RESEARCH ARTICLE

Tissue Binding Affects the Kinetics of Theophylline Diffusion Through the Stratum Corneum Barrier Layer of Skin

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ABSTRACT: New data sets on both (i) equilibrium theophylline (TH) partitioning/binding in stratum corneum and (ii) transient TH diffusion through human epidermis are explained by an extended partition–diffusion model with reversible binding. Data conform to a linear binding isotherm within the tested concentration range (0–2000 μg/mL) with an equilibrium ratio of bound-to-free solute of approximately 1.4. The permeability coefficient for TH is 4.86×10^{-5} cm/h, and the lag time is 20.1 h. Binding occurs as a slow process, significantly affecting the kinetics of dermal penetration. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

Most topical and transdermal drug applications, as well as occupational chemical exposures, occur in a transient mode.[1–3] For realistic estimation of dermal absorption, it is therefore important to employ rigorous mathematical models accounting for time-dependent diffusion[1–4] (not just steady-state permeability), and to include in these models all physicochemical phenomena that can affect penetration kinetics. For water diffusion through the stratum corneum (SC, barrier) layer of skin, Anissimov and Roberts[4] have demonstrated recently that apparent inconsistencies between parameter sets fitted to penetration versus desorption experiments using a standard two-parameter partition–diffusion model are removed by incorporation of a slow reversible binding process. The purpose of this paper is to report new experiments with a model hydrophilic compound, theophylline (TH), addressing both (i) partitioning/binding in SC and (ii) transient penetration through human epidermis. Both data sets support the extended model with binding.

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MATERIALS AND METHODS

Experiments

Buffer

Buffer was HEPES-buffered Hanks Balanced Salt Solution (Gibco®, Invitrogen, Carlsbad, California), with 50 mg/L of gentamicin. For TH, a weak acid with a p*K*a of 8.81,[5] buffer at pH 6.0 was used for the dosing solution to assure that virtually all the drug was in the unionized state. For other uses, the pH of the buffer was adjusted to 7.4. Buffer was filtered (0.2 μg pore size, Nalgene, Thermo Fisher Scientific, Rochester, New York) and degassed prior to use by warming to 40°C and stirring under laboratory vacuum.

Tissue

Caucasian female skin from breast reduction surgery was obtained from the West Virginia University Tissue Bank. Our use of this tissue was deemed "not human subject research" and therefore not subject to Institutional Review Board approval. Skin was obtained fresh on the day of surgery. Heat-separated epidermal membranes (HEMs) were obtained by submersing the skin in buffer at 60°C for 45 s. Epidermis was teased from dermis using cotton swabs. Epidermal discs (1.59 cm diameter) were cut and stored

frozen (−85°C) on gauze saturated with buffer plus 10% glycerol. SC was isolated by incubating thawed epidermal discs in 0.01% type II-S porcine pancreatic trypsin (Sigma–Aldrich, Saint Louis, Missouri) in buffer overnight at 37°C with gentle agitation, followed by multiple rinses with ultrapure H_2O. SC was stored at room temperature under laboratory vacuum in a desiccator until used within 2 days. For studies on TH permeation and binding, skin from a single donor was used to eliminate interindividual variability in the measured variables.

Steady State SC–TH Partitioning/Binding Study

Isolated SC membranes were used, preweighed in a desiccated state. Incubation solutions (0–2000 μg/mL) consisted of defined amounts of TH in buffer and 5 μCi/mL of ^{14}C-labeled compound (8–^{14}C TH, 50 mCi/mmol; Moravek Biochemicals, Brea, California). Triplicate membranes were used for each concentration. They were incubated separately in 2 mL of solution for 60 h at 37°C with gentle agitation. SC samples were removed, blotted dry on tissues, rinsed twice in fresh buffer, then solubilized in 1 mL Soluene® 350 (PerkinElmer, Waltham, Massachusetts) at 55°C for 4 h. TH concentrations in SC (mass/mass dry SC) and incubation solutions (mass/volume) were quantified using liquid scintillation counting. Complete removal of surface TH was confirmed as the rinses exhibited approximately background activity.

