0022-3565/96/2771-0366$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 277, No. 1
Printed in U.S.A.

JPET 277:366–374, 1996

The Effect of Protein Binding on the Deep Tissue Penetration and Efflux of Dermally Applied Salicylic Acid, Lidocaine and Diazepam in the Perfused Rat Hindlimb[1]

SHEREE E. CROSS, ZHENYU WU and MICHAEL S. ROBERTS
Department of Medicine, The University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland, Australia 4102
Accepted for publication December 4, 1995

ABSTRACT

Doses of radiolabeled water, salicylic acid, lidocaine and diazepam were applied in 2 ml of phosphate buffer saline to the exposed dermis on the thigh of a perfused rat hindlimb. Hindlimbs were perfused with Krebs-Heinseleit buffer at 37°C containing 4% albumin or 2.5% dextran 40 at a constant rate of 4 ml/min. Clearance of solutes from dermal sites was monitored by frequent sampling at the dermal site and of the outflowing perfusate. In addition, the concentration of solutes in the various tissues below the treated and contralateral sites were determined at the end of a perfusion after dissection, solubilization and scintillation counting of the individual tissues. Estimates of the absorption rate constant from the dermis and the elimination rate constant from the perfused limb were made by the simultaneous fitting of absorption and efflux data with a one-compartmental pharmacokinetic model. Diazepam was cleared fastest through the dermis, followed by water, salicylic acid and lidocaine. The efflux of diazepam and salicylic acid from the hindlimb was significantly enhanced by the presence of albumin in the perfusate relative to no albumin ($P < .05$), whereas the efflux of water and lidocaine were unaffected by changes in albumin content. Analysis of individual tissues at the end of the perfusion showed that water had the highest relative tissue concentrations with diazepam the least, high concentrations appearing to partition into and remain associated with the dermis. The presence of albumin in the perfusate significantly increased the concentration of diazepam found in contralateral skin samples ($P < .05$) and showed the same trend for salicylic acid and lidocaine. These findings show that protein binding significantly affects the deep tissue penetration and distribution of dermally applied solutes and that this effect is more prominent for highly protein-bound solutes.

There are numerous advantages to the local topical treatment of deep tissue injury, including the ability to 1) concentrate drug molecules around the injury site, 2) reduce the risk of systemic side effects by minimizing plasma concentrations, 3) provide a sustained release of drug at the site and 4) to avoid first pass metabolism and gastric irritation associated with oral therapy. A number of drugs are presently administered by topical application to treat local painful and/or inflammatory conditions, such as local anesthetics (McCafferty *et al.*, 1988) and nonsteroidal anti-inflammatory agents (Hadgraft, 1989). It has been generally assumed that the dermal circulation acts as a sink, removing the majority of a topically absorbed solute once it has traversed the upper skin layers, allowing for the targeting of topically applied solutes into the systemic circulation. The mechanism by which solutes reach deeper tissues after topical administration has been investigated by Singh and Roberts (1993a, 1994a) and McNeill *et al.* (1992). The dermal blood supply has been shown to play an important role in the clearance of drugs after topical application (Siddiqui *et al.*, 1989; Singh and Roberts, 1990, 1994a; Cross and Roberts, 1993). Singh and Roberts (1994b) showed that coadministration of the vasoconstrictor phenylephrine can significantly increase quantities of solutes delivered to local tissues after dermal application in the anesthetized rat by limiting removal of solutes by the dermal circulation, thus allowing greater diffusion into deeper tissue sites. Interestingly, McNeill and his co-workers found high concentrations of piroxicam in the underlying musculature after topical administration to the shoulder region of young rats that could not be attributed solely to systemic delivery or simple diffusion, which suggests that a more localized deep tissue delivery mechanism must exist (McNeill *et al.*, 1992). In a later study, it was demonstrated that the local tissue distribution of piroxicam applied topically to pigs was dependent on the orientation of local microvascular networks, allowing the solute to be carried into deeper tissues from the application site when applied over muscular sites (Monterio-Riviere *et al.*, 1993).

Most studies assessing the deep tissue penetration of solutes have been undertaken *in vivo* where systemically ab-

Received for publication April 6, 1995.
[1] Supported by the National Health and Medical Research Council (S.E.C., M.S.R.), the Charles and Silvia Viertel Charitable Foundation (S.E.C.), the Queensland Cancer Fund and Australian Government Overseas Postgraduate Research Scholarship scheme (Z.W.) and the Queensland and Northern New South Wales Lions Foundation (M.S.R.).

sorbed solute is recirculated and redistributed into tissues throughout the animal. Singh and Roberts (1994b) developed a mathematical model to describe the local tissue penetration of dermally applied solutes which took into account the redistribution of solutes by the systemic blood supply, a process that may overshadow the events controlling the deep tissue penetration of solutes by localized means. To examine more fully the deep tissue penetration of solutes in the absence of redistribution by the systemic circulation, isolated perfused tissue preparations such as the perfused rat hindlimb (Cross *et al.*, 1994) and the perfused porcine skin flap (Riviere *et al.*, 1992) have been used. The perfused hindlimb preparation used in the present study was developed and validated in this laboratory by Wu *et al.* (1993) and has been used extensively to study the distribution of systemically, dermally and iontophoretically applied solutes (Wu *et al.*, 1993, 1995; Cross *et al.*, 1994).

In the present study we have investigated the effect of solute plasma protein binding on the deep tissue penetration of solutes after dermal application with the isolated perfused rat hindlimb model. The binding of drugs to various blood and tissue proteins has been known for some time to influence their therapeutic, pharmacodynamic and toxicological actions (Gillette, 1973; Koch-Weser and Sellers, 1976; Vallner, 1977) and to exert profound effects on drug distribution in whole animal studies (Hirate *et al.*, 1989). Owen *et al.* (1994) recently showed that the efflux of highly protein-bound solutes from synovial fluid occurred by two mechanisms: diffusion of unbound drug and the lymphatic transport of protein-bound drug. A key aim of the present study was to examine whether selective deep tissue penetration, as distinct from systemic absorption, was related to plasma protein binding. Consequently, four solutes with differing protein-binding characteristics at physiological pH (an anion, salicylic acid; a cation, lidocaine; a lipophilic neutral solute, diazepam and water) were applied topically to a single pass rat hindlimb preparation with dextran or a bovine serum albumin solution as perfusates. The topical absorption kinetics was studied by the monitoring of changes in concentration of applied solute with time, solute recovery in outflowing perfusate was determined to allow estimation of the amount of solute in the tissues of the hindlimb together with measurement of actual solute tissue concentrations below the treated site and in the contralateral tissues at the end of the study.

