Improved Method for Determining Partition and Diffusion Coefficients in Human Dermis

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Received 31 December 2009; revised 28 March 2010; accepted 15 April 2010

Published online 8 June 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22216

ABSTRACT: Accurate values of partition and diffusion coefficients within the lower skin layers are among the information required to estimate skin concentrations of permeants following topical application. For highly lipophilic compounds these parameters also play a significant role in determining systemic absorption rates. In this study methodology is described for making more accurate *in vitro* measurements of partition and diffusion coefficients in dermis. Isolated human dermis obtained from surgical reduction was mounted in side-by-side diffusion cells in the presence and absence of a dialysis membrane (5000 MW cut-off) placed between the dermis and the donor solution. Permeation of *N*,*N*-diethyltoluamide (DEET) and diclofenac across the composite membrane system, as well as steady-state skin concentrations, were measured by radiochemical techniques. For the highly protein bound compound, diclofenac, dermis permeability, and partition coefficient determined in the presence of the dialysis membrane were significantly higher than those determined in its absence. No significant differences were observed for the moderately protein bound DEET. The results show that, in the absence of a stratum corneum barrier, attention must be given to the diffusion of soluble proteins in order to obtain accurate estimates of transport and partitioning parameters for highly protein bound solutes in dermis. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association
J Pharm Sci 99:4928–4939, 2010

Keywords: albumin; diffusion; drug transport; *in vitro* models; passive diffusion/transport; permeability; protein binding; membrane transport; skin; tissue partition

INTRODUCTION

It is well established that the stratum corneum dominates the skin's barrier to transport of polar and moderately lipophilic compounds due to its unique brick and mortar structure including a continuous lipid barrier. However, highly lipophilic compounds (those with an octanol/water partition coefficient greater than about 1000) experience significant diffusive resistance in the lower skin layers during absorption due to the water-continuous structure of these layers.[1] Representing these layers as an unstirred aqueous layer of about 200 μm thickness has proven to be satisfactory to describe many features of steady-state skin permeation of lipophilic compounds.[1–4] However, this representation fails to account for the magnitude of lipophilic permeant concentrations in the dermis following topical administration. High concentrations arise due to binding of these compounds to lipids and proteins within the tissue,[5–7] including a substantial contribution from hair follicles.[8–10] It is of interest to better estimate skin concentrations of topically applied compounds for several applications including dermatological drug development[11] and skin sensitization risk assessment.[4]

Capillary clearance profoundly affects permeant concentrations in dermis *in vivo*, leading to steady-state concentration profiles that approximate exponential decays.[7,12,13] *In vitro* experiments with isolated dermis can determine solute diffusivities and partition coefficients in this skin layer in the absence of capillary clearance. The combination of a steady-state permeability experiment with an equilibrium partitioning experiment suffices to yield both parameters.[7,14]

Binding of solutes to extravascular albumin and other soluble proteins in the dermal matrix is thought to contribute appreciably to the partitioning of lipophilic compounds.[5,7,15] The total concentration of serum proteins in dermis is 11 mg/g, based on the measurements of Bert et al.[16] in postmortem human dermis. The concentration in the albumin accessible regions (approximately 32% of the fluid fraction)[17] has been estimated to be 2.7% (w/v).[7] It is furthermore

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Journal of Pharmaceutical Sciences
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evident that albumin and other serum proteins can migrate slowly through the tissue; otherwise, they would accumulate in dermis rather than being cleared in the lymph. This phenomenon presents a challenge to conducting accurate experiments *in vitro*. In particular, if albumin or other soluble proteins migrate into the donor solution during a side-by-side diffusion cell experiment, and the test permeant binds to the protein, then an artificially low-dermis permeability would be obtained. A similar limitation applies to equilibrium partition coefficients determined by immersing tissue samples in an aqueous buffer. The method described in this article removes this uncertainty by preventing the diffusion of macromolecules into the donor solution. The cost is that the permeability of the barrier membrane providing this function must be accurately known in order to estimate the dermis permeability. The method is illustrated using DEET, a moderately lipophilic compound that binds moderately to albumin, and diclofenac, an ionizable and therefore water-soluble drug that is nevertheless highly protein bound.

MATERIALS AND METHODS

Materials

Dialysis membrane (5000 Da cut-off) was purchased from Bel-Art Products (Pequannock, NJ). Pharm-Elast™ medical grade silicone (PDMS) membrane, 0.020 in. (~500 μm) in thickness, was obtained from SF Medical (now Trelleborg Sealing Solutions US, Bloomfield Hills, MI). Additional dialysis membrane (3500 Da cut-off) and dialysis tubing (100–500 Da cut-off) were purchased from SpectrumLabs (Rancho Dominguez, CA). Unlabeled DEET (97.3%) and diclofenac were purchased from Sigma–Aldrich (Atlanta, GA). [Carbonyl-^{14}C] DEET (52 mCi/mmol, radiochemical purity >99%) was purchased from Vitrax (Placentia, CA). [Carbonyl-^{14}C] diclofenac (55 mCi/mmol, radiochemical purity >99%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Ultima Gold™ XR scintillation cocktail and Soluene®-350 were purchased from Perkin–Elmer (Boston, MA). A Micro BCA™ protein assay kit was purchased from Thermo Scientific (Rockford, IL). Skin from abdominoplasty (three donors) and mammoplasty (four donors) was obtained from the University of Cincinnati Academic Health Center. Phosphate-buffered saline (PBS), bovine serum albumin (BSA) and sodium azide were purchased from Sigma Chemicals.

Barrier Membrane and Aqueous Boundary Layer Studies

Dialysis membrane (5000 Da cut-off) and PDMS sheets were considered as possible barrier membranes. Membranes were mounted in water-jacketed side-by-side diffusion cells (1.77 cm^2) maintained at a temperature of 37°C. Multiple membranes were mounted in some experiments to probe the contributions of aqueous boundary layers. Each donor compartment (6 mL) of the diffusion cells was filled with PBS solution to which sodium azide (0.02%) had been added to inhibit microbial growth. The receptor compartments (6 mL) were filled with either PBS or 2% BSA–PBS solution, both supplemented with sodium azide. Both compartments were magnetically stirred using synchronous motors operating at 600 rpm. After a 24-h equilibration the donor compartment was spiked with 100 μL of ^{14}C-DEET + unlabeled DEET in ethanol yielding a 10 μg/mL DEET solution containing 0.56 μCi of ^{14}C DEET. The donor and receptor compartments were sequentially sampled (0.1 mL donor; 0.5 mL receptor), and the receptor solution was replenished with an equal volume of fresh solution after each sampling. More consistent values for the initial donor concentrations were obtained by extrapolation of later time points (1, 1.5, and 6 h) back to time zero than by taking early time points. All collected samples were mixed with scintillation cocktail (5 mL) and analyzed by liquid scintillation counting. For dialysis membrane similar experiments were carried out using 7.5 μg/mL (0.56 μCi) of ^{14}C-diclofenac in the donor solution.

