diffuse through AVA vessel walls and ultimately diffuse through the skin layers. The possibility of this occurring seems unlikely due to the differences in the structure of AVA vessels (thick walls and rapid blood flow) and capillaries. This notion is supported by experimentation which has shown that arteriovenous shunting was poorly correlated with A-V oxygen difference (Cronenwett and Lindenauer 1979). A plausible explanation for the lack of association between phenylephrine-mediated vasoconstriction and amount of isoflurane permeation may be found in the work of O'Neill and coworkers (1982). They showed that when AVA flow was reduced with norepinephrine, there was a marked increase in capillary blood flow. This explanation would then imply that LDF could be measuring both capillary and AVA blood flow.

Leukotriene D_4 also had a significant effect, equal in

magnitude and direction to minoxidil, on the amount of isoflurane permeating through the skin. The mean (± SEM) Kp_{eff} of 0.0554 ± 0.0058 cm/hr was 160% and 199% of the mean Kp_{eff} of normal and control experiments, respectively. The observed reduction in skin blood flow, which was greater than with phenylephrine, did not appear to be influential in reducing the Kp_{eff}. The significant increase in isoflurane diffusion may be explained by the increased plasma leakage from postcapillary venules resulting from the contraction of endothelial cells by LTD_4 (Dahlen et al. 1981). A very plausible explanation for the apparent discordance of decreased relative cutaneous blood flow and increased transcutaneous isoflurane permeation originates from the methodology used to measure blood flow. Laser Doppler flowmetry relies on the volume and velocity of red blood cells to produce a relative blood flow measurement. LTD_4, in addition to producing plasma leakage, produces vasoconstriction. The decreased relative blood flow, as measured by LDF, arose from the vasoconstrictive property of LTD_4. However, the LDF was not capable of measuring the increased plasma flow, which carried the bulk of the isoflurane, and resulted in a marked increase in transcutanous permeation.

The striking effect on disruption of the stratum corneum barrier was produced by both the chemical (ether-ethanol) and mechanical (tape-stripping) alterations. Pre-

treatment of the skin with ether-ethanol resulted in a mean (± SEM) Kp_{eff} of 0.0656 ± 0.0082 cm/hr, which was 189% of normal experiments. Visual observation of ether-ethanol treated sites did not lead one to speculate that skin permeability could be affected to such an extent. The sites appeared different from un-treated sites (shriveled and dried out), but the skin structures were still intact. However, as shown in the photomicrographs, the whole epidermal layer appeared to be disassociated from the underlying skin and apparently could no longer fully function as an effective barrier to permeation.

Removal of the stratum corneum by tape stripping had the most substantial effect on the amount of isoflurane permeating through the skin. The mean (± SEM) Kp_{eff} of 0.1017 ± 0.0097 cm/hr was 293% of unperturbed (normal) skin experiments. An observed Kp_{eff} of such magnitude approached the permeability constants of benzene vapor (0.152 cm/hr, McDougal et al. 1990) and permanent gases (Scheuplein and Blank 1971).

SUMMARY AND CONCLUSIONS

The major objective of this project was to develop an in vivo animal model for investigating the contributions of skin capillary physiology, viable epidermis, and the stratum corneum to the outward migration of a probe compound through the skin. It was hypothesized that perturbations of the aforementioned barriers to skin permeation could be separately quantified and related to changes in the amount of outward flux of the marker compound. This hypothesis was based on the additive nature of the anatomically serial diffusional resistances of the individual barriers and their relation to the overall effective permeability coefficient. It was envisioned that the in vivo model could be so devised as to maintain all but one barrier at its basal functional level while perturbing that one barrier to its maximum or minimum diffusional resistance. Refinement of the model could then lead to mathematical parsing out the contribution of each barrier to the total diffusional resistance.

Isoflurane, a general inhalational anesthetic agent, was used as a probe of xenobiotic permeability because of its physicochemical properties. Its low molecular weight, high vapor pressure, relatively high lipid solubility,

nonflammability, good molecular stability and low blood and tissue solubility made it an ideal anesthetic agent and an excellent candidate probe compound for investigating skin permeation. The demanding quantitative aspects of this project (dose administered, isoflurane blood concentration and isoflurane egressing through the skin) necessitated the development of a novel, intravenous route for administering isoflurane. Liquid isoflurane was solubilized in a commercially available intravenous fat emulsion to an approximate 12% (w/v) concentration. This novel formulation facilitated the use of a precise infusion pump to deliver the isoflurane at a constant rate and rapidly achieve and maintain a steady-state blood concentration.

