Convective transport of highly plasma protein bound drugs facilitates direct penetration into deep tissues after topical application

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

Many products are applied to human skin for local effects in deeper tissues. Animal studies suggest that deep dermal and/or subcutaneous delivery may be facilitated by both dermal diffusion and transport via the cutaneous vasculature. However, the relationship between the extent and pathways of penetration, drug physicochemical properties and deeper tissue physiology is not well understood.

WHAT THIS STUDY ADDS

We have used a physiologically based pharmacokinetic model to analyze published human cutaneous microdialysis data, complemented by our own *in vitro* skin penetration studies. We found that convective blood, lymphatic and interstitial flow led to significant deep tissue concentrations for drugs that are highly plasma protein bound. In such cases, deeper tissue concentrations will occur earlier and may be several orders of magnitude greater than predicted by passive dermal diffusion alone.

AIMS

To relate the varying dermal, subcutaneous and muscle microdialysate concentrations found in man after topical application to the nature of the drug applied and to the underlying physiology.

METHODS

We developed a physiologically based pharmacokinetic model in which transport to deeper tissues was determined by tissue diffusion, blood, lymphatic and interstitial flow transport and drug properties. The model was applied to interpret published human microdialysis data, estimated *in vitro* dermal diffusion and protein binding affinity of drugs that have been previously applied topically *in vivo* and measured in deep cutaneous tissues over time.

RESULTS

Deeper tissue microdialysis concentrations for various drugs *in vivo* vary widely. Here, we show that carriage by the blood to the deeper tissues below topical application sites facilitates the transport of highly plasma protein bound drugs that penetrate the skin, leading to rapid and significant concentrations in those tissues. Hence, the fractional concentration for the highly plasma protein bound diclofenac in deeper tissues is 0.79 times that in a probe 4.5 mm below a superficial probe whereas the corresponding fractional concentration for the poorly protein bound nicotine is 0.02. Their corresponding estimated *in vivo* lag times for appearance of the drugs in the deeper probes were 1.1 min for diclofenac and 30 min for nicotine.

CONCLUSIONS

Poorly plasma protein bound drugs are mainly transported to deeper tissues after topical application by tissue diffusion whereas the transport of highly plasma protein bound drugs is additionally facilitated by convective blood, lymphatic and interstitial transport to deep tissues.

Introduction

A number of topical drugs are applied to target deeper dermal, subcutaneous and muscle layers, including non-steroidal anti-inflammatory drugs, topical anaesthetics, steroids, anti-infective and antifungal agents [1]. For such drugs, understanding the penetration behaviour into deep dermal tissue layers is critical for therapeutic success and early work has shown that direct and significant penetration of therapeutic solutes deep into human skin after topical application can occur [2]. In general, therapeutic concentrations of drugs being targeted to deeper tissues after topical application in man are assessed either using cutaneous microdialysis [3–5] or by biopsy [6, 7]. In contrast, deep dermal transport is probably undesirable for drugs being applied in transdermal delivery systems for systemic effects, as transport into deep dermal tissue layers may be associated with sequestration, metabolism and a lack of therapeutic advantage. Indeed, human cutaneous microdialysis studies of topically applied nicotine for smoking cessation suggest that nicotine is not delivered to deeper tissues in high concentrations [8]. The relationship between the extent and mechanisms of drug penetration into deep dermal tissue after topical application, solute properties and skin physiology is controversial [9]. We and others have deduced from rat wound deposition studies that diffusion governs penetration into deep tissue and that drug molecular weight is the key physicochemical determinant of underlying tissue concentrations [10–12]. Other studies in rats [13] and in pigs [14] suggest that penetration of topically applied drugs into deep skin layers also involves the cutaneous microvasculature. Our own work suggests that methyl salicylate-induced increase in cutaneous blood flow can promote the deeper penetration of salicylate from topically applied methylsalicylate [15].

Our goal in this study is to understand the mechanisms whereby topically applied drugs can (or cannot) reach high microdialysate concentrations in dermal, subcutaneous and muscle tissue in man. We sought to separate out the relative importance of dermal diffusion and dermal binding as determinants of dermal transport by conducting *in vitro* penetration experiments of six drugs of varying lipophilicity and protein binding affinity using excised human dermis. We then compared the *in vitro* lag times obtained to those reported *in vivo* in the dermis and deeper tissues. We developed a physiological pharmacokinetic model to describe the time dependence of diclofenac and nicotine transport *in vivo* using human microdialysis data from Muller *et al.* [16, 17]. The model describes drug diffusion in the extravascular tissue space, tissue and vascular binding, axial (into the tissue) vascular, lymphatic and interstitial convection transport and constant radial (clearance) vascular transport, with the assumption of high capillary permeability (Figure 1). We also measured transport of various solutes in the human

dermis and used published *in vivo* drug penetration data for human and animal dermis, subcutaneous and muscle tissue (Table 1) to show that deep tissue drug transport can be related to the physicochemical properties of the drug molecules (lipophilicity and protein binding affinity) and tissue physiology.