Purified Keratin–TH Covalent Binding Study

Purified keratin protein derived from human epidermis (Sigma–Aldrich) was incubated (0.5 mg protein/mL) in 2000 μg/mL TH in pH 6.0 buffer or buffer alone (control) for 24 h at 37°C with gentle agitation. One hundred microliter aliquots were diluted to 1 mL in 50 mM NH_4HCO_3. Trypsin was added at a 40:1 (w:w) protein:trypsin ratio and the solutions were incubated overnight at 37°C.

One microliter aliquots of the resulting trypsin digest solutions of keratin control and keratin incubated with TH were analyzed by ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). UPLC was performed on a nanoACQUITY (Waters, Milford, Massachusetts) system utilizing a 100 μm × 150 mm BEH C_{18} capillary column (1.7 μm particle size). Reverse-phase chromatography was performed at a flow rate of 250 nL/min and a gradient from 99/1 A/B to 40/60 A/B over 60 min, where A (0.1% formic acid in ultrapure H_2O) and B (0.1% formic acid in acetonitrile) were the aqueous and organic phase solvents, respectively.

Eluent from the UPLC entered the orthogonal nanospray assembly of a quadrupole time-of-flight tandem mass spectrometer (qTOF-MS/MS; SYNAPT, Waters) capable of mass resolution $m/\Delta m > 12{,}000$ and mass accuracy of 5 ppm or better. [Glu]^1-fibrinopeptide B was sprayed 90° off axis from the eluent spray to continually supply a mass standard, so that the data could be continually calibrated during an acquisition. Both capillary emitters were maintained at a voltage of 3.5 kV for the duration of the experiment. The instrument cone voltage was set to 40 V, and dry N_2 gas was supplied at 0.5 bar (Nitroflow Lab, Parker Hannifin, Cleveland, Ohio). Data were acquired in an "MS^e" fashion,[6] where alternating low- and high-energy collision gas (Ar) was applied. Low-energy scans were performed at 4 eV, whereas high-energy collision gas was applied utilizing a ramp of 15–35 eV per 1 s scan. Data were analyzed with ProteinLynx Global Server v.2.4 and BioPharmaLynx v.1.2 (Waters).

TH Permeation Study

Epidermal membranes were mounted on Franz-type (static) diffusion cells (PermeGear, Inc., Hellertown, Pennsylvania) with dialysis tubing used as a membrane support. The cells were maintained at 37°C via a recirculating water bath, which maintained skin surface temperature at 32°C. Skin discs were equilibrated overnight with pH 7.4 buffer in receptor compartments, and H_2O in donor compartments to assure constant and complete hydration of SC prior to applying the aqueous donor solution, which was TH (2000 μg/mL; Sigma–Aldrich) in pH 6.0 buffer. Time courses of TH accumulation in the receptor compartments over 36-h infinite dose exposures were obtained for four skin samples. Donor solution was replaced periodically to assure "infinite dose" conditions. One HEM sample was an obvious outlier and was not considered in subsequent analysis.

Theophylline concentrations were determined using high-performance liquid chromatography (HPLC). An Agilent 1100 series HPLC was used with a C_{18} column (Phenomenex Onyx Monolithic, Torrance, California) operating at 30°C with ultraviolet detection at 272 nm. The mobile phase (0.5 mL/min, pH 2.84) was 10% methanol and 90% 1% acetic acid in H_2O. The retention time for TH was 11.0 min. The calibration curve for this experiment was linear ($r^2 = 0.999$) down to the lowest (nonzero) measured concentration.