Methods

Hindlimb perfusions were prepared in a manner similar to that we have described previously (Wu *et al.*, 1993; Cross *et al.*, 1994). Briefly, rats (male Wistar 300 ± 25 g) were anesthetized, the abdomen opened and the right femoral artery cannulated (PE50) *via* the dorsal aorta. A second cannula (PE205) was placed in the dorsal vena cava, and the hindlimb perfused in a humidicrib with oxygenated (95%O_2/5% CO_2) Krebs-Heinseleit Buffer (pH 7.4, 37°C), containing 4% bovine serum albumin (Fraction V, Sigma Chemical Co, Australia) or 2.5% dextran 40 (Sigma Chemical Co, Australia). In an attempt to attenuate the allergic response of rats to dextran, animals were injected i.p. with 1 ml of 2.5% dextran in phosphate-buffered saline 24 hr before perfusion studies (Perez-Trepichio *et al.*, 1991). Once the perfusions were established, rats were killed by creating a pneumothorax and turned over so that glass diffusion cells could be attached to the perfused limb. The perfused preparation has been shown to be stable for up to 2 hr, as validated by testing inflowing and outflowing perfusate samples for pH, dissolved oxygen concentrations, lactate dehydrogenase and creatinine kinase as markers of cell damage (Wu *et al.*, 1993). Perfusion flow rates of 4 ml/min, actual rates 3.97 ± 0.22 ml/min, were controlled by a graduated peristaltic pump and were measured at the beginning and end of each perfusion.

Perfusate oncotic pressure was determined with a IL186 WEIL Oncometer System to establish the concentration of dextran 40 that corresponded to the oncotic pressure produced by 4% (bovine serum albumin (BSA). Oncotic pressures of samples of Krebs' buffer containing 0 to 7% BSA or 0 to 4% dextran 40 were determined with human serum albumin as a control.

Glass diffusion cells (8 cm high, 1.8 cm internal diameter), identical to those used for our *in vivo* transdermal penetration studies (Cross and Roberts, 1993; Cross *et al.*, 1994; Singh and Roberts, 1994a, b), were fixed to the skin on the posterior of the hindlimb with adhesive. The application area on the hindlimb was first depilated with commercial Nair™ hair-removal cream and the epidermis removed with a dermatome. Solutions (2 ml) containing 1.5 μCi of the radiolabeled solute(s) of interest: ^{14}C-salicylic acid (DuPont NEN [Australia] Ltd.), ^{3}H-water (Sigma Chemical Co., Australia), ^{14}C-lidocaine (DuPont NEN [Australia] Ltd.) or ^{3}H-diazepam (DuPont NEN [Australia] Ltd.), in phosphate-buffered saline (pH 7.4) were introduced into the cell at time zero. Samples (10 μl) were removed from the cell at 15-min intervals through a sampling port, added to 5 ml of Emulsifier safe liquid scintillation fluid and counted in a liquid scintillation counter (1600TR Liquid Scintillation Analyser, Packard, Canberra, Australia). Absorption studies were conducted for 90 min, after which the cell contents were drawn out with a pipette and the cell removed. The fraction remaining in the cell (F_t) at various times were regressed as a semilogarithmic plot to obtain the absorption rate constant k_a:

$$F_t = \exp(-k_a t) \tag{1}$$

The clearance of solutes from the cell was then determined from the relationship:

$$\text{Clearance (ml/min)} = k_a \cdot V \tag{2}$$

where V is the volume applied (Siddiqui *et al.*, 1985). The permeability coefficient for each solute is calculated as:

$$\text{Permeability coefficient (cm/min)} = \text{clearance/surface area} \tag{3}$$

Outflowing perfusate from the venous catheter was collected in 5-min sample periods into 25-ml plastic vials for determination of solute concentrations. A 200-μl aliquot of each sample was added to 10 ml Emulsifier safe liquid scintillation cocktail and counted in a liquid scintillation counter, as described previously. The cumulative amount of solute eluted in the perfusate (M_{eluted}) at various times was calculated from the product of perfusate flow rate (Q_p) and the cumulative AUC (area under the curve) of the plot of concentration (C) in the perfusate against time (minutes) by the trapezoidal rule, *i.e.*,

$$M_{eluted} = \int_0^t Q_p \cdot C \, dt = Q_p \cdot \text{AUC} \tag{4}$$

The total amount of solute in the tissues (M_{tiss}) at time t, was estimated from:

$$M_{tiss} = M_{cell} - M_{eluted} \tag{5}$$

where M_{cell} is the amount of solute lost from the absorption cell to time t (fig. 1).

Elimination rate constants (k_{el}) were estimated from the cumulative amount of solute in the outflowing perfusate, assuming the

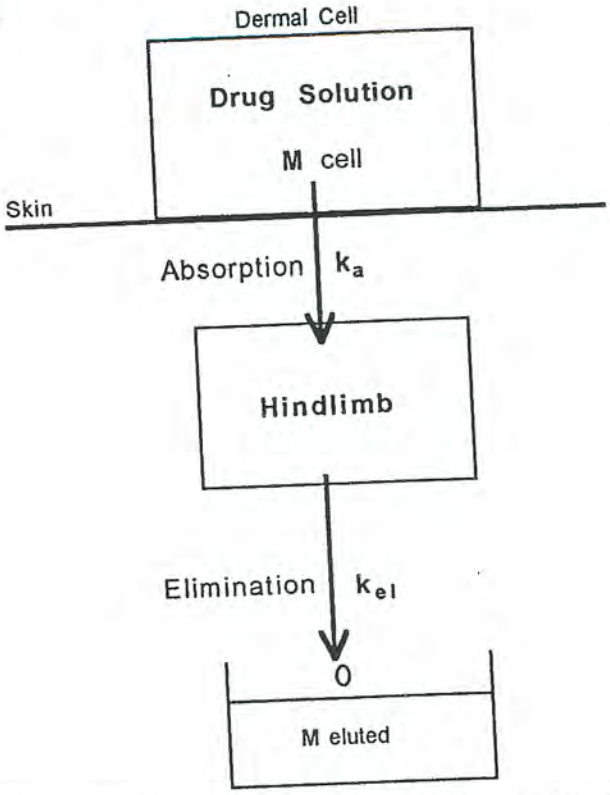

Fig. 1. Diagrammatic representation of the kinetic modeling used for the absorption and elimination of topically applied solutes in the perfused rat hindlimb preparation. M_{cell}, amount lost from the dermal absorption cell; k_a, absorption rate constant; k_{el}, elimination rate constant; M_{eluted}, amount eluted and recovered in the outflowing perfusate.

hindlimb behaves as a single compartment with a first-order dermal input rate constant, k_a (equation 1, fig. 1) (Cross *et al.*, 1994).