Methods

Chemicals

The drugs used were diclofenac acid (Amoli Organics, Mumbai, India), fluconazole, ibuprofen, lidocaine HCl (Sigma-Aldrich, Sydney, NSW, Australia), (–)-nicotine (BDH Laboratory Supplies, Poole, England) and propranolol HCl provided by Imperial Chemical Industries PLC Pharmaceutical Division. For the HPLC assays, additional chemicals used were mepivacaine HCl provided by Astra Pain Control, acetic acid from BDH Laboratory Supplies, fluconazole (Diflucan) from Pfizer, paracetamol, phosphate buffer saline (PBS), sodium acetate, sodium dihydrogen phosphate (NaH_2PO_4) and triethylamine from Sigma-Aldrich (Sydney, NSW, Australia), bovine serum albumin (BSA) from Bovogen (Melbourne, VIC, Australia), acetonitrile, sodium hydroxide and methanol from Lab-Scan Analytical Sciences (Seven Hills, NSW, Australia), and potassium dihydrogen phosphate (KH_2PO_4) from Ajax Finechem (Taren Point, NSW, Australia).

Preparation of dermal tissue

Female abdominal skin was obtained from two donors following abdominoplasty in accordance with human ethics approval from the Princess Alexandra Hospital Human Ethics Committee. Fat and subcutaneous tissues were removed using blunt dissection. The epidermis and the dermis were separated using the heat separation technique (immersion in water at 60°C for 1 min) [18].

In vitro penetration study

Skin penetration studies were carried out using side-by-side diffusion cells immersed in a water bath at 36°C, $n = 6$ for each drug. The donor and receptor volumes were 1.5 ml and the mean surface area of penetration was $1.12 \pm 0.03 \text{ cm}^2$. Both sides of the dermis were first equilibrated at 36°C for 4 h with 1.5 ml PBS at pH 7.4 with 4% BSA in the donor and receptor compartments of the side-by-side cells. After equilibration, the PBS with 4% BSA was removed from both compartments. It was replaced on the donor side of the cells with 1.5 ml of the drug under consideration in PBS at pH 7.4 for fluconazole ($890 \mu\text{g ml}^{-1}$), lidocaine ($1190 \mu\text{g ml}^{-1}$) and propranolol ($880 \mu\text{g ml}^{-1}$) and PBS with 4% BSA at pH 7.4 in the cases of diclofenac ($690 \mu\text{g ml}^{-1}$), ibuprofen ($820 \mu\text{g ml}^{-1}$) and nicotine ($5300 \mu\text{g ml}^{-1}$). The receptor side of the cells was filled with 1.5 ml of either PBS or PBS with 4% BSA at pH 7.4. The presence of BSA in both the donor and receptor phases

Figure 1

Physiological pharmacokinetic model for topical drug transport processes in deeper skin. (A) Blood vessels, lymphatic vessels and interstitial convection in dermis. (B) Schematic of physiological pharmacokinetic model used to analyze human cutaneous microdialysis data at two depths, the concentration–time profile in the superficial microdialysis probe being used as an input to predict the deeper microdialysis probe concentration–time profile

mimicked the physiological conditions of the dermis *in vivo* (we assumed it did not alter the dermis transport properties). It is also a standard addition to receptor solutions for *in vitro* skin penetration studies of lipophilic compounds [19]. Unfortunately, it was not possible to develop simple HPLC assays for fluconazole, lidocaine and propranolol with BSA present. For this reason we used PBS as the solvent and receptor solution for the skin penetration experiments involving these drugs. The penetration experiments were conducted for 24 h. Donor and receptor solutions were continuously stirred with magnetic stirrers. Samples with volumes of 200 μ l were taken from the

receptor solution of each cell at fixed time points, and the receptor solution was topped up with 200 μ l of fresh PBS at pH 7.4 with 4% BSA. At the end of the experiment the donor and receptor solutions were removed and the cells dismantled. The donor and receptor sides of each piece of dermis were sampled using one dry cotton swab for each side. The swabs were put in 500 μ l acetonitrile for extraction. The area of exposed dermis was cut from the rest of the piece of skin and weighed. The thickness of the dermis was measured between two microscope glass slides using an electronic calliper. To measure the solute concentration remaining in the skin, the dermis pieces were cut in smaller

Table 1

Human and animal dermis and deep tissue drug penetration studies. All references to published *in vivo* experiments are microdialysis studies except [2] and [15]