A gas chromatographic assay technique was developed to analyze isoflurane blood concentrations, isoflurane concentrations in the fat emulsion mixture and the amount of isoflurane egressing through skin. The assay technique employed pesticide grade hexanes (to negate interference with the electron capture detector) as the extracting solvent for all samples. The chromatographic column used was able to separate isoflurane from the biological matrix of blood, the various components of the commercially prepared fat emulsion and the numerous other compounds emanating from the skin surface and collected by the transdermal vapor collection device (TVCD). The assay was log-linear in the range of 299.2 to 29,920 pcg/μl injected on column and its specificity,

sensitivity, precision, accuracy and reproducibility were acceptably high.

The fuzzy rat was selected as the animal species for this project as an acceptable in vivo model to study skin permeability. The lack of fur on this species diminished the need for extensive skin preparation which could alter normal skin permeability characteristics. Furthermore, in vitro experiments (Bronaugh and Stewart 1986) have shown that the permeability of fuzzy rat skin to a variety of test chemicals is similar to that of human skin.

Our understanding of skin permeability emanates primarily from in vitro studies, and is substantially based on the extensive investigations of Scheuplein and others from the 60's followed by more recent elaborations. The bulk of these studies have looked at inward permeation (xenobiotic applied to the outer stratum corneum surface) which is associated with several limitations, the two main ones being a.) the thickness and variable viability of the skin employed and b.) the lack of a functioning vasculature to remove the permeating molecule. The in vivo model developed in this project incorporated the notion that skin capillary circulation is an integral component of total skin permeability. Transcutaneous oxygen and carbon dioxide monitoring, in order to be quantitative, necessitates cutaneous blood vessel dilation by heating of the skin surface. However, only recently has it been appreciated that

alterations in skin capillary blood flow rate can have a significant impact on the total diffusional resistance of other compounds.

The skin permeability studies of this project were an extension of the novel methodology developed by Peck (1987) and served as an example for United States Patent No. 4,909,256. TVCD's affixed to the skin of the rats were used to quantitatively collect the permeating isoflurane. The measurements of isoflurane blood concentrations over time enabled the calculation of the area-under-the-curve, which, in effect, is the integrated steady-state isoflurane blood concentration. The steady-state isoflurane blood concentration in skin capillaries was the driving force for the permeation through the skin and its relationship to the integrated flux of isoflurane through the skin (amount collected in the TVCD's) enabled the calculation of an effective permeability coefficient (Kp_{eff})(Peck et al. 1988). The Kp_{eff}, which was determined from these experiments was composed of the diffusional resistances of stratum corneum, epidermis, capillary wall and capillary blood flow and can be expressed mathematically as the sum of the individual resistances[10] in the following equation:

$$Kp_{eff} = \frac{1}{1/Kp_{sc} + 1/Kp_{ve} + 1/Kp_{cap} + 1/Q}$$

[10] Diffusional resistance is the reciprocal of the permeability coefficient as described in Introduction (Scheuplein and Blank 1971).

where Kp_{sc}, Kp_{ve} and Kp_{cap} are the individual permeability coefficients of stratum corneum, viable epidermis-dermis and capillary wall and Q is the capillary blood flow. The Kp_{eff} of isoflurane through normal, unperturbed fuzzy rat skin was 0.0347 $\pm$ 0.0028 (mean $\pm$ SEM) cm/hr, which was similar to that observed in dermal absorption studies using Fischer-344 rats (McDougal et al. 1990).

Physiologic perturbations were performed to investigate the effects of minoxidil-mediated vasodilation, phenylephrine-mediated vasoconstriction and leukotriene D_4-mediated increased capillary permeability on the outward flux of isoflurane. Laser Doppler flowmetry was used to measure skin blood flow in order to associate the physiologic changes produced by the aforementioned agents. Minoxidil produced a substantial increase in relative skin blood flow, compared to baseline, and a Kp_{eff} of 0.0527 $\pm$ 0.0037 (mean $\pm$ SEM) cm/hr, which was over 50% increase of unperturbed skin. Phenylephrine produced a near 50% reduction in relative skin blood flow, however, the observed Kp_{eff} of 0.0336 $\pm$ 0.0037 (mean $\pm$ SEM) cm/hr was essentially the same as unperturbed skin. LTD_4 markedly reduced relative skin blood flow, which contrary to the effects of phenylephrine, persisted for the duration of the experiments. However, the purported effect of LTD_4 on increasing capillary wall permeability apparently predominated, as the observed Kp_{eff} of 0.0554 $\pm$ 0.0058 (mean $\pm$ SEM) cm/hr was 60% greater than unperturbed skin. It is well