The cumulative amount of solute in the outflowing perfusate, M_{eluted}, expressed as a fraction, A_t, at various times, t, was defined as:

$$A_t = \frac{M_{eluted}}{M_{cell\ t=0}} = 1 + \frac{k_a e^{-k_{el}t}}{k_{el} - k_a} - \frac{k_{el} e^{-k_a t}}{k_{el} - k_a} \quad (6)$$

Estimates for k_a and k_{el} were obtained by the simultaneous nonlinear regression of F_t and A_t *vs.* t with the computer program MINIM on a Macintosh Classic II. The half-life for the elimination of solutes from the limb was then defined as $t_{1/2}$ el = $0.693/k_{el}$ The time for the peak amount of solute in the tissues was estimated from a one-compartment model with first-order absorption:

$$t_p = \frac{1}{k_a - k_{el}} \cdot \log \frac{k_a}{k_{el}} \quad (7)$$

The tissues from beneath the position of the cell and at a contralateral site were dissected sequentially after swabbing the site of application with an alcohol-soaked absorbent tissue at the end of each perfusion. Samples were placed into preweighed vials for weight determination and solubilization (NCS-II tissue solubiliser, Amersham Australia) for determination of radioactivity content by liquid scintillation counting (OCS organic scintillant, Amersham Australia) with the preset channels of a Minaxi β Tri-carb 4000 series liquid scintillation counter (Packard Instrument Co., Meriden, CT). The concentration of radioactivity in each of the tissue samples was expressed as a fraction of the initial amount applied in the diffusion cells.

The fraction of each of the solutes unbound (f_u) in 4% BSA and 2.5% dextran 40 Krebs' buffer (pH 7.4) was determined by equilibrium dialysis with a Spectra/Por® molecular porous membrane (Spectrum Medical Industries, Los Angeles, CA) against Krebs' buffer saline (pH 7.4) rolled in a water bath at 37°C for 20 hr. The radiolabeled solute concentration on each side of the membrane was determined by scintillation counting, and f_u defined as the concentration ratio of solute in Krebs' buffer to that in albumin perfusate.

Octanol-Krebs' buffer partition coefficients were determined for each solute as follows: octanol was saturated with buffer, pH 7.4, by rotating 10-ml 1:1 solutions of each on a chuck wheel at room temperature overnight. After centrifugation, 1-ml aliquots of octanol were drawn off with a Pasteur pipette and added to 1-ml aliquots of the buffer to which the radiolabeled solute of interest had been added. Solutions were then rotated on a chuck wheel for 16 hr at room temperature before centrifugation and determination of radioactivity in the organic and buffer layers. Partition coefficients, Poct, were expressed as the ratio of solute in the organic to the water layer.

Data were compared by a one-way or two-way ANOVA, with significance taken at $P < .05$, and differences between groups identified with the Tukey test.

Results

The 2.5% concentration of dextran 40 used in the perfusions was estimated from the point on the plot of oncotic pressure against dextran 40 concentration that corresponded to the oncotic pressure generated by 4% BSA. It was noted that the perfused hindlimb weight, relative to body weight, was increased after dextran perfusions and the subcutaneous layer of dextran-perfused limbs appeared to be slightly edematous, whereas no difference was noted in the appearance of other limb tissue groups.

Octanol/buffer partitioning showed that the most highly lipophilic compound was diazepam, 129.2 ± 8.2, followed by lidocaine, 81.4 ± 0.6 and salicylic acid, 0.245 ± 0.01. The fraction unbound, f_u, for each solute in 4% BSA as determined by equilibrium dialysis was 0.1 for salicylic acid, 0.14 for diazepam, 0.43 for lidocaine and 1.0 for water. There was negligible binding of all solutes to the dextran perfusion medium and f_u was assumed to be unity. With the exception of lidocaine, in which higher values were obtained during dextran perfusions, neither clearance or permeability coefficients were affected by the extent of solute binding (table 1).

Significantly lower amounts of salicylic acid ($P < .05$) and diazepam ($P < .05$) were recovered during dextran perfusions compared with the amount eluted with protein present in the perfusate (fig. 2). There was no change in the amount of lidocaine eluted between dextran and BSA perfusions. Similarly, there was no significant difference in the amount of water recovered in the perfusate between dextran and BSA perfusions whether coadministered with salicylic acid or lidocaine.

The fitting of experimental data to equations 1 and 6 yielded the predicted fractions of solute in the donor cell, hindlimb tissues and outflowing perfusate for each of the individual solutes during dextran and BSA perfusions as shown in figure 3. The regression coefficient, R^2, for all solutes exceeded 0.98. Water was rapidly eluted from the perfused limb with a lag time of about 15 min for both dextran and BSA perfusions (fig. 3A); the estimated peak tissue concentrations and half-lives in the system were shorter than the other solutes in both BSA and dextran perfusions (table 1). Conversely, diazepam showed a high rate of sequestration in the tissues and low rate of efflux in the perfusate (fig. 3D, table 1). The recovery in perfusate of salicylic acid was much

TABLE 1

Summary of the kinetic parameters (mean ± S.E., n = 3, except water n = 6) obtained for each of the solutes studied in the perfused rat hindlimb preparation

	Albumin	Dextran	P Value
Clearance (μl/min)			
Water	1.47 ± 0.2	1.82 ± 0.2	N.S.
Salicylic acid	1.33 ± 0.3	1.56 ± 0.2	N.S.
Lidocaine	0.89 ± 0.1	1.36 ± 0.2	<.05
Diazepam	2.07 ± 0.2	2.14 ± 0.1	N.S.
Fraction of absorbed dose in perfusate			
Water	0.693 ± 0.06	0.694 ± 0.05	N.S.
Salicylic acid	0.617 ± 0.08	0.277 ± 0.06	<.05
Lidocaine	0.550 ± 0.04	0.507 ± 0.06	N.S.
Diazepam	0.175 ± 0.05	0.033 ± 0.002	<.05
Elimination half-life from limb (min)			
Water	27.64 ± 5.8	26.46 ± 6.3	N.S.
Salicylic acid	30.43 ± 9.0	124.41 ± 42.5	<.05
Lidocaine	37.26 ± 9.2	49.33 ± 4.5	N.S.
Diazepam	244.62 ± 74.6	952.57 ± 52.3	<.05
Time to estimated peak tissue concentration (min)			
Water	35.45 ± 4.7	31.22 ± 5.6	N.S.
Salicylic acid	39.34 ± 6.4	84.35 ± 11.8	<.05
Lidocaine	56.39 ± 9.8	53.79 ± 2.3	N.S.
Diazepam	95.17 ± 10.9	190.98 ± 9.5	<.05

faster for perfusate containing protein than for that with dextran present (fig. 3B), with a correspondingly longer half-life in the protein-free perfused hindlimbs (table 1). Similar rates of recovery and tissue accumulation were observed for lidocaine between perfusions containing BSA and dextran (fig. 3C).

The relative concentrations of each solute found in tissues after dextran and BSA perfusions are compared in figure 4, which shows that the penetration of solutes into deeper muscle layers was of the order water > lidocaine ≥ salicylate > diazepam for both BSA and dextran perfusions. Tissue concentrations for each solute were highest in the dermis and decreased with the depth of tissue sample. Tissue concentrations of diazepam were much higher in the skin and much lower in the deeper muscle layers than any other solute during both dextran and BSA perfusions. The most unexpected difference in the profiles shown in figure 4B is a higher concentration of all solutes in the contralateral skin after BSA perfusions compared with those with dextran. This difference reached statistical significance for diazepam ($P < .05$).