Drug	Molecular weight	Logarithm octanol/water partitioning coefficient (Reference)	In vitro data		In vivo data		Mean lag time (min)	Reference
			Fraction unbound to protein	Mean lag time (min)	Application site and depth*	Concentration levels		
Diclofenac	296	4.3 [10]	0.05	537	This study	Human thigh 3.2 mm (subcutis) 9.1 mm (muscle) Human thigh Subcutaneous adipose tissue Skeletal muscle tissue	Nearly identical 90 min after application Nearly identical 20 min and 600 min after application.	<30 <60 [17, 57]
Ibuprofen	206	3.6 [58]	0.11	216	This study	Human thigh 4–5 mm (subcutis) 25–30 mm (muscle)	6–15 times lower in muscle than in subcutis after 240 min. In 50% of volunteers a large increase in muscle tissue.	41 (subcutis) 104 (muscle) [36]
Fluconazole	306	0.50 [37]	0.85	26	This study	Rat dorsal region 0.23 to 0.46 mm (dermis)	AUC constant to 0.35 mm in depth, below AUC decreases with increasing depth.	30 [37]
Lidocaine	234	2.33 [59]	0.90	44	This study	Human arm ≈1.0 mm (dermis)	Inter-individual differences in skin barrier function is a greater determinant of observed variability in cutaneous drug concentrations after topical application than probe depth.	110 [26]
Nicotine	162	1.2 [60]	0.91	44	This study	Human peri-umbilical region 2.0 mm (subcutis) to 9.5 mm (muscle) Human thigh 0.8 mm (dermis)	Significant differences at each depth, 90 min after application Uniform for shallow distance	35 180 [16] [8]
Propranolol	259	3.4 [61]	0.57	56	This study	Human arm 0.3 to 2.2 mm (dermis-subcutis). Iontophoretic delivery.	Uniform	9.6–10.5 [23]
Ethanol	46	0.19 [62]	~1.0† [63]	73	[64]	Human arm 1.07 (dermis) and 2.37 mm (subcutis)	Up to 200 times lower in subcutis than in dermis	20, 70 [65]
5-fluorouracil	130	–0.95 [66]	–0.90	180–540	[67]	Rat dermal tissue	In vivo steady-state concentrations are 40-fold lower than in vitro.	60–120 [67]
Ketoprofen	254	2.8 [68]	0.05† [69]	360–900	[70]	Human arm 0.64 to 0.87 mm (dermis)	Uniform	15 [50]
Methyl salicylate (MeSA)	152	2.46 [71]	0.14†	~10‡	–	Rat abdomen Dog joint 0.42 mm (dermis) and 19.5 mm (muscle)	Average AUC in tissue is 1 to about 4 times greater than in equivalent contralateral sites in deep tissue. 2-fold higher concentration of MeSA and salicylic acid in treated vs. untreated sites, where concentrations are equivalent to systemic plasma concentrations.	30 60 [15, 46]
Salicylic acid	138	2.25 [71]	0.14† [10]	10	[55]	Human arm 1.0 mm (dermis) Rat side of trunk 0.5 to 1.2 mm (dermis)	Uniform AUC About 70-fold difference between shallowest and deepest depth	10 10 [3, 33]
Salicylic compounds	137–211	1.3–4.6	0.14†	~10‡	–	Rat side of trunk 0.7 to 1.1 mm (dermis)	Uniform	10 [34]
Trolamine salicylate	287	–	0.14†	~10‡	–	Human knee Dog knee	Absorption into joints. Synovial fluid concentrations are about 60% of the values obtained by oral administration. High concentrations in joints may be due to recirculation. Synovial fluid concentrations are about 80% of the values obtained by oral administration, while tendon concentrations are about 10 times greater.	/ / [2]

*The skin layer characterization (dermis, subcutis, muscle) corresponding to the cited microdialysis probe depths is obtained from information in [72, 73]. †Fractions unbound to plasma protein. ‡Lag times into dermis of all salicylate compounds are expected to be similar to that of salicylic acid.

pieces and placed in 500 µl acetonitrile for at least 12 h for extraction.

Determination of fraction unbound to albumin

The fraction of permeant unbound to BSA was determined by ultrafiltration. Diclofenac and nicotine solution in PBS (500 µl) were put in the donor compartment of Microcon centrifugal filter devices (Millipore Corporation, Bedford, MA, USA) with a cutoff of 30 000 Da, vortexed and ultrafiltered at 13 000 rev min⁻¹ for 10 min. The partitioning coefficient $K_{d/de}$ was calculated from the permeant concentrations in the donor, c_d , and in the dermis, c_{de} , at the end of the penetration experiment (24 h), assuming a linear decrease in the tissue concentration with depth (consistent with steady-state diffusion in the absence of elimination): $K_{d/de} = 2c_d/c_{de}$.

HPLC analysis

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a Shimadzu LC-10AD pump; a Shimadzu SIL-6B auto injector; a C18 column (Waters Symmetry C18, 5 µm, 150 × 3.9 mm for diclofenac, propranolol and fluconazole, Phenomenex Luna 5 µm 150 × 4.6 mm for ibuprofen and nicotine and Sorbax Eclipse Plus C18, 3.5 µm, 150 × 4.60 mm for lidocaine) as well as a guard column (Phenomenex C18 4 × 3 mm), a Shimadzu UV detector with detection wavelengths of 276 nm, 215 nm, 259 nm, 205 nm, 375 nm and 210 nm for diclofenac, ibuprofen, nicotine, lidocaine, propranolol and fluconazole, respectively.

The mobile phase used was acetic acid : acetonitrile : water 2:62:36 (pH 4.35) for diclofenac, methanol : 0.5% acetic acid 75:25 (pH = 3) for ibuprofen, acetate buffer (0.03 M, pH = 6) : 0.02 M sodium acetate in acetonitrile 80:20 for nicotine, 0.05 M KH₂PO₄ : acetonitrile : triethylamine 75:25:1 (pH = 6) for lidocaine, 0.01 M KH₂PO₄ : acetonitrile 75:25 (pH = 3) for propranolol and 0.02 M NaH₂PO₄ : acetonitrile 80:20 for fluconazole. A flow rate of 1 ml min⁻¹ was used for all the assays.