recognized that the concentration in the "blood compartment" which is driving the permeation of isoflurane is not identical to that measured from the distal femoral artery. However, it is the change in blood flow and capillary permeability in the capillaries of the skin beneath the TVCD's which resulted in the significant observed changes in Kp_{eff} from the effects of minoxidil-mediated vasodilation and LTD_4-mediated increased capillary permeability.

The dominant resistance to permeation of the stratum corneum, even for a rapidly permeating substance like isoflurane, has been confirmed. Pre-application of an ether-ethanol solution to the TVCD sites resulted in a Kp_{eff} of 0.0657 ± 0.0082 (mean $\pm$ SEM) cm/hr which was approximately 90% greater than that of unperturbed skin. The gross visual appearance of the ether-ethanol application sites revealed a dried, shriveled skin surface which on microscopic examination appeared to exhibit complete detachment of the epidermal layer from the underlying dermis. However, even in view of the microscopically observed, extensive damage to the epidermal barrier layer, the increase in observed Kp_{eff} was substantially less than from the effects of tape stripping away the stratum corneum. A possible explanation may be due to the epidermal layer not being completely removed, albeit substantially altered, by the ether-ethanol application, and thereby still providing a minimum residual barrier. Alternately, this could be due to the epidermal reservoir effect.

Tape-stripping of the stratum corneum layer with ordinary Scotch tape resulted in the most dramatic increase in the permeation of isoflurane. The observed Kp_{eff} of 0.1017 ± 0.0097 (mean ± SEM) cm/hr was nearly 300% that of unperturbed skin. It is apparent that removal of the stratum corneum eliminates both a dominant barrier to permeation and/or a reservoir (or "sink") that isoflurane could be sorbed (dissolved) in.

Stoelting and Eger (1969) suggested that the transfer across skin of the anesthetic gases (which they studied), was limited more by diffusional resistance than by cutaneous blood flow. However, they observed a significant increase in the percutaneous loss of nitrous oxide after heating the skin, which they postulated was due to opening of additional capillaries nearer the skin surface.

Scheuplein and Blank (1971) theorized that for a compound with diffusion comparable or faster than the blood perfusion rate, the transfer of molecules into capillaries may control percutaneous absorption. The studies described in this dissertation, outside of studies with permanent gases, have for the first time corroborated their theory and have shown that increased cutaneous blood flow and increased capillary permeability, can significantly impact the outward flux of the probe compound, isoflurane.

This research and application of the model has important potential for future studies to develop a better

understanding of in vivo skin barrier function. Refinement of the model is a necessity in order to better distinguish between changes in capillary permeability and changes in blood flow. This may be accomplished by investigating other capillary permeability modifiers such as other leukotrienes or other chemicals which may have different effects on blood flow. Other blood flow modifiers could be investigated including physical perturbations such as extremes of hot and cold and other chemical vasodilators and vasoconstrictors. Repeating capillary permeability and blood flow experiments after removal of the resistance of stratum corneum would yield more accurate estimates of their contributions to skin permeability. Other refinements of the model could include improved methods of quantifying changes in capillary permeability (e. g., measure Evans blue in vivo on viable skin using reflectance spectroscopy) and blood flow. Isolation of the barrier property of viable epidermis may be attempted by trying alternate probes such as other gaseous compounds with varying water/fat solubility.

In summary, this project was novel in several ways. First and foremost, an in vivo animal model was devised and demonstrated to be capable of quantitative investigations of perturbations of skin permeation to a probe compound. The influential barrier function of stratum corneum on isoflurane efflux has been confirmed and it has been shown that intracutaneously administered minoxidil and LTD_4 can have a

demonstrable effect on the outward flux of isoflurane, presumably by altering blood flow and capillary permeability. This necessitated the development of an intravenous formulation of isoflurane enabling a new and controlled route for its administration, the employment of a novel transdermal vapor collection device, the establishment of quantitative assays for isoflurane in several media and advanced data analysis procedures.

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