Figure 5 shows that there was a significant increase ($P < .05$) in the concentration of water, salicylic acid and lidocaine in the subcutaneous tissue layer after dextran perfusions compared with those with BSA ($P < .05$). Also the concentration of lidocaine remained higher in the superficial muscle (M1) layer ($P < .05$) after dextran perfusions. The tissue concentrations of solutes tended to be higher after dextran perfusions directly below the application site, with the exception of diazepam, although values failed to reach significance. The tissue concentrations of diazepam followed a slightly different trend from that seen with the other solutes; higher tissue concentrations of diazepam in the upper tissue layers, and more significantly in the contralateral tissues, were achieved after BSA perfusions.

Discussion

The penetration of a topically applied drug into underlying superficial and deep tissue sites is a prerequisite for the local treatment of painful musculoskeletal disorders with topically applied anti-inflammatory agents and other products. Much of our earlier work was carried out by assessing drug concentrations in tissues below the dermal application site *in vivo* (Singh and Roberts, 1991, 1993a, b) and attempted to estimate the contribution of systemic recirculation on the deeper tissue concentrations. However, the estimations relied upon determined tissue concentrations in contralateral tissues and assumed that the blood flow to each tissue compartment was constant throughout the course of the study. *In vitro* examination of solute permeability through human and animal skin (Koch *et al.*, 1987; Kushla and Zatz, 1989; Neubert *et al.*, 1990) complements the dermal studies undertaken here in that the rates of absorption obtained can be convoluted with *in vivo* dermal absorption kinetics to predict the likely tissue penetration of solutes under varying topical application conditions. The isolated perfused limb model allows, in theory, assessment of the deep tissue penetration in the absence of this recirculation and has previously been shown to offer many benefits over *in vitro* systems without the innate variability and lack of control over physiological parameters, such as tissue perfusion rates, inherent in *in vivo* studies (Cross *et al.*, 1994).

To determine the absorption, distribution and elimination kinetics of solutes in the absence of protein, and given the need for an adequate oncotic pressure to maintain a viable hindlimb preparation and to avoid edema (Wu *et al.*, 1993), dextran 40 Krebs-Henseleit solution was chosen as a protein-free buffer for comparison with the BSA perfusate studies. Hindlimbs that showed any visible signs of excessive edema during 2.5% dextran perfusions were not included in the results presented. Small amounts of edema caused during dextran perfusions, due to an allergic type reaction by rats to dextran, has been documented previously (Harris *et al.*, 1967; Flaim *et al.*, 1978).

The movement of solute molecules from the dermal site of application to underlying and other tissues depends on (1) tissue diffusion and binding and (2) removal and distribution by the local blood supply (Roberts, 1991). In the present

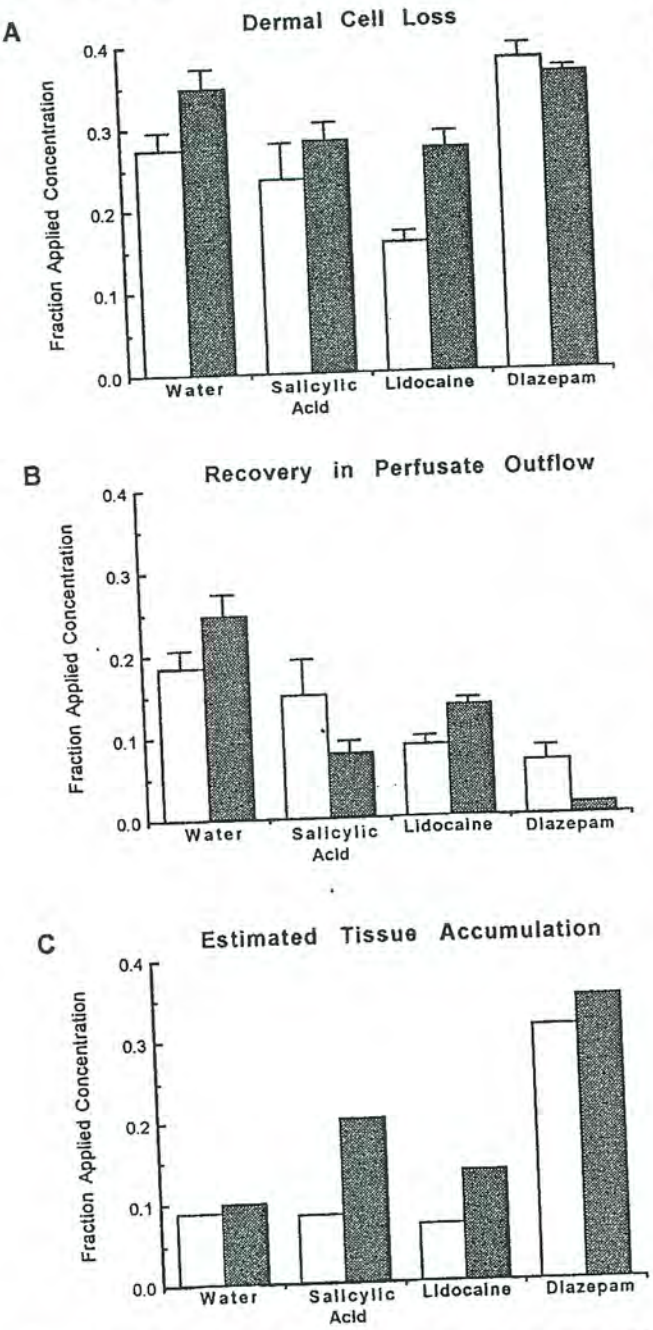

Fig. 2. Total fraction of the applied concentration of solutes (A) lost from the dermal absorption cell, (B) recovered in the outflowing perfusate and (C) estimated to be distributed in the tissues after 4% BSA (open columns) and 2.5% dextran (closed columns) perfusions. Mean ± S.E., $n = 3$, except water $n = 6$.

study the permeability coefficients obtained for all the solutes tested were similar, ranging from 0.35 to 0.85 $\times 10^{-3}$ cm/min. The solutes were applied to the dermis in a protein-free aqueous solution and so the fraction unbound for each solute was unity. The observed permeability coefficients are a reflection of the rate of uptake of the solutes from an aqueous solution into an aqueous medium (dermis), and hence will be dependent on the partitioning properties of the solutes and not their protein binding. Singh and Roberts (1994a, b) have reported comparable dermal permeability coefficients for a range of solutes and showed that the dermal clearance in anesthetized animals is about twice that for sacrificed animals. Cross *et al.* (1994) have shown that a doubling of perfusate flow increases the dermal permeability coefficient by only 10 to 30% in the perfused hindlimb model. In this work, the use of protein-containing or protein-free perfusate had little effect on the dermal clearance of protein-bound drugs. It is, therefore, concluded that the sink conditions below the dermal site (determined by blood flow and perfusate protein binding) are less significant determinants than solute diffusion in the dermis.

