Title page

Kinetics of cellular retention during Caco-2 permeation experiments: Role of lysosomal sequestration and impact on permeability estimates

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Running title page

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Abbreviations:

$P_{app}$, apparent permeability through cell monolayer

$P_{app,sink}$, estimated apparent permeability through cell monolayer assuming sink conditions

$P_{app,nonsink}$, estimated apparent permeability through cell monolayer without sink condition assumption

A to B and B to A, apical to basal and basal to apical directions, respectively
\( M_D \) and \( M_R \), amount of test compound in donor and receiver compartments, respectively

\( V_D \) and \( V_R \), volumes of donor and receiver compartments, respectively

UWL, unstirred water layer

\( P_{api} \), permeability coefficient through barrier between apical and cytosolic compartments (unstirred water layer and cell membrane)

\( P_{baso} \), permeability coefficient through barriers between basal and cytosolic compartments (unstirred water layer, filter support and cell membrane)

\( M_{api} \), \( M_{baso} \), \( M_{cyto} \), \( M_{lyso} \), amounts in apical, basal, cytosolic and lysosomal compartments, respectively

\( V_{api} \), \( V_{baso} \) and \( V_{cell} \), volumes of apical (500 \( \mu \)l) and basal (1500 \( \mu \)l) compartments and cell monolayer (~2.3 \( \mu \)l), respectively

\( A \), area of the cell monolayer, 113.1 mm\(^2\)

\( h \), height of the cell monolayer, approximated using cross section microscopy, 20 \( \mu \)m

\( K \), apparent cell-buffer distribution coefficient

\( K_{max} \) and \( K_{min} \), higher and lower limits for apparent cell-buffer distribution coefficient, respectively

\( EC_{50} \), the free cytosolic concentration resulting \( K \) to be average of \( K_{max} \) and \( K_{min} \)

\( Q_{12} \) and \( Q_{21} \), first order rate coefficients for mass transfer from cytosol to lysosome and lysosome to cytosol, respectively

**Recommended Section Assignment:** Metabolism, Transport and Pharmacogenomics
Abstract

The permeability estimation from cell monolayer permeation data is usually based on 100 %
recovery assumption. However, poor recovery is often seen in such experiments in practice, but
often neglected in data interpretation. In the present study, the cellular retention kinetics during
Caco-2 permeation experiments of three passively transported compounds (weakly basic
propranolol, weakly acidic ibuprofen and neutral testosterone) were determined. Further, the effects
of cellular retention kinetics on apparent permeability were evaluated and the role of lysosomal
sequestration in cellular retention of propranolol was explored. The cellular retention profiles were
observed to be direction and concentration dependent, which may cause erroneous directionality
and concentration dependence in permeability estimates. Further, the lysosomal sequestration was
demonstrated to contribute to the extent and kinetics of the cellular retention of propranolol.
Introduction

Cell monolayer permeation experiments are commonly used to predict intestinal absorption potential of drug molecules (Artursson et al., 2001) and to screen compounds for their interactions with active transporters (Polli et al., 2001). In addition, cell monolayer permeation experiments are often used to study the mechanisms of drug transfer (Artursson et al., 2001).

Typically, in in vitro permeation experiments the permeability estimates are based on the appearance kinetics of the test compound to the receiver compartment. Additionally, samples from both donor and receiver sides are often collected at the final time point of the experiment to determine the recovery of the test compound. The analysis and interpretation of permeation data are often based on the assumption that the cell monolayer behaves as a single barrier for the solute transfer and that the whole mass of the studied compound is in donor and receiver compartments, i.e. the recovery is 100%. However, physiologically the permeation barrier in cell monolayer experiments consists of various serial and parallel barriers (Ho et al., 1999) and, further, reduced recovery is often observed in permeability experiments (Polli et al., 2001). Incomplete recovery is attributed to metabolism, drug binding to the plastic surfaces and/or to cellular retention of drug (Fisher et al., 1999; Östh et al., 2002; Palmgren et al., 2006). The possible bias in permeability estimates caused by poor recovery is generally acknowledged but still often neglected in the data interpretation.

The mechanisms causing poor recovery and, consequently, the kinetics of apparent loss of test compound in permeation experiments may be specific to compound, experimental apparatus and permeation barrier (Östh et al., 2002; Tran et al., 2004). Thus, accurate universal correction terms for poor recovery in permeation experiments cannot be applied. However, in some reported studies the poor recovery have been taken into account by correcting the calculations with experimental recovery at a single time point, practically either at the end of the experiment (Youdim et al., 2003).
or after initial 'cell loading' period in the beginning of the experiment (Korjamo et al., 2008; Tran et al., 2004).

In Caco-2 cells, drug metabolism is limited due to low expression levels of cytochrome P450 metabolizing enzymes (Engman et al., 2001; Korjamo et al., 2006; Prueksaritanont et al., 1996). Further, an earlier study in our lab suggested that in the presence of buffer the loss of various drugs to cell culture plastics is minimal whereas the cellular retention may be substantial (Palmgren et al., 2006). Therefore, retention into the cellular structures is likely to be the principle cause of poor recovery for many compounds in cell-based permeation experiments.

Cellular retention may be caused by partitioning into the cellular lipids due to high lipophilicity (Sawada et al., 1999), specific or nonspecific binding to the cellular protein or due to electrostatic binding to the charged structures of the cell. Further, pH gradient driven sequestration of the ionisable compounds into intracellular organelles may contribute to cellular retention. Intracellular sequestration mechanisms are more thoroughly reviewed elsewhere (Duvvuri and Krise, 2005).