For all experiments the cumulative amount of solute passing through unit area of membrane $M(t)$ was plotted versus time. The steady-state flux J_{ss} was calculated as the slope of the linear portion of the graph, and the time lag T_L was calculated as the intercept of the regression line on the time axis. The permeability coefficient k_p and its reciprocal, the total diffusive resistance R_{tot}, were calculated as

$$k_p = \frac{1}{R_{tot}} = \frac{J_{ss}}{\Delta C} \approx \frac{J_{ss}}{C_d} \quad (1)$$

where C_d is the donor solution concentration. The approximation $\Delta C \cong C_d$ was justified since the concentration of permeant in the receptor solution never exceeded 13% of that in the donor solution.

The assumption was made that the membrane system under study was surrounded by two potentially unsymmetrical, unstirred aqueous layers (aqueous boundary layers or ABLs) having resistances R_1 (donor solution) and R_2 (receptor solution). The membrane resistance was determined by subtracting their sum from R_{tot}

$$R_{mem} = R_{tot} - (R_1 + R_2) \quad (2)$$

The value of $R_1 + R_2$ for the symmetrical case in which both donor and receptor solutions contained PBS (thus, $R_1 = R_2$) was determined from a ^{14}C-DEET experiment in which $N = 1$, 2, and 3 dialysis membranes were mounted in series. A linear regression

was performed on a plot of R_{tot} versus the number of membranes in each cell according to Eq. 3:

$$R_{tot} = (R_1 + R_2) + NR_{Dial} \quad (3)$$

The boundary layer thickness h_{ABL} was then calculated from Eq. 4 using the Wilke–Chang relationship (Eq. 5) to estimate the aqueous diffusivity D_{aq} for DEET.

$$h_{ABL} = D_{aq}R_1 \quad (4)$$

$$D_{aq}(cm^2/s) = \frac{7.4 \times 10^{-8} T(M \times \phi)^{1/2}}{\eta V_A^{0.6}} \quad (5)$$

In Eq. (5), T is temperature in Kelvin, M is solvent molecular weight (18.01 for water), ϕ is the solvent association parameter (2.27 for water), η is solvent viscosity in centipoise (0.67 cP for water at 37°C) and V_A is the molar volume of the solute at the normal boiling point in cm^3/mol. V_A was estimated using Schröeders method.[18] Eq. (5) refers to the diffusivity of unbound permeant. For bound permeant, the diffusivity would be closer to that of albumin as explained in Appendix 1.

For experiments involving other permeants, and for those in which BSA was included in the receptor solution, the values of R_1 and R_2 were recalculated according to Eqs. (6) and (7),

$$R_1 = \frac{h_{ABL}}{D_{aq}} \quad (6)$$

$$R_2 = f_u \frac{h_{ABL}}{D_{eff}} \quad (7)$$

In these relationships, D_{aq} is the aqueous diffusivity of the test permeant estimated from Eq. (5) and h_{ABL} is aqueous boundary layer thickness determined from Eq. (4), a suitable choice since the viscosity of a 2% BSA solution is not appreciably different than that of water[19] and the aqueous diffusivities of the test permeants are comparable.[20] In Eq. (7), f_u is the unbound fraction of test permeant in the receptor solution and D_{eff} is the effective diffusivity of the test permeant in the receptor solution,

$$D_{eff} = f_u D_{free} + (1 - f_u) D_{bound} \quad (8)$$

Here D_{free} is the aqueous diffusivity of unbound permeant (equivalent to D_{aq} in Eqs. 5 and 6) and D_{bound} is that of permeant bound to albumin. The value of D_{bound} was taken to be 9.29×10^{-7} cm^2/s, the estimated diffusivity of BSA in water at 37°C.[21] A justification for Eqs. (7) and (8) is given in Appendix 1.

Protein Binding Studies

These studies were carried out using dialysis membranes (5000 Da cut-off) mounted in side-by-side diffusion cells as described above with the exception that they were continued for 96 h to ensure equilibrium conditions. The donor solution contained radiolabeled DEET (10 μg/mL) or diclofenac (7.5 μg/mL) in PBS and the receptor solution contained PBS + 2% BSA. Both solutions were preserved with 0.02% sodium azide. The value for DEET was obtained in a previous study.[22] The fraction of the drug unbound to protein f_u was calculated as the ratio of donor solution concentration to receptor solution concentration at 96 h.

An additional study was carried out in which donor solutions obtained from a protein diffusion experiment employing a dialysis membrane (5000 Da cut-off—see description below) in series with dermis were dialyzed against PBS using dialysis tubing (100–500 Da cut-off). This study was conducted in order to determine whether protein fragments, which passed through the 5000 Da membrane were capable of binding diclofenac. The donor solution was placed inside the dialysis tubing, which was immersed in PBS solution. ^{14}C-diclofenac (7.5 μg/mL) was added to either the PBS or donor solution. The solutions were dialyzed for 31 days.

Dermis Transport Studies

Dialysis membranes (5000 Da cut-off) were prepared as previously described. In most cases skin was stored frozen at −80°C and allowed to thaw before use. Limited testing of fresh versus frozen skin from the same donor did not show significant differences in transport parameters for DEET; however, the supply of fresh skin was not sufficient to rigorously test this finding. Skin samples from abdominoplasty included some subcutaneous fat, which was cut away using surgical scissors prior to freezing. Skin samples from mammoplasty were thinner and contained no visible fat. The skin was then rinsed thoroughly in PBS solution to remove any traces of blood and cut into approximately 2.5 cm × 2.5 cm pieces. Any skin samples with visible tears were discarded. The epidermis was separated from the dermis by heat separation[23] using 57°C water for 2 min. Excess water was then removed from the samples, which were placed on weighing article and accurately weighed. A trace was made of each sample and used to quantify its area. The thickness of the sample was then determined assuming a density of 1.075 g/cm^3.[24] The dermis sample was mounted in the diffusion cell in series with a dialysis membrane placed between the dermis and the donor solution. Test permeants were ^{14}C-DEET (10 μg/mL) and ^{14}C-diclofenac (7.5 μg/mL). The receptor solutions were either PBS or 2% BSA–PBS solution, both supplemented with sodium azide. The BSA was added to maintain a physiological environment within the dermis. All DEET studies were carried out for 6 h and diclofenac studies were carried out for 48 h to attain steady-state conditions. The remainder of the experiment was conducted as

previously described. Each dermis sample was dissolved in Soluene® (1 mL) and placed in an autoclave oven overnight. All collected samples and dissolved skin samples were analyzed by LSC. A separate study was conducted using dermis samples mounted without the dialysis membrane, and the results were compared.