The differences observed in elimination half-lives of the solutes is consistent with albumin enhancing the redistribution of highly bound solutes, shown by the increased recovery of salicylic acid and diazepam in outflowing perfusate in the presence of albumin (fig. 3). The differences in estimated time to peak tissue concentrations with and without albumin in the perfusate is also consistent with the strong influence of protein binding on drug distribution, with diazepam appearing to have a longer elimination half-life and time to peak tissue concentration than other solutes. Given the high partition coefficient for diazepam relative to other solutes, sequestration in the fat pad which accounts for 3% of the hindlimb (Wu *et al.*, 1995) is likely to be additive to any protein-binding effects.

The distribution of water into tissues was, as expected, more ubiquitous than other solutes studied during both BSA and dextran perfusions, which reflects its ready diffusion into tissue spaces and its negligible binding to proteins. Similar water tissue distribution patterns after BSA and dextran perfusions is consistent with an unchanged fraction unbound in both perfusates; the water molecules partitioned and diffused equally well through the hindlimb in the presence of both protein and protein-free perfusate.

The significantly greater recovery of salicylic acid in the perfusate during BSA perfusions reduced the amount of the unbound solute available for tissue distribution. This effect of solute removal by plasma protein is increased in the perfused hindlimb preparation; because the perfusate is not recirculated, tissues are constantly perfused by solute-free solution and an equilibrium between free and bound solute is never reached, binding solutes are constantly drawn to the perfusate protein. Decreases in the distribution of salicylic acid throughout the hindlimb by the protein-free perfusate were reflected by the increased half-life and time to reach peak tissue concentration and reduced recovery in outflowing perfusate.

The higher tissue concentrations of salicylic acid measured beneath the application site after dextran perfusions (fig. 4) suggest that protein binding of this solute reduced its concentration in the upper tissue layers by increasing clearance in the perfusate and so limiting its diffusion to deep muscle sites. A second explanation of the observed effect could be accounted for by the fact that, in the absence of binding in the perfusate, a higher fraction of the salicylic acid entering the upper tissue layers was available to bind to tissue albumin and proteins, thus increasing the association of the solute with the tissues and slowing its clearance. Hirate *et al.* (1989) found an increase in the apparent volume of distribution of salicylic acid when administered systemically to albuminemic rats compared with controls. These authors suggested that, as it is generally accepted that only the free or unbound form of the drug in the vascular system is capable of under-

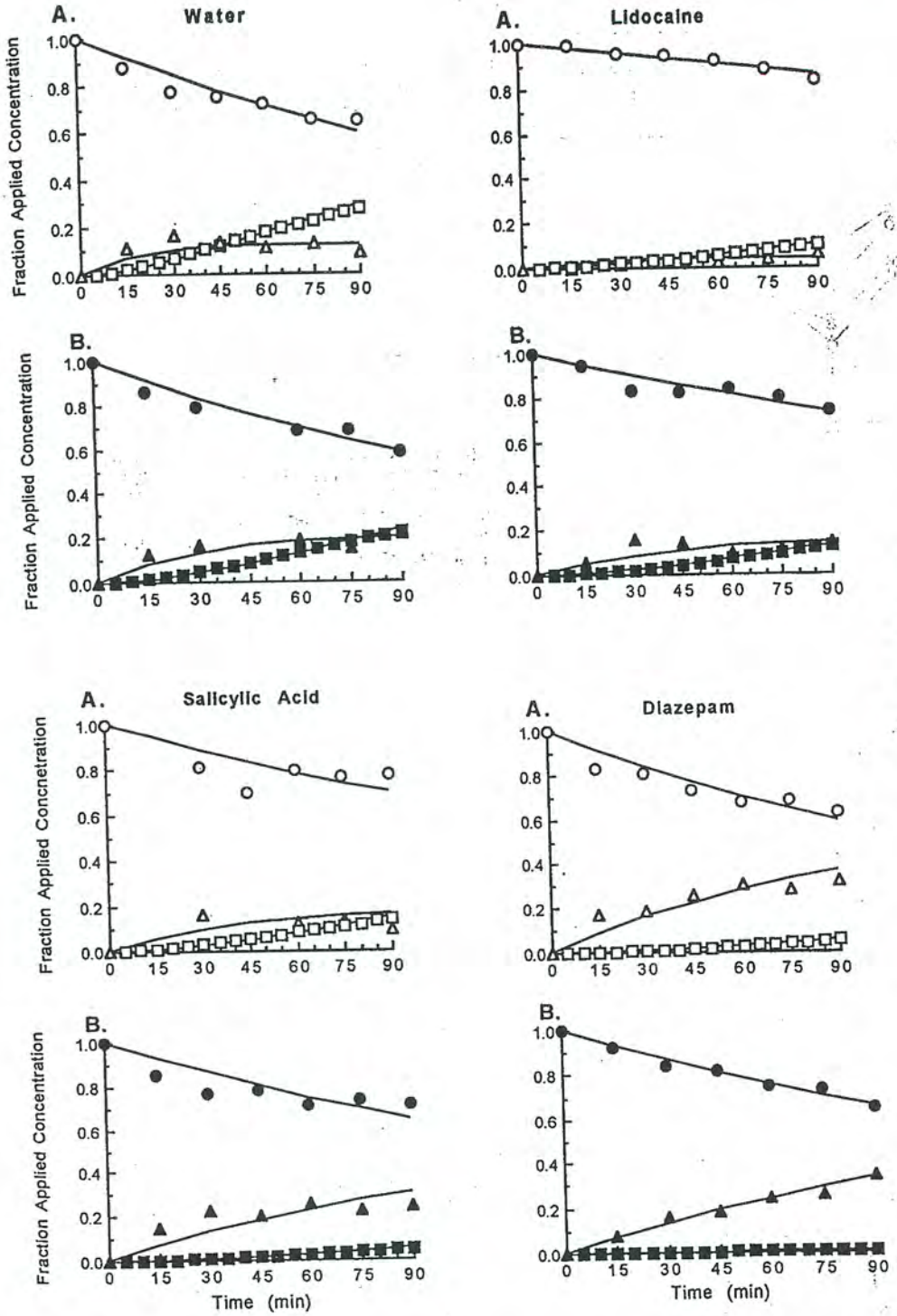

Fig. 3. Kinetic profiles of solutes generated from regression of equations 1 and 5 to experimental data for each solute, showing cumulative absorption from diffusion cells (●), cumulative efflux in perfusate (▲) and fraction calculated to be remaining in tissues (■) during 4% BSA (A) and 2.5% dextran (B) perfusions. Mean ± S.E., $n = 3$, except water $n = 6$.

going distribution, the lower plasma levels of salicylic acid measured and associated larger volume of distribution were a result of enhanced levels of free drug passing into the tissue spaces. The present study suggests that salicylic acid remains associated with the tissue sites and that some form of binding may be involved to reduce the clearance of the solute from the tissue. If no tissue binding was present, the free solute would pass as easily back out of the tissue space as into it. The increased concentration of salicylic acid in the subcutaneous layer after dextran perfusions was probably a result of the slight edema in the area as seen with the distribution of water.