The standards and samples were prepared for HPLC analysis according to the following procedure: 1) Diclofenac, ibuprofen, nicotine and propranolol: 100 µl aliquots of receptor and ultrafiltration samples were mixed with 10 µl of 30% paracetamol and 200 µl of acetonitrile; 2) Lidocaine: 100 µl aliquots of receptor and ultrafiltration samples were mixed with 20 µl of 50 mg ml⁻¹ mepivacaine HCl; 3) Fluconazole: 50 µl aliquots of receptor and ultrafiltration samples were mixed with 20 µl of 100 µg ml⁻¹ paracetamol. The mixture was vortex-mixed, centrifuged and injected into the HPLC column. The injection volume was 35, 75, 75, 50, 50 and 20 µl for diclofenac, ibuprofen, nicotine, lidocaine, propranolol and fluconazole respectively.

The assays proved to be sensitive and linear over the range 3.13–100 µg ml⁻¹ for diclofenac, 3.13–200 µg ml⁻¹ for ibuprofen, 6–100 µg ml⁻¹ for nicotine, 1.56–200 µg ml⁻¹ for lidocaine, 0.8–10 µg ml⁻¹ for propranolol and 0.39–100 µg ml⁻¹ for fluconazole. The inter-sample reproducibility

was 2.2%, 0.7%, 3%, 1.7%, 3% and 2.3% for diclofenac, ibuprofen, fluconazole, lidocaine, nicotine and propranolol, respectively.

Calculation of in vitro diffusion coefficients

The *in vitro* dermis diffusion coefficient D_{de} is a measure of the 'speed' of penetration into the tissue, as shown by the relationship $t_{lag} = h_{de}^2/6D_{de}$, where t_{lag} is lag time for the penetration of a solute across excised dermis, and h_{de} is the dermis thickness. The diffusion coefficient is thus determined using the relation $D_{de} = h_{de}^2/6t_{lag}$, once t_{lag} and h_{de} have been determined experimentally. The lag times t_{lag} are obtained by extrapolating the linear part of the *in vitro* cumulative amount penetrated vs. time curve (data not shown) to the time axis. The parameter h_{de} is the dermal thickness measured at the end of the 24 h experiment.

Physiologically based pharmacokinetic model used to analyze in vivo diclofenac and nicotine human microdialysis data

In this analysis, we used the physiological pharmacokinetic model that we have recently described [6] using the particular form of the model in which the permeability of blood and lymphatic vessels was not rate limiting in solute transport. We have previously shown that such a model adequately described the distribution and clearance of highly protein bound solutes in a perfused hind limb after topical application [20]. An important addition to the model is the recognition of interstitial convection associated with capillary flow and draining of the interstitial space (Figure 1A). Here, our detailed model analysis used serial cutaneous microdialysis concentration–time profiles available at two depths in the skin of individual human volunteers [16, 17] below the epidermis (Figure 1B). Importantly, our analysis was unaffected by the nature of solute penetration through the stratum corneum. Stratum corneum thickness, and therefore the penetration lag time to the epidermal-dermal junction and beyond, can vary with body site [21]. In Muller *et al.*'s microdialysis studies, diclofenac was applied to the thigh and nicotine was applied to periumbilical skin. In our analysis we have circumvented these details by using the concentration–time profile in a superficial probe in the dermis as an input function for our physiologically based pharmacokinetic model, in which the deeper microdialysis probe concentration–time profiles were used as the effect site function (Figure 1B). In particular, we used Muller *et al.*'s [16, 17] data for the superficial microdialysis probe depths of $z_1 = 3.2$ mm (diclofenac) and 2.0 mm (nicotine), the microdialysate concentration–time profiles $c(z_1, t)$ and fitted them as an empirical bi-exponential input function:

$$c(z_1, t) = c_1 = A(e^{-b_1 t} - e^{-b_2 t}) \quad (1)$$

where A , b_1 and b_2 are arbitrary constants. This reduced physiological pharmacokinetic model also includes drug

diffusion in the extravascular tissue space (D_{diff}), unbound dermal binding (f_{uD}) and unbound vascular binding (f_u), dispersion due to the additional transport in blood and lymphatic vessels as well as interstitial convection (D_{disp}) and elimination at a given depth, expressed as radial (clearance) vascular transport (k_e), with the assumption of high capillary permeability (Figure 1B). The governing partial differential equation describing transport into the dermis and subcutaneous tissue, approximated as a homogenous tissue medium in which the various pharmacokinetic parameters are constant over time and with distance, is

$$\partial c(z, t) / \partial t = (D_{disp} + f_u D_{diff}) (\partial^2 c(z, t) / \partial z^2) - k_e c(z, t) \quad (2)$$

The boundary conditions are $c(z_1, t) = c_1$ (equation 1) and $c(z \rightarrow \infty, t) = 0$ (approximating the skin as a semi-infinite medium). The time-dependent model solution c_2 at a depth $z_2 > z_1$ is calculated in the Laplace domain as

$$c_2(s) = A / [(s + b_1)(s + b_2)] \cdot \exp\left[-\sqrt{(s + k_e) / (D_{disp} + f_u D_{diff})} (z_2 - z_1)\right] \quad (3)$$

By fitting Muller *et al.*'s deep tissue concentration profiles at the microdialysis probe depths of $z_2 = 9.1$ mm (diclofenac) and 4.5 mm (nicotine) to the numerical inverse Laplacian of equation 3, we obtained estimates for D_{disp} and k_e for each drug. The *in vivo* elimination half-lives are calculated from [20]:

$$t_{1/2} = 0.693 / k_e \quad (4)$$

All data fits and SDs were obtained using SCIENTIST (Micro-Math Scientific Software, Salt Lake City, UT, USA).