Permeation data were analyzed according to Eqs. (1) and (2). The value of R_{mem} represented either the dermis resistance R_{de} or the sum of dermis plus dialysis membrane resistances $R_{de}+R_{Dial}$, depending on the type of study conducted. Permeant concentrations in the receptor compartment for DEET never exceeded 10% of the donor concentration. Those for diclofenac at the end of the study averaged 16% (dermis only) and 34% (dermis + dialysis membrane) of the donor concentration. The consequences of this level of accumulation are discussed later. For dermis + dialysis membrane studies, the value of R_{de} was thus calculated as:

$$R_{de} = R_{tot} - R_1 - R_2 - R_{Dial} \quad (9)$$

The product of dermis diffusivity and partition coefficient $D_{de}K_{de}$, often termed permeability P_{de}[7] was calculated from R_{de} and the thickness h_{de} of each sample according to Eq. 10:

$$P_{de} = D_{de}K_{de} = \frac{h_{de}}{R_{de}} \quad (10)$$

The value of the dermis/donor solution partition coefficient K_{de} was obtained from the average concentration measured in the dermis tissue sample $\overline{C_{de}}$ according to Eq. 11, which is justified in Appendix 2:

$$K_{de} = \frac{2\overline{C_{de}}}{C_d}\left(\frac{R_{tot}}{R_{tot} - R_1 - R_{Dial} + R_2}\right) \quad (11)$$

Dermis diffusivity D_{de} was then calculated as

$$D_{de} = \frac{P_{de}}{K_{de}} \quad (12)$$

For studies in which the dermis dominated the time lag T_L, D_{de} was also calculated from Eq. 13:

$$D_{de} = \frac{h_{de}^2}{6T_L} \quad (13)$$

Comparisons between diffusivities obtained from Eqs. (12) and (13) are discussed later. Corresponding formulas for dermis only experiments also employed Eqs. (9)–(13) except that the value of R_{Dial} was set to zero.

Protein Diffusion Studies

Human dermis was prepared and mounted in side-by-side diffusion cells as in the dermis transport studies. In some cells a dialysis membrane (either 3500 or 5000 Da cut-off) was inserted between the dermis and the donor solution. The donor solution was PBS and the receptor solution was PBS + 2% BSA. Both solutions contained 0.02% sodium azide to inhibit microbial growth. Five minutes after mounting the cells, and periodically thereafter, samples (200 μL) were withdrawn from the donor solution (with replacement buffer added) and 150 μL of the sample was placed in a 96-well plate. In the case of the dermis only treatment, the sample was diluted by a factor of 5 prior to assay. The collected samples were analyzed for protein content using the Micro BCA™ assay kit. This method utilizes bicinchonic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. It is a nonspecific assay for protein. The assay was calibrated using solutions of BSA in PBS with varying concentration. Results were calculated as BSA-equivalents/mL and expressed following conversion by the cell parameters as BSA-equivalents/cm^2.

Statistical Analysis

For all experiments, results were calculated individually for each diffusion cell, and then averaged to obtain a mean and standard error. Transport and partitioning parameters obtained by different methods were compared via two-way ANOVA using donor as a blocking variable. The pairwise comparison test used was the Holm–Sidak method. $p < 0.05$ were considered to be significant. For comparisons involving only two groups, a Student's t-test was employed. All tests were conducted using SigmaStat version 3.10 (SYSTAT, Chicago, IL).

RESULTS

Aqueous Boundary Layer Study

Permeation profiles for ^{14}C-DEET across 1, 2, or 3 dialysis membranes into PBS are shown in Figure 1a. Total diffusive resistances calculated from these profiles based on the slopes from 0–3 h postdose are plotted versus the number of membranes in Figure 1b. Linear regression of these data according to Eq. (3) yielded $R_1 + R_2 = (3950 \pm 120)$ s/cm and $R_{Dial} = (21740 \pm 50)$ s/cm, with a squared correlation coefficient $r^2 = 0.995$. Since the boundary layers were symmetrical in this study, $R_1 = R_2 = 1975$ s/cm. Further analysis according to Eqs. (4) and (5) led to $D_{aq} = 8.54 \times 10^{-6}$ cm^2/s and $h_{ABL} = 0.0169$ cm or about 170 μm. The total unstirred layer thickness relevant to the diffusion cells is thus $2 \times h_{ABL} \approx$ 340 μm. For diclofenac, Eqs. (5) and (6) yield $D_{aq} = 7.08 \times 10^{-6}$ cm^2/s and $R_1 = 2390$ s/cm for this slightly larger and more slowly diffusing permeant.

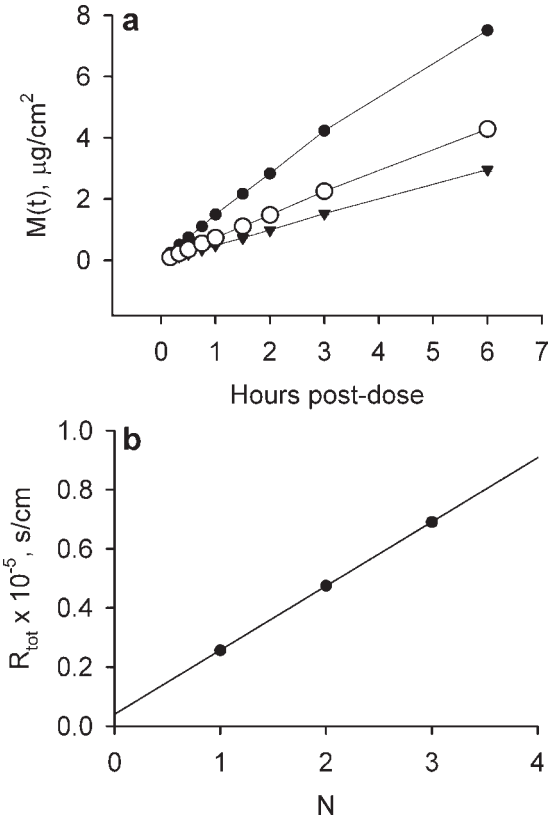

Figure 1. (a) Results of aqueous boundary layer study for ^{14}C-DEET permeating across 1 (●), 2 (○), or 3 (▼) dialysis membranes placed in series in the diffusion cells. Data shown are the mean ± SE of nine determinations. (b) Analysis of data shown in panel (a) according to Eq. (3). In both panels, the error bars are smaller than the size of the symbols.