To support the use of HEM for a 36-h permeation experiment, we subjected a set of HEMs from a different skin donor to a 52-h permeation experiment with diethyl phthalate as donor. In our experience, this permeant yields linear mass accumulation following a lag time of approximately 1–2 h. Prior to the exposure, HEMs were equilibrated overnight, mounted on diffusion cells with H_2O in the donor compartments. From the average of five skin discs, linear regression of data points for times exceeding three lag times yielded a correlation coefficient (r^2) of 0.9997. This result suggests that membrane deterioration (which

would cause a slow increase in membrane permeability evidenced as curvature in the data) does not occur even after 52 h. Thus, the TH permeation experiments are unlikely to be biased by membrane deterioration.

Data Analysis

SC Volume and Thickness

A reasonable nominal value for SC hydrated volume is 3.518 cm^3 hydrated volume per gram of original dry tissue.[7] Together with the measured dry mass of tissue (mean ± SD = 0.914 ± 0.106 mg/cm^2), this volume implies an average SC thickness (h) of 32.2 μm.

Free Solute Partitioning

Theophylline has a molecular weight of 180.17 and $\log_{10}K_{\text{octanol/water}} = -0.02$.[8] Mobile (unbound) solute potentially partitions into both intercellular lipid (comprising ~3% of hydrated SC volume) and corneocytes (~97%). In the latter, approximately 80% of the volume is accessible water around the keratin filaments. By a recent correlation of partitioning data,[7] these factors yield the estimate

$$K = 0.756 \tag{1}$$

for the tissue-average partition coefficient of free (unbound) TH relative to an aqueous solution maintaining the unionized form[9] (see Appendix A, Eqs. A5–A7 of this reference). According to this analysis, intercellular lipid accounts for only approximately 2% of free solute holdup in the SC.

Equilibrium Binding

Binding of TH to keratin (and possibly other corneocyte constituents such as the cornified cell envelope)[7,10] is viewed in macroscopic (tissue-average) terms and assumed to follow the linear reversible binding rate law

$$\text{rate of binding} = k_f C - k_r B\,, \tag{2}$$

where C and B denote tissue-average concentrations of free and bound TH, and k_f and k_r denote the forward ("on") and reverse ("off") binding rate constants. On the basis of this model, the equilibrium amount of total (free + bound) solute is quantified by an auxiliary partition coefficient[4]

$$K_{f+b} = K\left(1 + \frac{k_f}{k_r}\right). \tag{3}$$

Theoretical Model for Permeation

Data analysis assumed partitioning into the SC and diffusion therein, with the additional phenomenon of binding following Eq. 2, as described by the equations

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - (k_f C - k_r B) \tag{4}$$

$$\frac{\partial B}{\partial t} = +(k_f C - k_r B)\,, \tag{5}$$

posed with boundary and initial conditions

$$\begin{aligned} &C(0,t) = KC_0\,, \quad C(h,t) = 0\,,\\ &C(x,0) = 0\,, \quad B(x,0) = 0\,, \end{aligned} \tag{6}$$

governing the free and bound TH concentrations $C(x,t)$ and $B(x,t)$, respectively.[4,9] As are the parameters K, k_f, and k_r, the diffusion coefficient D is likewise regarded as a macroscopic average property of the tissue. C_0 is the (constant) donor TH concentration (here, 2000 μg/mL). For given parameters, values of TH accumulation in the receptor compartments at each time were obtained from the Laplace-domain solution presented by Anissimov and Roberts[4] by numerical inversion using the software package Scientist (MicroMath Scientific Software, Saint Louis, Missouri).

Strategy for Fitting Parameters

An overall data set for the time course of TH permeation was constructed by averaging the measured accumulation at each time over all three skin samples. K was assigned the value given by Eq. 1; the ratio $k_f/k_r$was held fixed at the value implied by the steady-state TH/SC binding studies, and best-fit values of D and k_r were then obtained by least-squares regression of the permeation data, also using Scientist. For comparison, a simplified model assuming no binding (Eq. 4 with $k_f = k_r = 0$) was used to obtain best-fit values of the parameters K and D from these same permeation data. Sub-SC tissue (viable epidermis) has a small mass transfer resistance; thus, HEM reflects the permeation kinetics of SC.[11]