The relative concentrations for diclofenac and nicotine in a probe at a depth of 4.5 mm below a superficial probe, in which the drug concentration is a constant value of $c_{ss,i}$, is given by the boundary conditions $c(z_1, t) = c_{ss,i}$, so that the relative time-dependent model concentration $c_2 / c_{ss,i}$ at a depth of 4.5 mm below z_1 is calculated in the Laplace domain as

$$c_2(s) / c_{ss,i}(s) = [1/s] \cdot \exp\left[-\sqrt{(s + k_e) / (D_{disp} + f_u D_{diff})} (4.5 \text{ mm})\right] \quad (5)$$

At long times (steady-state), the inversion of equation (5) is given by $s \cdot f(s)$ where $s \rightarrow 0$, i.e., the steady-state relative concentrations are given by

$$c_2 / c_{ss,i} = \exp\left[-\sqrt{(s + k_e) / (D_{disp} + f_u D_{diff})} (4.5 \text{ mm})\right] \quad (6)$$

As the expression for time lag in a diffusion-reaction system is quite complex [22] we have defined lag time in the deep probe for a constant input concentration in the superficial probe as the time taken to reach 0.01 of that input concentration as defined by equation 5.

Results

Comparison of *in vitro* dermis and *in vivo* dermis/deep tissue penetration lag times

Table 1 compares the *in vitro* lag times for diclofenac, fluconazole, ibuprofen, lidocaine, nicotine and propranolol obtained from our dermis penetration experiments with published *in vivo* data. Diclofenac and ibuprofen are highly BSA bound ($f_{uBSA} = 0.05$ and 0.11, respectively). The *in vitro* lag times determined in our experiments are 5 to 18 times larger than the observed *in vivo* lag times in subcutaneous and muscle tissue (Table 1). The fraction of propranolol unbound to BSA was determined to be 0.57, and the mean *in vitro* lag time we calculated was 5 to 6 times greater than that reported by Stagni *et al.* [23] for the iontophoretic delivery of propranolol into dermal and subcutaneous tissue. On the other hand, fluconazole and nicotine remain mostly unbound to BSA ($f_{uBSA} = 0.85$ and 0.91, respectively). These drugs yielded *in vitro* lag times which are on the same order of magnitude as the observed *in vivo* lag times into dermis and deeper tissues. The *in vitro* lag time of mostly unbound ($f_{uBSA} = 0.90$) lidocaine from an aqueous solution was 44 min, similar to that reported for unionized lidocaine across dermatomed human skin [24], to the observed *in vivo* human skin lag times of about 70 min for a eutectic topical product [25] as well as to the lower value of 45 min for a microemulsion formulation [26].

The relative penetration of diclofenac and nicotine in deep subcutaneous skin layers

We used our results for the *in vitro* penetration of these drugs through excised human dermis (Table 1) to analyze the reported *in vivo* deep tissue penetration of diclofenac and nicotine [16, 17]. Figure 2A and B show regressions obtained using diclofenac and nicotine data sets using our physiological pharmacokinetic model at different cutaneous microdialysis depths (3.2 and 9.1 mm for diclofenac; 2.0 and 4.5 mm for nicotine). When transport was assumed to be by tissue diffusion and constant elimination alone, the model poorly described the diclofenac data (Figure 2A) but adequately described the nicotine data (Figure 2B). In contrast, a satisfactory regression fit to the microdialysis concentration profile of diclofenac at a depth of 9.1 mm was obtained assuming transport by a model that included convective blood, lymphatic and convective flow transport (Figure 2A). This latter regression yielded an apparent *in vivo* dermal dispersion coefficient of $5.3 \pm 1.2 \times 10^{-4} \text{ cm}^2 \text{ s}^{-1}$ and an *in vivo* elimination half-life into the blood leaving the subcutaneous tissue of 185 ± 120 min. Our *in vitro* experiments show that diclofenac is highly protein bound, with the unbound fractions in 4% BSA solution equal to 0.04 ± 0.005 . This value is consistent with a published value of fraction unbound to 2% albumin solution, 0.029 [10]. Our penetration studies with excised dermis yielded an *in vitro* dermal diffusion coefficient of $3.8 \pm 0.8 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. The high apparent *in vivo* dispersion

Figure 2

Human cutaneous microdialysis data and fitting. (A) Diclofenac data at (●) 3.2 mm and (■) 9.1 mm and regression lines for empirical fit model at 3.2 mm (—), convective flow + diffusion model at 9.1 mm (---) and diffusion only model at 9.1 mm (---). (B) Nicotine microdialysis data at (●) 2.0 mm and (■) 4.5 mm and regression lines for empirical fit model at 2.0 mm (—), convective flow + diffusion model at 4.5 mm (---) and diffusion only model at 4.5 mm (---). (C) Predicted concentration–depth profiles for diclofenac (—) and nicotine (---) at 120 min

coefficient for diclofenac suggest that it is being transported about 1400 times faster *in vivo* than would be predicted by the *in vitro* dermis diffusion coefficient. As elucidated more fully in the discussion, a high apparent *in vivo* dispersion coefficient most likely reflects blood convection, lymphatic and convective dispersion caused by the variation in flows and vasculature orientation as originally described as a carriage mechanism modifying convective blood transport of drugs in the liver by Roberts & Rowland [27, 28] and, most recently, in the dermis by Anisimov & Roberts [6] where dispersion was also suggested to arise from lymphatic flow.