Protein Binding Studies

The percentages of ^{14}C-DEET and ^{14}C-diclofenac bound in a 2% BSA solution (mean ± SE, $n = 4$–5) were 81.1 ± 0.4% and 96.0 ± 0.5%, respectively, based on equilibrium concentrations at 96 h postdose. Thus, $f_u = 0.19$ for DEET and 0.04 for diclofenac. These results were used in combination with those in the previous section and a published value of BSA diffusivity in water[21] to estimate the aqueous boundary layer resistance in receptor compartments containing PBS + 2% BSA. Under these conditions, Eqs. (7) and (8) yield $R_2 = 1370$ s/cm for DEET and 580 s/cm for diclofenac.

Equilibration of ^{14}C-diclofenac between donor solutions containing small protein fragments (<5000 Da) derived from dermis diffusion studies dialyzed against PBS using dialysis tubing (100–500 Da cut-off) was a slow process that was not complete within 31 days. However, since the approach to equilibrium was followed from both directions (i.e., the radiolabel was placed either inside or outside the dialysis tubing), limits to the true equilibrium could be established. An upper limit to binding of ~11% and a lower limit of ~2% were obtained, so that f_u for the donor solutions fell within in the range 0.89–0.98 with a likely equilibrium value of ~0.94 (data not shown). This study thus established that diclofenac was not strongly bound in donor solutions separated from dermis by a dialysis membrane.

Barrier Membrane Studies

The results for dialysis membrane permeability studies are shown in Figure 2 and Table 1. No significant difference was observed between the permeability coefficients or dialysis membrane resistances for permeants tested with or without BSA in the receptor solutions. However, the amount of diclofenac permeated at 6 h with BSA in the receptor solution was significantly higher than that with PBS, suggesting that the decreased boundary layer resistance for the BSA solution did measurably impact transport. The average ratio of dialysis membrane resistances for DEET and diclofenac calculated from the values in Table 1, Column 2 (0.85), was very close to the inverse ratio of aqueous diffusivities calculated from the Wilke–Change relationship, Eq. (5) (0.83).

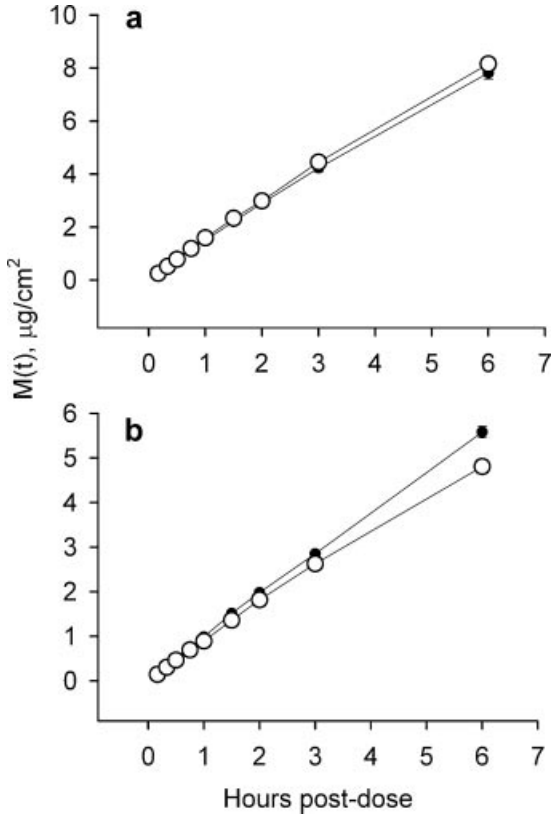

Figure 2. Permeation of (a) ^{14}C-DEET or (b) ^{14}C-diclofenac across dialysis membrane in the presence (●) and absence (○) of BSA in the receptor solutions. The data shown represent the mean ± SE of 13–19 replicates (DEET) and 4–5 replicates (diclofenac). Error bars are smaller than the size of the symbols.

 DOI 10.1002/jps

Table 1. Transport Parameters (Mean ± SE) for DEET and Diclofenac in Dialysis Membrane Obtained by Analyzing the Data Shown in Figure 2

	n^a	$k_p \times 10^5$ (cm/s)	$R_{Dial} \times 10^{-5}$ (s/cm)	T_L (h)
DEET				
PBS	7 (34)	4.09 ± 0.09	0.203 ± 0.004	−0.06 ± 0.03
PBS + 2% BSA	3 (13)	4.00 ± 0.14	0.220 ± 0.009	−0.05 ± 0.03
Diclofenac				
PBS	1 (4)	3.35 ± 0.09	0.252 ± 0.008	−0.03 ± 0.01
PBS + 2% BSA	1 (5)	3.63 ± 0.08	0.247 ± 0.006	−0.02 ± 0.01

[a]Reported as n = No. of studies (total no. of replicates).

This result is consistent with the expectation that diffusion through dialysis membrane occurs via aqueous channels.

The average value $R_{Dial} = 21200$ s/cm for DEET is within 3% of the value $R_{Dial} = 21740$ s/cm obtained in the aqueous boundary layer study. These values are not significantly different. For the remainder of the analysis we used the average value obtained from the barrier membrane studies, $R_{Dial} = 21200$ s/cm for DEET due to the higher number of replicates and $R_{Dial} = 24900$ s/cm for diclofenac.

Results with PDMS membranes were not as consistent as those with dialysis membranes (data not shown). Although the permeability coefficient and associated diffusive resistance of PDMS membrane were comparable to dialysis membrane, the standard deviation of these values was higher and an additional time lag of approximately 0.4 h was introduced into the system. A plot of total diffusive resistance versus number of membranes for PDMS membranes placed in series was not as linear as that for dialysis membranes (cf. Fig. 1b). We hypothesized that imperfect adhesion of the PDMS membranes to one another may have led to the variability. We tried to improve the results by coating the apposed PDMS surfaces with a thin layer of silicone grease; however, this approach was not effective. Consequently dialysis membrane was chosen as the barrier membrane for the dermis transport studies.