Lidocaine and other local anesthetics are commonly applied as topical formulations for local effects (Adriani *et al.*, 1963). The absorption of solutes through the dermis was not affected by the constitution of the perfusion medium except in the case of lidocaine. The most likely explanation for the increase is the higher water content of dermis due to slight edema formation. Such an increase would favor both an increased partitioning of water into the dermis together with

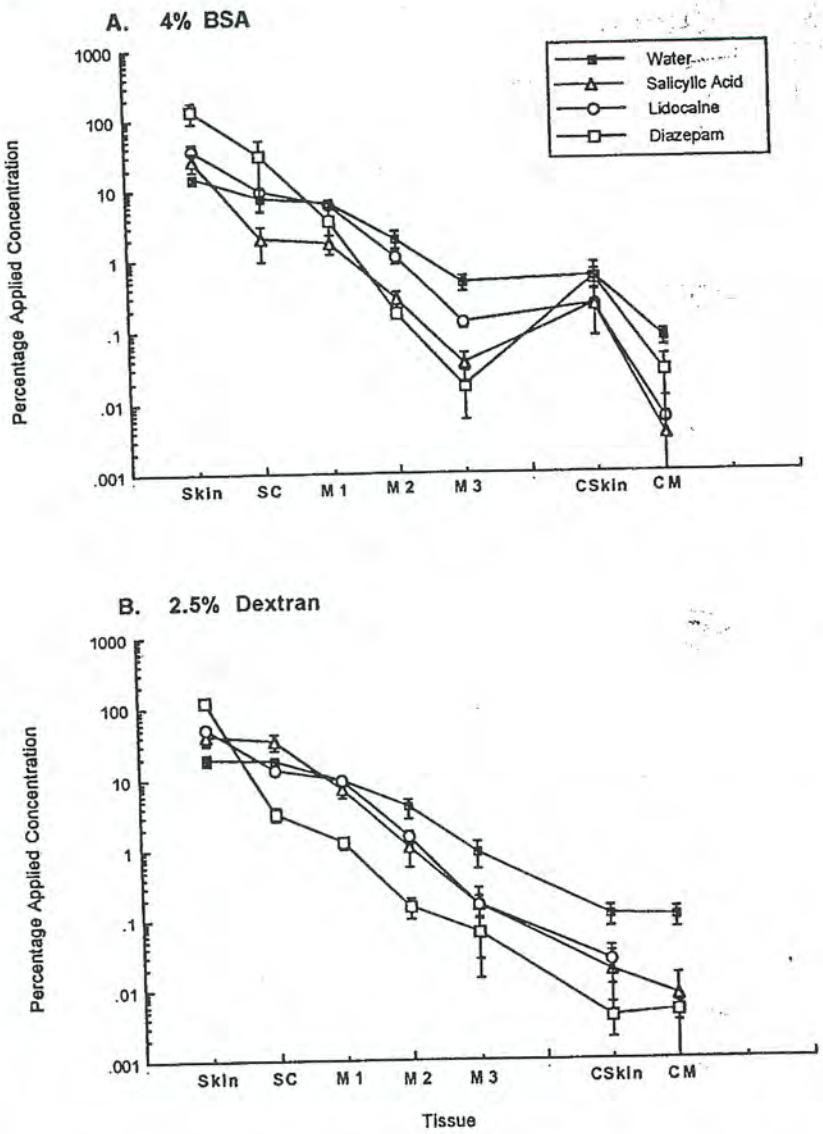

Fig. 4. Percentage of the applied concentration of solutes determined in tissue samples after 90 min perfusion with 4% BSA (A) and 2.5% dextran (B). SC, subcutaneous tissue; M1, superficial muscle; M2, muscle; M3, deep muscle; CSkin, contralateral skin (underneath the limb); CM, contralateral muscle. Mean ± S.E., $n = 3$, except water $n = 6$.

an increased diffusivity of solutes in the dermis. Both effects would result in an increased permeability coefficient for lidocaine in the dermis.

The lack of dependency of lidocaine tissue distribution and recovery in the outflowing perfusate on perfusate composition could have been anticipated because the major binding protein for this solute in plasma is not albumin but, as discussed later, α_1-glycoprotein. The efflux profiles generated suggest that the removal of lidocaine in the perfusate was limiting its accumulation in the tissue (fig. 3C). The increased concentration of lidocaine in the subcutaneous and superficial muscle tissue after dextran perfusions was assumed, as with other solutes, to be a result of the slight edema in this area caused by the dextran.

It should be born in mind during the interpretation and application of the results presented in this study that only the protein binding of solutes to albumin is considered. In human plasma, albumin constitutes approximately 62% of the total protein content, and globulins (34%) and fibrinogen (4%) constitute the remaining plasma protein fraction (Guyton, 1991). This effect is particularly important for the basic solute lidocaine which is known to bind predominantly to α_1-glycoproteins in the blood (Piafsky and Knoppert, 1978; Routledge *et al.*, 1980), therefore the plasma binding determined in this study is underestimated relative to *in vivo* values. The binding of solutes to albumin alone was used to minimize the number of factors influencing perfusate binding and so allow for better interpretation of the results obtained.

A comparison of the dermal clearance of solutes showed that the lipophilic, uncharged and highly protein-bound solute diazepam was absorbed fastest from the application site. Increases in half-life and time to reach peak tissue concentration reflect the reduced elimination of diazepam from the hindlimb and its reduced distribution throughout the hindlimb, which suggests that the protein present in the perfusate was responsible for the transport of diazepam to tissues in the hindlimb beyond the area through which the solute could diffuse directly from the absorption cell. The distribution of diazepam in the skin and subcutaneous tissue layers with very little in the deep muscle layers, together with the relatively small increase in fraction recovered in outflowing perfusate in the presence of protein, even though protein binding is high, suggests that diazepam penetrates into the