In contrast, regression using the *in vivo* nicotine concentration profile at a depth of 4.5 mm leads to a much smaller apparent *in vivo* dermal dispersion coefficient of $1.8 \pm 0.9 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, with an elimination half-life into

the blood leaving the subcutaneous tissue of $19.6 \pm 13.0 \text{ min}$ (Figure 2B). This is about four times the *in vitro* diffusion coefficient of excised dermis, $4.4 \pm 2.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Our experimental data suggest that nicotine exists mainly in the unbound form in both the plasma (fraction of drug unbound in a 4% BSA solution: 0.90 ± 0.02) and in the dermis (fraction unbound: 0.72 ± 0.02), consistent with literature findings (plasma fraction unbound: 0.92 to 0.95 [29, 30]). The *in vitro* nicotine concentration profile, predicted assuming dermal transport by diffusion with irreversible clearance into the vasculature but no convection to deeper tissues by vascular, lymphatic and interstitial convective transport, is comparable to the reported *in vivo* data at a skin depth of 4.5 mm (Figure 2B). Figure 2C shows the predicted diclofenac and nicotine concentration–depth profiles for times of 0 to 120 min. The

diclofenac profile is relatively flat whereas the nicotine concentrations decrease exponentially with depth.

The importance of dispersion as a determinant of transport is most evident under input steady-state conditions. Under such conditions the *in vivo* lag time and relative concentration measured in two microdialysis probes separated by a distance of 4.5 mm are 1.1 min and 0.86 for the highly plasma protein bound diclofenac. For the poorly protein bound nicotine, the *in vivo* lag time and relative concentration are 30 min and 0.08. Under steady-state conditions, the *in vitro* lag times would be 1500 min (25 h) for diclofenac and 125 min for nicotine. The *in vitro* relative concentrations for a microdialysis probe separation of 4.5 mm would be 0.02 for both diclofenac and nicotine.

Discussion

A major finding in this work is that transport of highly plasma protein bound drugs into deeper dermal tissues occurs several orders of magnitude faster than predicted by passive dermal diffusion. There appears to be no concentration–depth gradient for highly bound drugs in the papillary dermis adjacent to the application site, and only a small gradient in the reticular dermis. In contrast, a substantial concentration–depth profile is apparent for poorly protein bound drugs and predicted for highly bound drugs using *in vitro* dermis diffusion coefficients. Convective transport apparently increases the concentration in deep tissues compared to values predicted from dermal diffusion alone. The nature of the convective blood, lymphatic and interstitial flow affecting drug distribution is different in the papillary and reticular dermis (Figure 3A).

The majority of cutaneous blood vessels are located in the top 1 to 2 mm of the skin [31]. Adjacent lymphatic capillaries begin deeper and drain vertically into lower dermal and upper subcutaneous vessels, without anastomosing with blood vessels [32]. Figure 3B shows that the distribution of blood vessel surface area to total skin surface area is effectively constant down to 2 mm, then decreases quasi-exponentially. This structure supports a ‘well-stirred’ mixing behaviour below 2 mm, with no concentration–depth gradient for highly bound drugs. Contrary to Muller *et al.* [16], Hegemann *et al.* [8] reported no correlation between nicotine concentration and probe depth (0.57 to 1.22 mm), possibly because of higher blood vessel density in superficial skin layers (Figure 4). The high inter-subject variability in human *in vivo* studies may obfuscate a concentration–probe depth correlation. Similar studies with inbred rat species [33] have revealed such correlations, where inter-subject variability is lower. No such correlation was found in Simonsen *et al.* [34], perhaps due to different distances between probes.

Relatively uniform concentrations for all solutes are often seen to a depth of about 0.8 mm (Table 1), suggesting that dispersion attenuates diffusion-associated con-

centration gradients. Roberts & Rowland [28] reported a similar phenomenon for the hepatic elimination of drugs, using a convection-dispersion model. Roberts & Anissimov and Anissimov *et al.* [27, 35] developed this model further, showing that convection-dispersion depends on the relative blood flows in direct, branching and interconnecting sinusoids. More recently, we have shown that dermal biopsy data for many compounds can only be described by convective-dispersive blood and lymphatic transport. Consistent with this proposed model, at deeper dermal tissue levels where there is a lower vessel density, fewer interconnections and less flow (Figure 3A,B), highly protein bound drugs appear to have weak concentration gradients, whereas poorly bound drugs show an exponential concentration gradient (see Table 1 for examples). Exceptions are ibuprofen ($f_{uBSA} = 0.11$) and lidocaine ($f_{uBSA} = 0.90$) (Table 1). The data for ibuprofen refer to muscle vs. subcutaneous tissue [36] and suggest that high blood clearance in the muscle is more significant than convective transport in the dermis and subcutaneous tissue. For lidocaine, a high skin barrier function inter-individual variability is observed, and the potential dependence of dialysate concentration on probe depth is not described [26].