Dermis Transport Studies

The results of these studies are shown in Figures 3 and 4 and Tables 2 and 3. For DEET, the dialysis membrane and dermis functioned together as a barrier in an approximately additive manner. Flux across the diffusion cell (Figs. 3 and 4a) and tissue concentrations in the dermis (Tab. 2) were lowered by the presence of the dialysis membrane by an amount consistent with its diffusive resistance. Transport and partitioning parameters for DEET in dermis calculated from the data in the presence and absence of dialysis membrane, or in the presence and absence of BSA in the receptor solution, were not significantly different (Tab. 3). Diffusivities calculated from the time lag (Eq. 13) averaged 40–50% higher than those calculated from permeability data (Eq. 12). These differences were significant. Breast and abdominal skin samples had comparable permeability ($P_{de} = D_{de}K_{de}$) to DEET; however, breast skin had a lower diffusivity D_{de} (Eq. 12, $p = 0.11$; Eq. 13, $p < 0.01$) and a higher partition coefficient K_{de} ($p < 0.01$) relative to abdominal skin. DEET diffusivity in breast and abdominal skin, as calculated from Eq. (12), averaged 42% and 69%, respectively, of the calculated aqueous diffusivity $D_{aq} = 8.54 \times 10^{-6}$ cm^2/s estimated from Eq. (5) at 37°C.

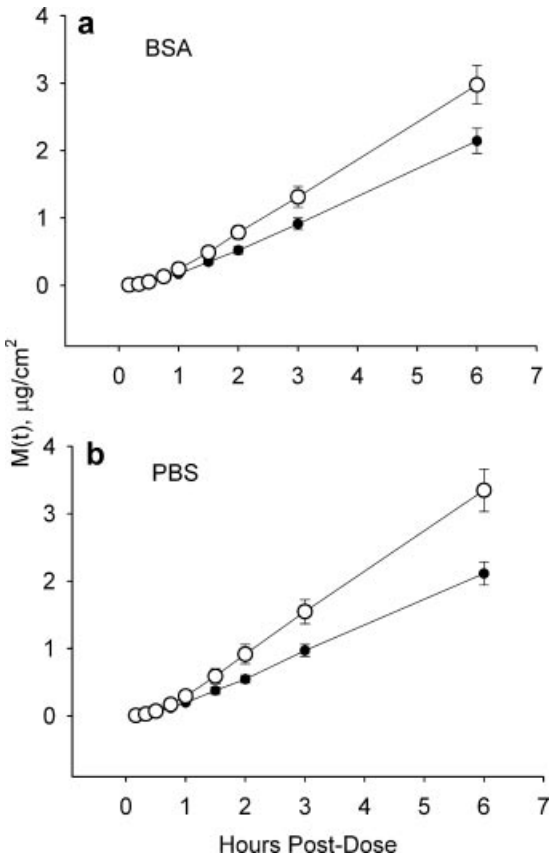

Figure 3. Results of breast skin dermis permeation studies with ^{14}C-DEET in the presence (●) and absence (○) of a dialysis membrane placed between the dermis and the donor solution. The donor solutions contained the permeants dissolved in PBS and the receptor solutions contained in either (a) PBS + 2% BSA or (b) PBS.

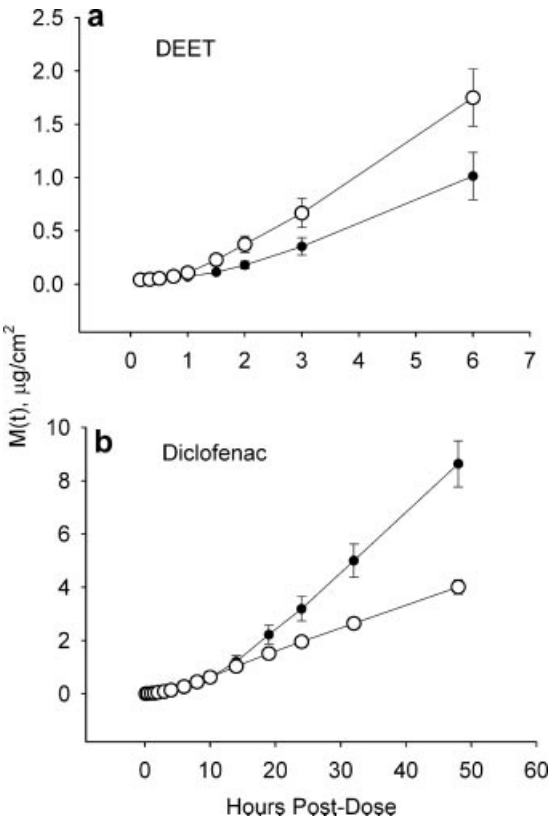

Figure 4. Results of abdominal skin dermis permeation studies in the presence (●) and absence (○) of a dialysis membrane placed between the dermis and the donor solution. The donor solutions contained the permeants dissolved in PBS and the receptor solutions contained PBS + 2% BSA.

The above results, in combination with literature reports involving skin and other biological membranes,[25–27] led us to focus further studies on combinations in which the receptor solutions were supplemented with BSA. These studies were confined to abdominal skin due to tissue supply. Based on the DEET results, the abdominal skin/BSA combination is suitable for study if adequate time is allowed for achievement of steady-state permeation. For the highly protein bound permeant, diclofenac, the study period was increased to 48 h.

Results for diclofenac were strikingly different from those for DEET. Flux across the diffusion cells was higher for the composite membrane (dialysis + dermis) compared to dermis alone (Fig. 4b). Average dermis tissue concentrations were 2.7-fold higher for the composite membrane (Tab. 2). All dermis transport and partition parameters calculated from these data were significantly different for the composite membrane and dermis only systems (Tab. 3). In general, the composite membrane system yielded higher dermis permeabilities P_{de}, with the major contributor to the difference being a higher dermis/donor solution partition coefficient K_{de}. Mean diffusivities for diclofenac in dermis estimated from the composite membrane data were 0.57×10^{-6} cm^2/s (Eq. 12) or 0.72×10^{-6} cm^2/s (Eq. 13). These values are 8% and 10%, respectively of the aqueous diffusivity $D_{aq} = 7.08 \times 10^{-6}$ cm^2/s estimated from Eq. (5).