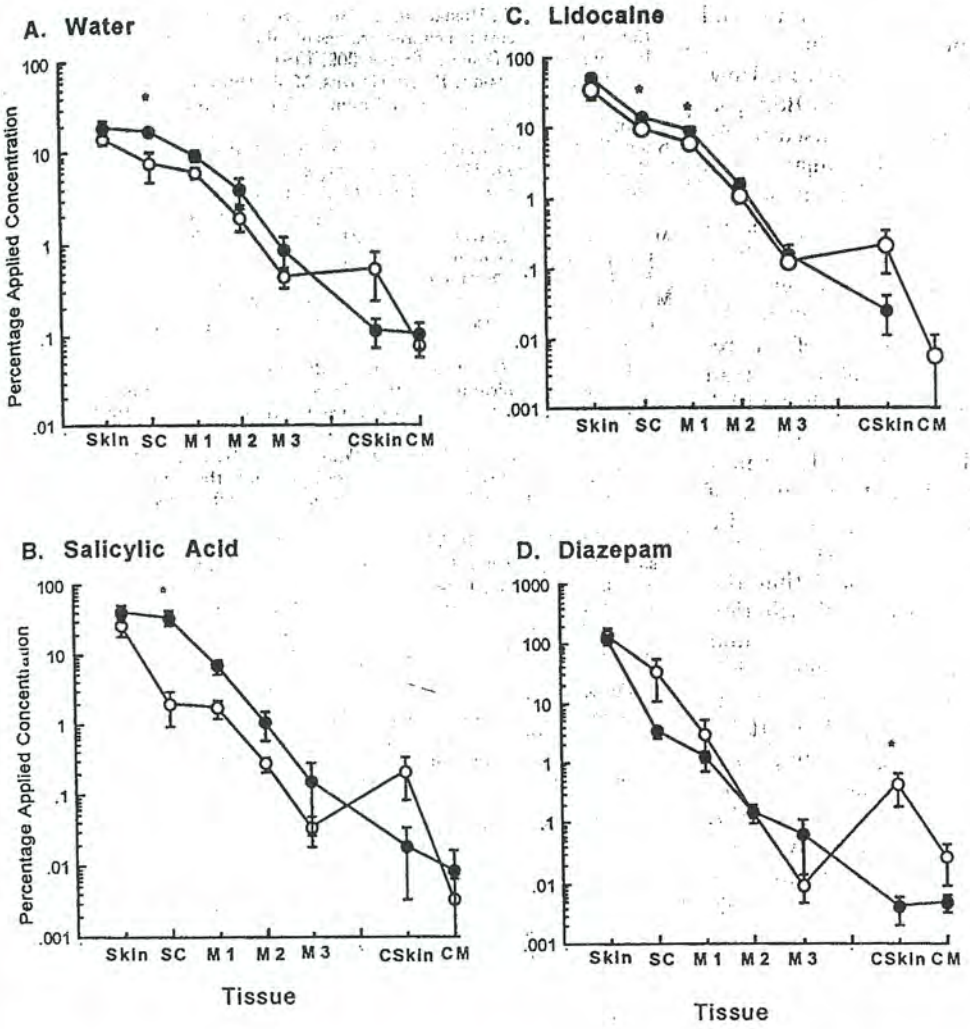

Fig. 5. Relationship between the percentage of the applied concentration of individual solutes determined in tissue samples after 90 min 4% BSA perfusions (○) and 2.5% dextran perfusions (●). Mean ± S.E., $n = 3$, except water $n = 6$.

upper dermis where it remains associated with tissue structures and has only a low rate of diffusion into deeper tissue layers. This finding is in accordance with the findings of Koch and co-workers (1987), who, in their study on the penetration of diazepam across hairless mouse and human skin, suggested that the presence of the dermis presented some barrier properties to diazepam diffusion and that *in vitro* the dermis may require complete saturation before diazepam could be detected in the receiving chamber. Hawkins and Reifenrath (1986) also observed the same phenomenon, the dermal reservoir effect increasing with the lipophilicity of solutes, while examining the penetration of various drugs through pig skin. The longer lag phase seen in recovery of diazepam before appreciable concentrations were detected in the perfusate suggests the presence of a lag between the disappearance of diazepam into the dermis and its emergence into the systemic circulation. These data show, in agreement with the suggestion of Koch *et al.* (1987), that the topical administration of diazepam for systemic administration would first require saturation of the dermis under the application site. The low k_{el} observed for diazepam during BSA and dextran perfusions reflects the sequestration of this solute into the tissues of the hindlimb. Due to its highly lipophilic character, diazepam would partition easily into fat around the hindlimb and easily cross the lipophilic membranes of cells. Solutes such as diazepam cannot be effectively applied topically for local deep muscle penetration. However, the lack of significant penetration of solutes with the physicochemical properties of diazepam into deeper tissues suggests that slow release into the systemic circulation could be achieved after a long lag phase with the advantage of reduced risk of local toxicity in underlying muscle tissue.

An interesting finding of this study was an increased concentration of solutes found in the contralateral skin samples beneath the perfused limb during BSA perfusions relative to dextran perfusions. It should be emphasized that the concentrations obtained at this site were very low, about 1% of the applied concentration, but comparable to that found in some of the deeper muscles. The most likely explanation for the findings is a significant partitioning of solutes from the albumin perfusate across the capillary or venule wall. The most substantive increase was observed for diazepam, a solute with both high protein binding and lipophilicity. Substantive protein binding allows solute delivery to more distant sites by increasing the effective amount of solute that can be carried by the perfusate. Being uncharged and with a high lipophilicity also allows diazepam to cross vessel walls readily. Consistent with this suggestion, salicylic acid yielded lower concentrations in the contralateral tissue despite having higher protein binding and higher fractions present in the perfusate. Salicylic acid is effectively completely ionized at physiological pH values and therefore would have greater

difficulty crossing vessel walls. The increases in water and lidocaine concentrations in the presence of albumin were not significantly greater than in the presence of dextran. However, the trend toward higher concentrations with BSA perfusate suggests that BSA itself may cause increased perfusion of the dermal region. The pattern of solute distribution could not have been caused by the diffusion of solutes through the hindlimb tissues from the application site, because much lower concentrations were found in intermediate tissues such as the deep and contralateral muscle layers. The possibility of contamination of contralateral skin during the procedure was discounted due to the consistency of this result and the lack of contamination seen in any of the dextran perfusions that were performed intermittently with the BSA experiments. In addition, negligible radioactivity was found on analysis of blank tissues from all regions. Solutes may also be transported in the protein perfusate from areas of relatively high concentration beneath the application site directly to the contralateral skin. Monterio-Riviere *et al.* (1993) and McNeill *et al.* (1992) have suggested that the orientation of local blood vessels influences the distribution of topically applied compounds. These investigators found that the deep tissue penetration of piroxicam applied topically to rats over muscular areas, known to contain complicated vascular networks, was greater than could be attributed to diffusion alone. The present study suggests that blood vessels pass either through the deep tissues of the leg directly to the opposite side and therefore shunt solutes across the muscle layers to the contralateral skin. Alternatively, blood vessels run in the skin beneath the application site and continue through the skin either 1) down the leg and back through the skin as part of the venous system or 2) across the leg and through the contralateral skin sampling site. Further investigations are underway to examine the vascular networks in the rat hindlimb to shed more light on this phenomenon.

References

Adriani, J., Zepernick, R., Arens, J. and Authement, E.: The comparative potency and effectiveness of topical anaesthetics in man. Clin. Pharmacol. Ther. **5:** 49–62, 1963.