In principle, convective transport of solutes to deeper tissues is a much faster process than tissue diffusion. In general, lag times for measurable microdialysate concentrations of protein bound drugs in deeper tissues are shorter than predicted from *in vitro* dermal diffusion alone (Table 1). The penetration from the papillary dermis into subcutaneous tissue is significantly faster *in vivo* than *in vitro* penetration into excised dermis. For strongly protein bound drugs, the *in vivo* : *in vitro* lag time ratios into dermis and deeper tissue are 1:5 for ibuprofen and <1:18 for diclofenac. For propranolol, which has similar lipophilicity as ibuprofen but is less protein bound, Stagni *et al.* [23] reported *in vivo* lag time values that were six times smaller than we observed *in vitro*, on average. This contrasts strongly with polar drugs with low protein binding: the *in vivo* : *in vitro* lag time ratio is about 1:0.9 for fluconazole and 1:1.3 for nicotine [37]. Lidocaine is mostly unbound to BSA, but our skin penetration experiments yielded an *in vitro* lag time of 44 min, similar to the shortest *in vivo* lag time into dermis reported by Kreilgaard *et al.* [26] and Anderson *et al.* [25] (the lag time varied highly between subjects and the nature of the product used in these studies). Aqueous solutions used in our *in vitro* experiments would likely have induced a higher skin hydration level than the various products of used by Kreilgaard *et al.* [26] *in vivo*, reducing the epidermal penetration lag time. *In vivo* lag times can be estimated from the first microdialysis time point at which drug is detected. This overestimates the actual lag time if significant drug is detected at this point (as seems the case for diclofenac, see Figure 2A), providing an upper bound for the *in vivo* : *in vitro* lag time ratio, but with little sense of its accuracy. Alternatively, our quantitative analysis of Muller *et al.*'s data predicts

Figure 3

(A) Spatial dependence of (A) transport of solutes into deeper skin by diffusion in the extravascular tissue space, binding to tissue proteins, collagen and blood proteins, clearance and vascular, lymphatic and interstitial convection. (B) ratio of the blood vessel surface area to the skin surface area as a function of depth as defined by constant + bi-exponential function extended to a depth of 9 mm (black line) based on data (●) from Cevc & Vierl [31]

in vivo:in vitro lag time ratios of about 1:1400 for diclofenac and 1:4 for nicotine. These are consistent with estimates taken directly from the experimental data and demonstrate our model's capacity for greater precision in lag time estimation.

Our proposed dermal permeation mechanisms for poorly bound and highly bound solutes are summarized in Figure 4A and B respectively. For all drugs, convective transport of bound and unbound drug in the interstitial space arises from microvascular pressure differences parallel to blood vessels, fluid reabsorption in postcapillary

venules perpendicular to it (both with a velocity up to $1.5 \mu\text{m s}^{-1}$ [38, 39]) and resorption of fluid by lymphatic vessels [32]. Lymphatic uptake and fluid transport facilitates micromixing. The dispersion parameter for dermal lymphatic transport of isothiocyanate-dextran (MW = 150 kDa) is more than twice the dermal diffusion for hydrocortisone [6]. The higher blood and lymphatic vessel availability below the application site (Figure 3) can increase the penetration of topically applied substances into deep cutaneous tissue layers [13, 14]. Consequently, the *in vivo* dermal nicotine dispersion coefficient deduced from

Figure 4

Contrasting penetration mechanism of diclofenac and nicotine transport in subcutaneous tissue *in vivo*. (A) First limiting form of the model for drugs with moderate lipophilicity and low plasma protein binding in which both convection (interstitial, lymphatic and vascular) and diffusion in the extravascular space are the main mechanisms for deep tissue penetration and (B) Second limiting form of the model for drugs with moderate lipophilicity and high plasma protein binding in which convection involving binding and/or partitioning into blood and lymphatic vessels travelling to deeper tissues as well as convective interstitial flow are the main mechanisms for deep tissue penetration

regression (Figure 2B) for the convection-diffusion model at 9.1 mm is about four times the *in vitro* diffusion coefficient of excised dermis.

Additional processes affect the transport of highly protein bound drugs, which can also bind to collagen and albumin in the dermis, the albumin concentration in the aqueous dermal phase being about 0.7 that in serum [40]. Binding to immobile collagen can impair dermal diffusion, and convective transport of albumin into the lymphatics facilitates deeper transport (Figure 4b). Blood capillary transport of such drugs is also important. The dermal transport of diclofenac and other highly plasma/dermal protein bound drugs does not appear limited by capillary permeability [6, 20], consistent with their rapid and reversible distribution between blood capillaries and the extravascular space after intravenous administration [41, 42]. We and others have previously advocated that lipophilicity and solute binding to tissue protein are important in the blood capillary entry, retention and clearance of drugs in the dermal/deep cutaneous tissues [9, 20, 43–45]. The fraction unbound of diclofenac in tissues fu_T is about 0.22, based on a fu of ~0.05 (Table 1), an apparent volume of distribution V of ~12 l/70 kg⁻¹ [42], a volume of plasma V_p of ~3 l and an extravascular water volume V_T of ~39 l [41]. Several other studies identify blood flow as a determinant of transport to deep/joint tissues for topically applied highly plasma protein bound drugs [2, 14, 15, 46]. Systemic recirculation has been shown to determine deep tissue/joint concentration [10], and shallower concentration at long times [9, 47–49].