Protein Diffusion Studies

Donor solution protein content following the mounting of human dermis in side-by-side cells is shown in Figure 5. In the absence of a dialysis membrane (5000 Da cut-off), protein concentrations in the donor solution 0.08 h (5 min) post-dose averaged 610 μg BSA-equivalents/cm^2. These values rose in a nonlinear pattern to approximately 870 μg BSA-equivalents/cm^2 at 10 h and 1230 μg BSA-equivalents/cm^2 at 24 h. The corresponding values for donor solution protein content in the presence of a dialysis membrane were 25, 110, and 140 μg BSA-equivalents/cm^2 at 0.08, 10, and 24 h. Thus, the total protein content in the donor solution was reduced by the dialysis membrane by a factor ranging from 8 to 24. No significant differences were

Table 2. Thicknesses, Permeability Coefficients, Time Lags, and Tissue Concentrations Associated With the Dermis Transport Experiments

Membrane	Receptor	n^a	h_{de} (cm)	$k_p \times 10^5$ (cm/s)	T_L (h)	$\overline{C}_{de}$ (μg/cm^3)
DEET—breast skin						
Dialysis + dermis	BSA	6 (21)	0.246 ± 0.015	1.10 ± 0.09	0.68 ± 0.05	2.27 ± 0.20
	PBS	5 (18)	0.205 ± 0.018	1.05 ± 0.09	0.50 ± 0.08	2.05 ± 0.29
Dermis only	BSA	1 (4)	0.250 ± 0.017	1.46 ± 0.14	0.53 ± 0.04	4.95 ± 0.16
	PBS	1 (5)	0.254 ± 0.012	1.72 ± 0.16	0.53 ± 0.07	4.92 ± 0.24
DEET—abdominal skin						
Dialysis + dermis	BSA	2 (3)	0.508 ± 0.007	0.580 ± 0.124	1.10 ± 0.05	2.06 ± 0.19
	PBS	2 (3)	0.499 ± 0.025	0.383 ± 0.042	1.70 ± 0.17	2.03 ± 0.52
Dermis only	BSA	2 (6)	0.462 ± 0.029	0.993 ± 0.127	0.99 ± 0.11	3.15 ± 0.48
	PBS	2 (4)	0.514 ± 0.019	0.588 ± 0.040	1.53 ± 0.70	3.12 ± 0.25
Diclofenac—abdominal skin						
Dialysis + dermis	BSA	4 (7)	0.379 ± 0.024	0.839 ± 0.081	9.07 ± 1.11	9.13 ± 1.62
Dermis only	BSA	4 (11)	0.386 ± 0.012	0.338 ± 0.023	−0.05 ± 2.20	3.42 ± 0.37

Results are reported as mean ± SE.
[a]No. of studies (total no. of replicates).

Table 3. Transport and Partitioning Parameters (Mean ± SE) Obtained by Analyzing the Dermis Transport and Concentration Data In Table 2

Membrane	Receptor	$R_{de} \times 10^{-5}$ (s/cm)	$P_{de} \times 10^{6}$ (cm^2/s)	K_{de}	$D_{de} \times 10^{6}$ (cm^2/s) Eq. (12)	$D_{de} \times 10^{6}$ (cm^2/s) Eq. (13)
DEET—breast skin						
Dialysis + dermis	BSA	0.81 ± 0.02	4.16 ± 0.53	0.97 ± 0.08	4.63 ± 0.82	4.37 ± 0.44
	PBS	0.79 ± 0.09	3.45 ± 0.46	1.26 ± 0.22	3.17 ± 0.46	4.48 ± 0.74
Dermis only	BSA	0.67 ± 0.06	3.77 ± 0.18	1.19 ± 0.04	3.03 ± 0.16	5.53 ± 0.52
	PBS	0.56 ± 0.05	4.66 ± 0.31	1.25 ± 0.03	3.49 ± 0.29	5.87 ± 0.59
Mean ± SE (All treatments)		0.71 ± 0.06	4.01 ± 0.26	1.17 ± 0.07	3.58 ± 0.36	5.06 ± 0.38
DEET—abdominal skin						
Dialysis + dermis	BSA	1.60 ± 0.37	3.58 ± 0.90	0.54 ± 0.08	6.51 ± 1.00	10.0 ± 0.4
	PBS	2.38 ± 0.28	2.13 ± 0.15	0.55 ± 0.09	4.05 ± 0.54	7.03 ± 1.28
Dermis only	BSA	1.10 ± 0.21	5.36 ± 0.76	0.70 ± 0.10	9.07 ± 1.96	11.7 ± 2.4
	PBS	1.69 ± 0.12	3.08 ± 0.20	0.76 ± 0.03	4.07 ± 0.34	8.13 ± 0.93
Mean ± SE (All treatments)		1.69 ± 0.26	3.54 ± 0.68	0.64 ± 0.05	5.93 ± 1.20	9.22 ± 1.03
Diclofenac—abdominal skin						
Dialysis + dermis	BSA	1.02 ± 0.16	3.95 ± 0.31	7.56 ± 0.67	0.57 ± 0.06	0.72 ± 0.11
Dermis only	BSA	3.18 ± 0.40	1.29 ± 0.09	1.21 ± 0.09	1.14 ± 0.08	NA[a]

[a]Not estimated due to lack of apparent time lag.

observed between studies employing 3500 and 5000 Da membranes (data not shown). Based on the stated cut-offs, it is likely the proteins in the donor solution for the cells containing a dialysis membrane were small peptides and protein fragments stemming from the dermis or the BSA solution. As described in the protein binding section, these fragments did not bind diclofenac to a significant extent.

DISCUSSION

Solute transport in dermis and the associated capillary and lymphatic clearance processes are key to the survival and health of skin tissue. Nutrients and oxygen delivered from the dermal capillaries diffuse into the dermis and overlying epidermis, waste products resulting from cellular metabolism are concurrently removed by the same capillaries. Larger cellular debris and serum proteins that have leaked into the tissue are removed by the skin lymphatics. These same mechanisms remove exogenous chemicals, beneficial or hazardous, that have permeated across the skin's outer defenses (i.e., the stratum corneum) following dermal exposure. Transient concentrations of these chemicals in the skin depend on the permeation and clearance rates and the partition coefficient of the chemical in the dermis tissue. In order to accurately predict these concentrations, the underlying transport and partition phenomena must be well understood.

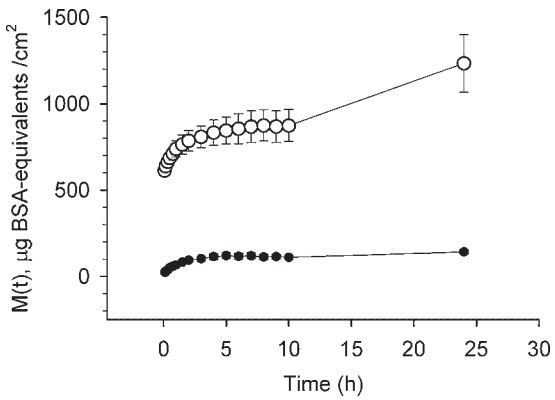

Figure 5. Results of protein assay studies in the presence (●) and absence (○) of a dialysis membrane (5000 Da cut-off) placed between the dermis and the donor solution. The donor solutions contained PBS and the receptor solutions contained PBS + 2% BSA. Data represent the mean ± SE of 10 determinations from one male donor.