RESULTS

Partitioning/Binding Study

Figure 1 shows the measured isotherm for total (free + bound) solute in the SC following 60 h incubation. Upon converting units, the regressed slope (6.385 mL/g) implies an overall (free + bound) partition coefficient

$$K_{f+b} = \frac{6.385\,\text{mL}}{1\,\text{g}} \times \frac{1\,\text{g}}{3.518\,\text{mL}} = 1.815\,. \tag{7}$$

The extent to which K_{f+b} exceeds K quantifies the amount of bound solute (cf. Eqs. 1 and 3), leading to

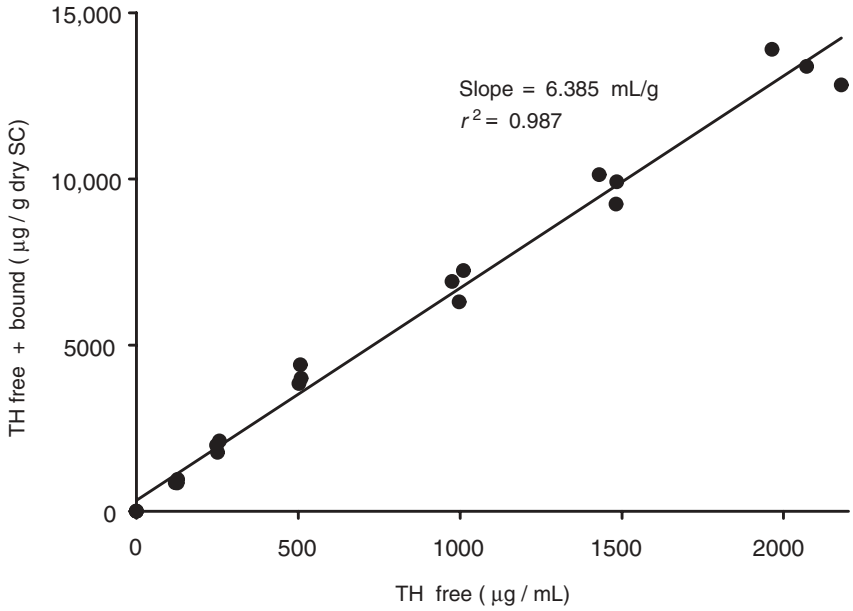

Figure 1. Measured isotherm quantifying equilibrium free + bound solute holdup in SC. The solid line represents the best-fit linear regression.

the result

$$\frac{k_f}{k_r} = 1.401\,. \tag{8}$$

the parameters

$$D = (2.069 \pm 0.019) \times 10^{-7}\,\mathrm{cm^2/h}\,, \tag{9}$$

$$k_r = 0.466 \pm 0.147\,\mathrm{h^{-1}} \tag{10}$$

with the constraint imposed by Eq. 8. The error estimates represent standard deviations of the parameters returned with the best fit by Scientist. The dotted curve represents the calculations with binding artificially switched off (conventional partition–diffusion model), for which the best-fit parameter estimates were found to be

$$D = (9.827 \pm 0.063) \times 10^{-8} \quad \mathrm{cm^2/h}\,, \tag{11}$$

$$K = 1.467 \pm 0.184\,. \tag{12}$$

Keratin–TH Covalent Binding Study

Tandem mass spectrometry data were analyzed for evidence of covalent binding of TH to keratin. Specifically, the retention time, relative abundance, and mass-to-charge ratio (*m/z*) of all eluting peaks were compared between the control and TH-incubated keratin. Covalently bound TH would have produced tryptic peptides shifted higher in *m/z* by 180.0647 u, whereas noncovalently bound TH would not be expected to survive the UPLC–MS experiment to be observed as a gas phase complex. Analysis of the two data sets demonstrated a very high degree of conformity between control and analyte spectra. No significant peaks were observed in the analyte spectra that were not present in the control (data not shown), demonstrating that the interaction of TH with keratin is not covalent. These data support the reversible binding model for TH permeation described by Eqs. 2, 4 and 5.