Cross, S. E., Wu, Z. Y. and Roberts, M. S.: Effect of perfusion flow rate on the tissue uptake of solutes after dermal application using the rat isolated perfused hindlimb preparation. J. Pharm. Pharmacol. **46:** 844–850, 1994.

Cross, S. E. and Roberts, M. S.: Subcutaneous absorption kinetics of interferon and other solutes. J. Pharm. Pharmacol. **45:** 606–609, 1993.

Flaim, S. F., Morris, Z. Q. and Kennedy, T. J.: Dextran as a radioactive microsphere suspending agent: Severe hypotensive effect in rat. Am. J. Physiol. **235:** H587–H591, 1978.

Gillette, J. R.: Overview of drug protein binding Ann. NY Acad. Sci. **226:** 6–17, 1973.

Guyton, A. C.: Textbook of Medical Physiology, 8th ed., p. 178, Saunders, Philadelphia, 1991.

Hadgraft, J.: Formulation of antiinflammatory agents. Pharmacol. Skin **2:** 21–43, 1989.

Harris, J. M., Luscome, D. K. and Poyser, R. H.: The influence of molecular weight and structure on the vascular permeability responses induced by glucose polymers in rats skin. Br. J. Pharmacol. Chemother. **29:** 16–24, 1967.

Hawkins, G. S. and Reifenrath, W. G.: Influence of skin source, penetration cell fluid and partition coefficient on in vitro skin penetration. J. Pharm. Sci. **75:** 378–381., 1986.

Hirate, J., Kato, Y., Horikoshi, I., Nagase, S. and Ueda, C.: Further observations on the disposition characteristics of salicylic acid in albuminemic rats. Biopharm. Drug Dispos. **10:** 299–309, 1989.

Koch, R. L., Palicharla, P. and Groves, M. J.: Diffusion of [2-^{14}C] diazepam across hairless mouse skin and human skin. J. Invest. Dermatol. **88:** 582–585, 1987.

Koch-Weser, J. and Sellers, E. M.: Binding of drugs to serum albumin. N. Engl. J. Med. **294:** 311–316, 1976.

Kushla, G. P. and Zatz, J. L.: Lidocaine penetration through human and hairless mouse skin *in vitro*. J. Soc. Cosmet. Chem. **40:** 41–50, 1989.

McCafferty, D. F., Woolfson, A. D., McClelland, K. H. and Boston, V.: Comparative *in vivo* and *in vitro* assessment of the percutaneous absorption. Br. J. Anaesth. **60:** 64–69, 1988.

McNeill, S. C., Potts, R. O. and Francoeur, M. L.: Local enhanced topical delivery of drugs: Does it really exist? Pharmaceutical Res. **9:** 1422–1427, 1992.

Monterio-Riviere, N. A., Inman, A. O., Riviere, J., McNeill, S. C. and Francoeur, M. L.: Topical penetration of piroxicam is dependent on the distribution of the local cutaneous vasculature. Pharmaceutical Res. **10:** 1326–1331, 1993.

Neubert, R., Partyka, D., Wohlrab, W., Dettlaff, B., Furst, W. and Taube, K. M.: Penetration of salicylic acid and salicylate into the multilayer membrane system and into the human horny layer. Dermatol. Monatsschr. **176:** 711–716, 1990.

Owen, S. G., Francis, H. W. and Roberts, M. S.: Disappearance kinetics of solutes from synovial fluid after intra-articular injecion. Br. J. Clin. Pharmacol. **38:** 349–355, 1994.

Perez-Trepichio, A. D., Salgado, A. V. and Jones, S. C.: Isovolumic hemodilution with dextran 40 in the rat: effect on the development of peripheral edema and various physiologic parameters. J. Pharmacol. Exp. Ther. **256:** 913–916, 1991.

Piafsky, K. M. and Knoppert, D.: Binding of local anaesthetics to a1-acid glycoprotein. Clin. Res. **26:** 836A, 1978.

Riviere, J. E., Riviere-Monteiro, N. A. and Inman, A. O.: Determination of lidocaine concentrations in skin after transdermal iontophoresis: effects of vasoactive drugs. Pharm. Res. 211–214, 1992.

Routledge, P. A., Barchowsky, A., Bjornsson, T. D., Kitchell, B. B. and Shand, D. G.: Lidocaine plasma protein binding. Clin. Pharmacol. Ther. **27:** 347–351, 1980.

Roberts, M. S.: Structure-permeability considerations in percutaneous absorption. *In* Predictions of Percutaneous Absorption, ed. by R. C. Scott, R. H. Guy and J. Hadgraft, IBC Technical Services, London, 1991.

Siddiqui, O., Roberts, M. S. and Polack, A. E.: Percutaneous absorption of steroids: Relative contributions of epidermal penetration and dermal clearance. J. Pharmacokin. Biopharm. **17:** 405–424, 1989.

Siddiqui, O., Roberts, M. S. and Polack, A. E.: Topical absorption of methotrexate: Role of dermal transport. Int. J. Pharm. **27:** 193–203, 1985.

Singh, P. and Roberts, M. S.: Deep tissue penetration of lignocaine after dermal application. Clin. Exp. Pharmacol. Physiol. **17:** suppl., 71, 1990.

Singh, P. and Roberts, M. S.: Dermal and underlying tissue pharmacokinetics of salicylic acid after topical application. J. Pharmacokin. Biopharm. **21:** 337–373, 1993a.

Singh, P. and Roberts, M. S.: Iontophoretic delivery of salicylic acid and lignocaine to local subcutaneous structures. J. Pharm. Sci. **82:** 127–131, 1993b.

Singh, P. and Roberts, M. S.: Dermal and underlying tissue pharmacokinetics of lignocaine after topical application. J. Pharm. Sci. **83:** 774–782, 1994a

Singh, P. and Roberts, M. S.: Effects of vasoconstriction on dermal pharmacokinetics and local tissue distribution of compounds. J. Pharm. Sci. **83:** 783–791, 1994b.

Vallner, J. J.: Binding of drugs by albumin and plasma protein. J. Pharm. Sci. **66:** 447–465, 1977.

Wu, Z., Cross, S. E. and Roberts, M. S.: Influence of physicochemical parameters and perfusate flow rate on the distribution of solutes in the isolated perfused rat hindlimb using the impulse-response technique. J. Pharm. Sci. **84:** 1020–1027, 1995.

Wu, Z. Y., Rivory, L. P. and Roberts, M. S.: Physiological pharmacokinetics of solutes in perfused rat hindlimb: Characterisation of the physiology with changing perfusate flow, protein content and temperature using statistical moment analysis. J. Pharmacokin. Biopharm. **21:** 653–688, 1993.

Send reprint requests to: Professor Michael S. Roberts, Department of Medicine, University of Queensland, Princess Alexandra Hospital, Brisbane, Qld 4102 Australia.