This study addresses a question posed in a recent modeling study of transport and partitioning of solutes in dermis.[7] In that study the investigators noted that conventional *in vitro* permeation and partition measurements of solutes in dermis could be confounded by diffusion of soluble proteins from the tissue. Strong binding of the solute to the diffusing protein would lead to inaccurate results. The present study confirms this to be the case for the highly protein bound drug, diclofenac ($f_u = 0.04$ in 2% BSA). Preventing the diffusion of large proteins, including albumin, into the donor solution in an *in vitro* permeation study involving human dermis resulted in a significantly higher permeability coefficient k_p (Tab. 2) and associated dermis permeability $P_{de} = D_{de}K_{de}$ (Tab. 3). The chief factor leading to this difference was the dermis/donor solution partition coefficient K_{de}. Blocking protein diffusion into the donor solution resulted in 2.7-fold higher tissue concentrations (Tab. 2) and a 6-fold higher calculated value of K_{de} for diclofenac (Tab. 3).

The same methodology applied to a moderately protein-bound solute, DEET ($f_u = 0.19$ in 2% BSA), did not reveal a significant difference between dermis transport or partition parameters determined in the presence and absence of a barrier membrane. The tests did, however, reveal significant differences between breast and abdominal skin samples with regard to DEET diffusivities and partition coefficients. Breast skin had lower values of D_{de} and higher values of K_{de}, but comparable permeability P_{de}, relative to abdominal skin. We suspect that these differences are related to the tissue thickness—abdominal samples were approximately twice as thick as breast samples. The 6-h time frame of the DEET permeation experiments, which was adequate for the breast skin studies (Fig. 3), may not have been quite long enough to achieve a steady state in abdominal skin (Fig. 4a). Such an error would lead to parameter differences in the observed direction. However, alternative explanations involving variation in tissue structure with site or depth cannot be ruled out.

The 48-h time frame of the diclofenac experiments was chosen to ensure achievement of a steady state. It is evident from Figure 4b that this goal was met. Accumulation of diclofenac in the receptor solution at 48 h exceeded the commonly accepted limit of 10% of the donor solution concentration; however, it may be seen from the figure that similar permeability coefficients and time lags would have been obtained had the experiment been terminated earlier. Any attempt to correct these data for nonsink conditions[28] would only accentuate the difference between the dermis only and dermis + dialysis permeation profiles, as the latter would have the larger (positive) correction. We refrained from applying such a correction, since the uncorrected data already establish the case that the dialysis membrane is an essential component of the system. Were a correction to be made, the resulting time lags would be slightly longer, and the Eq. (13) diffusivities slightly lower, than those reported in Table 3. Permeabilities and Eq. (12) diffusivities would be slightly higher. Thus, agreement between diffusivities calculated from Eqs. (12) and (13) would improve. In general, it is worth noting that, if the primary purpose of calculating a tissue diffusion coefficient is to estimate transport across the tissue, then the use of Eq. (12) (which directly invokes permeability) is a wiser choice than Eq. (13). The comparisons discussed below employ Eq. (12) diffusivities.

It is of interest to test the agreement of the data reported here with the model developed by Kretsos et al.[7], which represents the existing dermis permeability and partitioning database. For the case of delivery from a pH 7.4 donor solution into tissue at the same pH, Kretsos' formulas for diffusivity and partition coefficient can be summarized as follows:

$$K_{de/pH7.4} = 0.6 \times \text{BindingFactor} \quad (14)$$

$$D_{de} = \frac{10^{-4.15-0.655 \log \text{MW}}}{\text{BindingFactor}} \quad (15)$$

$$\text{BindingFactor} = 0.68 + 0.32/f_u + 0.001 f_{non} K_{oct} \quad (16)$$

Here, f_{non} is the fraction nonionized and K_{oct} is the octanol/water partition coefficient. Values for $K_{de/pH\ 7.4}$, D_{de} and P_{de} calculated from this model are shown in Table 4. For diclofenac a comparison with the dialysis + dermis results in Table 3 shows that the model underestimates K_{de}, D_{de}, and P_{de} by factors of 1.4, 2.9, and 3.9, respectively. For DEET comparison of these calculations with the averages of the values given for breast skin in Table 3 shows that K_{de} is overestimated by a factor of 1.3, whereas D_{de} and P_{de} are underestimated by factors of 4.0 and 2.9, respectively. Agreement of the calculation with the abdominal skin parameters is comparable for P_{de} (2.6-fold underestimate) but less satisfactory for D_{de} and K_{de}. In any case, it is evident that Eqs. (14)–(16) underestimate dermis permeability P_{de} for both DEET and diclofenac by 3- to 4-fold when compared to the experiments reported here. It seems likely from the above analysis that a substantial portion of this error is incurred in the estimation of D_{de} according to Eq. (15). It should be noted that Eq. (15) is based on a diffusion model in which bound permeant is considered to be immobile.[7] Replacement of this approximation with a model in which both free and bound permeant diffuse through the tissue (cf. Eq. 8) may yield better agreement. Better characterization of the binding proteins and their mobility within the tissue is required in order to complete this task.

Table 4. Transport and Partitioning Parameters Calculated from Eqs. (14)–(16)

Property	Units	DEET	Diclofenac
MW	Da	191.3	296.2
$\log K_{oct}$	—	2.18[29]	4.51[29]
pK_a	—	—	4.0
f_{non} at pH 7.4	—	1	3.98×10^{-4}
f_u	—	0.19	0.04
BindingFactor	—	2.52	8.69
$K_{de/pH\ 7.4}$	—	1.51	5.22
$D_{de} \times 10^{6}$[a]	cm^2/s	0.898	0.196
$P_{de} \times 10^{6}$	cm^2/s	1.36	1.02

[a] $P_{de} = K_{de/pH7.4} D_{de}$.

A recent study of the topical drugs pimecrolimus and tacrolimus highlights the complexity of protein

binding in skin.[15] These highly lipophilic macrolide anti-inflammatory drugs were found to bind extensively to skin and plasma proteins. Despite their lipophilic nature, the binding was largely to proteins (or, in blood, lipoproteins) other than albumin. In skin, the strongest binding was to an unidentified ~16 kDa protein which may have been the 12 kDa tacrolimus binding protein Macrophilin-12. Although, there are other compounds for which specific binding proteins in skin have been identified, e.g., retinoids and Vitamin A analogs, prediction of these highly specific interactions for the case of an arbitrary permeant is beyond our present capabilities. In the absence of detailed binding information, the use of 2% albumin as a surrogate for the complex milieu of soluble proteins in skin seems a reasonable approach to studying transport in the lower skin layers.