Permeation Study

Figure 2 shows the measured time course of TH accumulation in the diffusion cell receptor compartments. The solid curve represents the full partition–diffusion–binding model based on the best-fit estimates of

DISCUSSION

Our quantitative findings are embodied in the characterization of TH provided by Eqs. 8–10. We chose to characterize TH binding and transport within SC obtained from a single donor in the interest of eliminating interindividual variability. Thus, our results represent an accurate characterization of a representative example of SC.

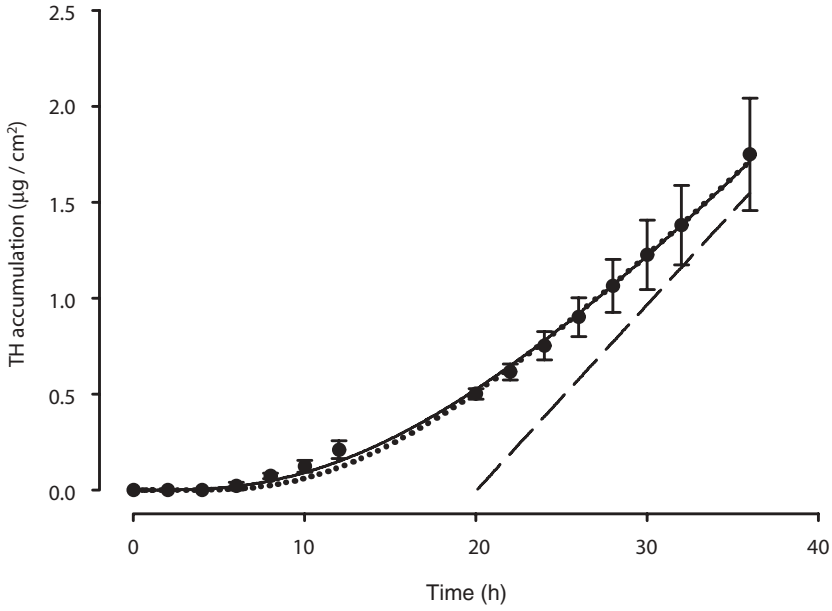

Figure 2. Accumulation of theophylline in the receptor compartments. Points with error bars represent measured values (mean ± SD) averaged over all three skin samples for each time. The solid curve shows the fit provided by the full *KD* + binding model. The dotted curve represents the fit provided by the conventional *KD* model with no binding, presented for comparison. The dashed line represents the ultimate asymptote for mass accumulation once diffusion has reached steady state and binding has equilibrated.

Explicit Representation of Bound Solute

Within the framework of a conventional partition–diffusion (*KD*) model of free solute transport, the steady state permeability coefficient of the SC membrane

$$k_p = \frac{KD}{h} \tag{13}$$

reflects the combined effect of molecular partitioning and diffusion factors, whereas the lag time for transient permeation

$$t_{lag} = \frac{h^2}{6D} \tag{14}$$

reflects only the latter. The long lag time for TH (as well as many other permeants) implies a small value of D, and consequently, a substantial value of K needed to match the observed k_p.[11,12] The numerical value in Eq. 1 represents solute holdup explainable by partitioning into the accessible volume of the SC. A very long apparent lag time making K larger than this value is readily explained in terms of additional holdup associated with solute binding to membrane components. The work reported here has quantified this phenomenon in detail for TH. The rigorous approach used involves matching transient permeation data with a transient diffusion model explicitly incorporating the kinetics of exchange between free and bound solute populations (*KD* + binding model, Eqs. 4–6). Such an approach is necessary because the conventional *KD* model—and any usual graphical constructions associated with it—miss the element of binding and are therefore incorrect