Inter-subject variability in blood vessel availability and a decrease in blood vessel density with depth (Figure 3) may explain the significant differences in maximum diclofenac concentrations observed by Muller *et al.* at skin depths of 4.0 and 8.7 mm [17]. We calculated the ratio of Muller *et al.*'s concentration values for each volunteer. The ratio is ~1 in some cases and significantly smaller in others. We hypothesize microdialysis sampling near a large subcutaneous blood vessel when the ratio is ~1 ('Sampling site I', Figure 5A), and sampling away from a blood vessel when the ratio is <0.4 ('Sampling sites II and III'). The deep : superficial tissue concentration ratio appears to decrease exponentially with distance from the vessels. Figure 5B compares our predictions of diclofenac and nicotine concentrations at a skin depth of 8.7 mm, as a function of distance from a blood vessel. We find an exponentially decreasing concentration-distance profile for diclofenac. Nicotine concentrations appear uncorrelated with blood vessel proximity. The exponential decrease in Muller *et al.*'s [17] diclofenac data is consistent with our model hypothesis of blood flow limitation for highly bound drugs, explaining why varying concentrations are observed for differing sampling sites in the same body region at the same site. In contrast, drugs whose transport is dominated by extravascular diffusion exhibit a minimal dependence of concentration on sampling site at a given distance below the site of application.

Figure 5

Analysis of sampling site in relation to distance from blood and lymphatic vessels at a given depth below the site of application. (A) The bars show the ratio of maximum diclofenac concentration in deep skin layers (8.7 ± 0.6 mm) to maximum diclofenac concentration in superficial skin layers (4.0 ± 0.5 mm), adapted from [17]. The differences in the ratios may be due to differences in blood and lymphatic vessel availability at different sampling sites. (B) Simulated *in vivo* diclofenac and nicotine concentration vs. distance from a blood/lymphatic vessel obtained using our physiological pharmacokinetic model (simulation conditions based on data in [17]).

Muller *et al.*'s probe depth errors may be greater than usual, as they used 7.5 MHz ultrasound rather than the more common 20 MHz [33, 50]. However, such errors are incorporated within our reported variability in Muller *et al.*'s mean diclofenac and nicotine concentrations (the only data available to us). We ignored lateral transport because of Muller *et al.*'s large area of drug application (30 cm²) but this might be relevant for smaller areas of application [51]. Muller *et al.* use only a single 'defined'

tissue layer for *in vivo* nicotine recovery calculations, but distinguish superficial and deep layers for diclofenac.

Increased blood perfusion has been observed within the first hour after probe insertion [52] and may induce faster drug transport. Muller *et al.*'s first sample time (at 30 min) may be too early to exclude this effect. Oedema resulting from probe insertion [53, 54] could mean increased solute diffusion. Microdialysis probes may increase solute transport through damage to blood and lymphatic vessels, although this has not been investigated in the literature.

Here we have used a physiologically based pharmacokinetic dispersion-diffusion-elimination model. In earlier papers on deep tissue penetration after topical application, we used a compartment-in-series model, defining transport between tissues by a range of inter-compartmental and elimination clearances [45, 55, 56]. A limitation in the current work is the implicit assumption of constant elimination with depth. In reality (see Figure 3) blood and lymphatic surface area decrease with depth. Our studies of various rat tissue blood flows using microspheres suggest that the blood flow in the subcutaneous tissue and fat pad is lower than in dermis, but blood flow in muscle tissue is higher [48]. Higher levels in the lower dermis may result from lower blood flow elimination, but higher levels in muscle tissue may arise from greater blood transport than predicted by our model. A disadvantage of the compartment-in-series model is the inability to find determinants for the various clearances obtained by regression. Consistent with the present model, vasoconstriction led to a greater reduction in the fascia and superficial muscle concentrations for the highly plasma protein bound salicylic acid than for the poorly bound lidocaine [49], whereas vasodilation promoted the skin penetration of methyl salicylate [15]. Deep tissue concentrations and the elimination of drugs with varying physiochemical properties depend on the relative binding to deep tissue and to plasma proteins [10, 20].

In conclusion, our analysis of available human cutaneous microdialysis data (Table 1, [16, 17]) and reanalysis of available animal data shows that while drugs are transported in deeper tissues by diffusion, transport of plasma protein bound and sufficiently lipophilic drugs can also occur through the blood and lymphatic systems as well as via interstitial convection, and that these transport routes may dominate in some cases. Such vascular transport can increase deep tissue concentrations beyond those resulting from extravascular diffusion alone, and decrease the drug's lag time into deep dermis, subcutaneous and muscle tissue.

Competing Interests

There are no competing interests to declare.

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