CONCLUSION

Binding of lipophilic permeants to soluble proteins in the dermis plays an important role in determining their partition coefficient and effective diffusivity within the tissue. In the absence of a stratum corneum barrier, attention to diffusion of these proteins out of the tissue is necessary in order to obtain accurate values of these parameters from *in vitro* studies involving highly protein bound permeants. For permeation studies involving isolated dermis, this can be achieved by isolating the tissue from the donor solution with a dialysis membrane and supplementing the receptor solution with 2% BSA.

APPENDIX 1: DIFFUSION OF A REVERSIBLY BOUND PERMEANT ON A MOBILE SUBSTRATE

Consider the case of a permeant diffusing in one dimension (x) across an aqueous membrane or layer containing a diffusing substrate, for example, a macromolecule. The permeant is rapidly and reversibly bound to the substrate according to a linear isotherm; thus $C_{\text{bound}} = KC_{\text{free}}$, where K is the binding constant. The total permeant concentration C_{tot} is the sum of $C_{\text{bound}} + C_{\text{free}}$. The assumption of linearity restricts the present analysis to low permeant concentrations. The unbound fraction of permeant f_u is equal to $1/(1+K)$, and the partition coefficient of the medium relative to water is $1+K = 1/f_u$. The flux of permeant at any point in the system, J, is equal to the sum of the free and bound fluxes. Assuming these fluxes to be governed by Fick's Law, one has

$$J = -D_{\text{free}}\frac{\partial C_{\text{free}}}{\partial x} - D_{\text{bound}}\frac{\partial C_{\text{bound}}}{\partial x} \tag{A.1}$$

Defining the effective diffusivity D_{eff} as the multiplier of the total concentration gradient that yields the observed flux, one has

$$\begin{aligned} D_{\text{eff}}\frac{\partial C_{\text{tot}}}{\partial x} &= D_{\text{free}}\frac{\partial C_{\text{free}}}{\partial x} + D_{\text{bound}}\frac{\partial C_{\text{bound}}}{\partial x} \\ &= (D_{\text{free}} + KD_{\text{bound}})\frac{\partial C_{\text{free}}}{\partial x} \\ &= (D_{\text{free}} + KD_{\text{bound}})\cdot f_u\frac{\partial C_{\text{tot}}}{\partial x} \end{aligned} \tag{A.2}$$

But $Kf_u = K/(1+K) = 1-f_u$, which is equivalent to the bound fraction f_b. Thus,

$$D_{\text{eff}} = f_u D_{\text{free}} + (1-f_u)D_{\text{bound}} \tag{A.3}$$

which is the same as Eq. (8) in the text. The diffusive resistance of a layer of this medium is equal to its thickness divided by the product of effective diffusivity and partition coefficient relative to water. Identifying this resistance as R_2, the thickness as h_{ABL}, and recalling that the partition coefficient of the medium is $1/f_u$ yields Eq. (7) in the text.

APPENDIX 2: CALCULATION OF K_{DE} FROM AVERAGE DERMIS CONCENTRATION

Assume the dermis is placed in series with a dialysis membrane in a side-by-side diffusion cell. Concentration C_d is maintained in the donor solution and sink conditions are maintained in the receptor solution. Unstirred aqueous boundary layers develop external to the composite membrane, as shown in Figure 6. The steady state the flux J_{ss} across this system is

$$J_{ss} = k_p(C_d - 0) = \frac{C_d}{R_{\text{tot}}} \tag{B.1}$$

where k_p is the permeability coefficient and $R_{\text{tot}} = R_{\text{Dial}} + R_{de} + R_1 + R_2$ is the total diffusive resistance.

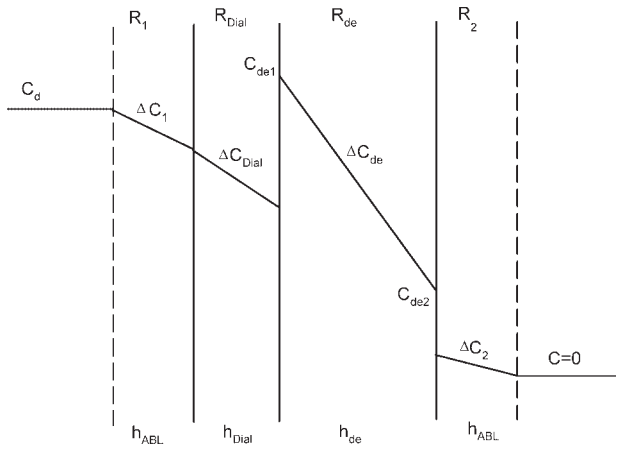

Figure 6. Steady-state concentration profiles for dermis + dialysis membrane system with aqueous boundary layers surrounding the composite membrane.

At steady state, the flux across each layer is identical such that

$$\frac{C_d}{R_{tot}} = \frac{\Delta C_1}{R_1} = \frac{\Delta C_2}{R_2} = \frac{\Delta C_{Dial}}{R_{Dial}} = \frac{\Delta C_{de}}{R_{de}} \tag{B.2}$$

Rearrangement of Eq. (B.2) yields

$$\Delta C_{de} = \frac{R_{de}}{R_{tot}} C_d = \frac{R_{de} C_d}{R_1 + R_{Dial} + R_{de} + R_2} \tag{B.3}$$

The sum of the concentration drops across each layer, normalized by partition coefficient, is equal to the donor concentration, that is,

$$C_d = \Delta C_1 + \Delta C_{Dial} + \frac{\Delta C_{de}}{K_{de}} + f_u \Delta C_2 \tag{B.4}$$

Thus, the average concentration in the dermis can be written as

$$\begin{aligned}
\overline{C_{de}} &= \frac{C_{de1} + C_{de2}}{2} \\
&= \frac{K_{de}(C_d - \Delta C_1 - \Delta C_{Dial}) + K_{de} f_u \Delta C_2}{2} \\
&= \frac{1}{2} K_{de}(C_d - \Delta C_1 - \Delta C_{Dial} + f_u \Delta C_2) \\
&= \frac{1}{2} K_{de} C_d \left(1 - \frac{R_1}{R_{tot}} - \frac{R_{Dial}}{R_{tot}} + \frac{R_2}{R_{tot}}\right) \\
&= \frac{1}{2} K_{de} C_d \left(\frac{R_{tot} - R_1 - R_{Dial} + R_2}{R_{tot}}\right)
\end{aligned} \tag{B.5}$$

Therefore, the partition coefficient for the dermis relative to the donor solution is

$$K_{de} = \frac{2\overline{C_{de}}}{C_d} \left(\frac{R_{tot}}{R_{tot} - R_1 - R_{Dial} + R_2}\right) \tag{B.6}$$

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