It is worthwhile to analyze the way in which the conventional *KD* model represents (imperfectly) the additional holdup associated with bound solute. In accordance with intuition, the fitted value of K it produces (Eq. 12) is intermediate between 0.756 (Eq. 1; no binding initially) and the value for total holdup given by Eq. 7 (binding close to equilibrium at long times). In the actual permeation process (and in the *KD* + binding model), the amount of bound relative to free solute in the membrane increases with time. In the *KD* model, bound solute is (mis)represented as additional free solute, and the bound/free ratio is effectively assigned a time-independent average value. Equations 11 and 12 yield a steady-state permeability coefficient $k_p = 4.48 \times 10^{-5}$ cm/h and lag time $t_{lag} =$ 17.6 h based on the *KD* model. These values are quite close to the actual results $k_p = 4.86 \times 10^{-5}$ cm/h and $t_{lag} = 20.1$ h emerging from the comprehensive *KD* + binding picture. The coefficient k_p now reflects only the contribution of free solute because bound solute does not contribute to steady-state flux. Also, we have used the result

$$t_{lag} = \frac{h^2}{6D}\left(1 + \frac{k_f}{k_r}\right) \tag{15}$$

for situations with reversible binding.[4,13] Anissimov and Roberts[4] have noted that the conventional *KD* model can offer a reasonable fit to data for a permeation experiment or a desorption experiment considered in isolation that is not necessarily worse than that provided by the *KD* + binding model. The key observation is that these two types of experiments yield inconsistent (and therefore inaccurate) parameter values, and that the only way to fit both with the same K and D is to incorporate the additional element of solute binding. In this study, we have used an independent equilibrium binding study (as opposed to a desorption experiment) as the second experiment that—together with the permeation study—determines the SC transport parameters of TH. This approach has the attractive feature of directly determining the ratio of k_f to k_r from the binding study (see Eq. 8).

We note in passing that the curve showing TH accumulation is quite far from the steady-state asymptote even after 36 h (Fig. 2). Thus, the only reliable way to determine lag times from permeation data is to regress the data with a diffusion model. One would not easily hit upon the correct asymptote by any graphical or "eyeball" procedure. This warning applies especially in cases of long lag times.

Partition Coefficient of Free Solute

Approximately 0.34 g of water per g of dry SC constituents in hydrated SC is bound[10] (cf. a similar estimate of 0.30 in the literature[14]). Anderson and Raykar[15] advanced the view that water in the SC—whether free or bound—possesses solvating properties essentially equivalent to those of bulk water. This is the position underlying Eq. 1. Indeed, any reasonable underlying physical picture would suggest a partition coefficient of this magnitude (i.e., unity minus a limited degree of volumetric exclusion) for partitioning of free hydrophilic solute into the predominantly aqueous environment of fully hydrated SC. Hansen et al.[10] argue that the bound water is unavailable for solvent partitioning. The bound amount is relatively minor compared with the total in fully hydrated SC, which contains around 2.75 g water per gram of dry SC constituents.[7] Thus, uncertainty on the question of water available for partitioning would affect the results by only $O(10\%)$. Nevertheless, it is worth emphasizing that Eq. 1 represents an upper limit on the holdup of free TH. Thus, binding is at least as important as represented by Eqs. 7 and 8, and might be slightly higher.

Quantity of Bound Solute

The measured level of binding is so substantial that there is more bound than free TH in the SC at equilibrium (bound/free ~1.4). Bound solute is often quantified in terms of an alternate partition coefficient PC_{pro} representing the equilibrium ratio (mass of bound solute per unit mass of SC protein)/(mass of solute per unit mass of water in adjacent solution). Referring to Eqs. 3 and B8 of Nitsche et al.,[7] Eq. 7 implies

$$PC_{pro} = 4.14 \tag{16}$$

for TH. This result is in profile with correlations given in the literature to estimate the amount of bound solute in human SC. For instance, on the basis of an analysis of a comprehensive database of SC–water partition coefficients, Nitsche et al.[7] suggested the formula

$$PC_{pro} = 5.4\left(K_{octanol/water}\right)^{0.27} \tag{17}$$

which represents a slight modification of a similar correlation advanced much earlier by Anderson and Raykar.[15] For TH, this formula gives $PC_{pro} = 5.33$, which is not far off. Equation 16 also agrees broadly with the value $PC_{pro} = 6.03 \pm 1.89$ reported by Wang et al.[16] for TH in porcine SC.

There is some question as to where in corneocytes binding occurs. It is clearly connected with interior keratin, although Hansen et al.[10] provide evidence that corneocyte envelope proteins may also play a role. Whatever the ultimate distribution of binding sites may be, substantial solute holdup in corneocytes—in excess of that explainable by partitioning into the water that hydrates them—is implicated. This fact strongly erodes the view of corneocytes as impermeable obstacles in the SC.

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REFERENCES

1. Frasch HF, Barbero AM. 2008. The transient dermal exposure: Theory and experimental examples using skin and silicone membranes. J Pharm Sci 97:1578–1592.
2. Ho C. 2004. Probabilistic modeling of percutaneous absorption for risk-based exposure assessments and transdermal drug delivery. Stat Methodol 1:47–69.
3. Roberts MS, Anissimov YG. 2005. Mathematical models in percutaneous absorption. In Percutaneous absorption: Drugs–cosmetics—mechanisms–methodology; Bronaugh R, Maibach H, Eds. 4th ed. New York: Marcel Dekker, pp 1–44.

4. Anissimov YG, Roberts MS. 2009. Diffusion modelling of percutaneous absorption kinetics: 4. Effects of a slow equilibration process within stratum corneum on absorption and desorption kinetics. J Pharm Sci 98:772–781.
5. Kortüm G, Vogel W, Andrussow K. 1960. Dissociation constants of organic acids in aqueous solution. Pure Appl Chem 1:187–536.
6. Chakraborty AB, Berger SJ, Gebler JC. 2007. Use of an integrated MS-multiplexed MS/MS data acquisition strategy for high-coverage peptide mapping studies. Rapid Commun Mass Spectrom 21:730–744.
7. Nitsche JM, Wang TF, Kasting GB. 2006. A two-phase analysis of solute partitioning into the stratum corneum. J Pharm Sci 95:649–666.
8. Hansch C, Leo A, Hoekman D. 1995. Exploring QSAR. Hydrophobic, electronic, and steric constants. Washington DC: American Chemical Society.
9. Nitsche JM, Frasch HF. 2011. Dynamics of diffusion with reversible binding in microscopically heterogeneous membranes: General theory and applications to dermal penetration. Chem Eng Sci, in press.
10. Hansen S, Naegel A, Heisig M, Wittum G, Neumann D, Kostka KH, Meiers P, Lehr CM, Schaefer UF. 2009. The role of corneocytes in skin transport revised–A combined computational and experimental approach. Pharm Res 26:1379–1397.
11. Wang TF, Kasting GB, Nitsche JM. 2007. A multiphase microscopic diffusion model for stratum corneum permeability. II. Estimation of physicochemical parameters, and application to a large permeability database. J Pharm Sci 96:3024–3051.
12. Barbero AM, Frasch HF. 2006. Transcellular route of diffusion through stratum corneum: Results from finite element models. J Pharm Sci 95:2186–2194.
13. Siegel RA. 2005. Characterization of relaxation to steady state in membranes with binding and reaction. J Membr Sci 251:91–99.
14. Kasting GB, Barai ND, Wang TF, Nitsche JM. 2003. Mobility of water in human stratum corneum. J Pharm Sci 92:2326–2340.
15. Anderson BD, Raykar PV. 1989. Solute structure-permeability relationships in human stratum-corneum. J Invest Dermatol 93:280–286.
16. Wang L, Chen L, Lian G, Han L. 2010. Determination of partition and binding properties of solutes to stratum corneum. Int J Pharm 398:114–122.