One major mechanism causing the compound sequestration into the intracellular organelles is ion trapping of weak bases into the organelles with acidic interior (Kaufmann and Krise, 2007). Several intracellular organelles possess acidic interior and intraorganelle pH may be as low as 4.5 in lysosomes, whereas cytosolic pH lies near neutrality (Asokan and Cho, 2002). It has been shown that weakly basic compounds with suitable pKa are sequestered into the acidic organelles, provided that the membrane permeability of the ionized and unionised forms differs substantially (Duvvuri et al., 2004). Further, lysosomes are the most acidic organelles in the cells and, therefore, most of the pH-partitioning based sequestration of weakly basic compounds is likely to be due to lysosomal sequestration. In addition, it is challenging to experimentally distinguish the true lysosomal sequestration from the possible sequestration into the other acidic vesicles, such as endosomes, in the cells. Therefore, for simplicity in this report term 'lysosome' is used to cover also the other acidic vesicles than lysosomes.
In this study, we have explored and modelled the cellular retention kinetics of basic propranolol, acidic ibuprofen and neutral testosterone during Caco-2 permeation experiments. Further, we studied the role of lysosomal sequestration in cellular retention of propranolol. Additionally, the effects of cellular retention kinetics on estimates of apparent permeability through the Caco-2 cell monolayer were evaluated.
Methods

Chemicals. Propranolol ((±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol) and ibuprofen (α-Methyl-4-(isobutyl)phenylacetic acid) were obtained from Sigma (St. Louis, MO, USA) and testosterone (17β-Hydroxy-4-androsten-3-one) from Fluka (Buchs, Switzerland). 3H-ibuprofen and 3H-testosterone were from American Radiolabeled Chemicals (St. Louis, MO, USA) and 3H-propranolol was from Perkin Elmer (Boston, MA, USA). Bafilomycin A1 was from LC Laboratories (Woburn, MA, USA). Fetal bovine serum was obtained from EuroClone (UK) and other cell culture reagents from BioWhittaker (Belgium).

Cell culture. Caco-2 cells (ATCC HTB-37, Manassas, VA, USA) were cultured and grown for experiments as earlier described (Korjamo et al., 2005). Briefly, Caco-2 cells (passages 43-55) were maintained subconfluent in cell culture flasks and subcultured twice a week. For the permeability and cellular retention experiments, 82 000 cells/cm2 were seeded onto Transwell inserts (Catalog number 3401, Corning Life Sciences, Corning, NY, USA) and were grown for 21–24 days prior to the experiments.

Permeability and cellular retention experiments. The physicochemical properties of the model compounds are given in Table 1. One basic, acidic and neutral compound was selected for the experiments. All the model compounds are known to be transported passively through Caco-2 monolayers when no pH gradient is present and are not significantly metabolized during Caco-2 permeation experiments (Engman et al., 2001; Korjamo et al., 2008).

Before the experiments, the excess of cell culture medium was washed from the cell monolayers and 0.5 ml and 1.5 ml of transport buffer (Hanks’ balanced salt solution buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) with or without 100 nM bafilomycin A1, a specific inhibitor of vacuolar type H+ -ATPase (Bowman et al., 1988), that inhibits the acidification of lysosomes (Yoshimori et al., 1991), was added on apical and basal compartments, respectively. The monolayers were preincubated for 30 minutes at 37 °C in a humidified incubator. The integrity
of the monolayers was checked by transepithelial electrical resistance measurement, only monolayers with resistance higher than 300 Ω·cm² were accepted for the experiments.

The donor solutions were spiked with the respective ³H-labelled compound. The initial donor concentrations were 1 µM, 50 µM and 300 µM for propranolol and ibuprofen and ~10 nM (blank transport buffer spiked with ³H-testosterone), 1 µM and 50 µM for testosterone. The stock solutions of unlabeled compounds were prepared in dimethylsulfoxide (DMSO) and the DMSO concentration did not exceed 1 % in final donor solutions.

The permeability and cellular retention experiments were conducted in both apical to basal (A to B) and basal to apical (B to A) directions on an orbital shaker at 320 rpm (Titramax 101, Heidolph, Schwabach, Germany) at 37 C° in a humidified incubator. The experiments were started by replacing the preincubation buffer with fresh transport buffer into the receiver compartment and donor solution into the donor compartment, both with or without 100 nM bafilomycin A1 as indicated. At each sampling time point (5, 15, 30, 60 and 90 minutes) 100µl samples were withdrawn from both chambers from three inserts. Promptly, these three inserts were washed with ice cold transport buffer and the cells were lysed with 300 µl transport buffer supplemented with 1 % Triton X 100 at least for 30 minutes at 37 C° in a humidified incubator. The cell lysates were carefully mixed and 100 µl samples were withdrawn for liquid scintillation counting.

For sample analysis, the samples were mixed with 500 µl of OptiPhase HiSafe scintillation cocktail (PerkinElmer Wallac) and the radioactivity was quantified using a MicroBeta liquid scintillation counter (PerkinElmer Wallac, Turku, Finland).

Kinetic modelling. The compartmental models were constructed, and data fitting and simulations were done with WinNonlin software (version 5.0.1, Pharsight Corporation, CA, USA).

In data fitting, 6 data sets (apical, basal and cellular amounts at every sampled timepoint) of corresponding test compound were simultaneously used; 3 concentrations (1, 50 and 300 µM for ibuprofen and propranolol and 0.01, 1 and 50 µM for testosterone) in both directions. All the
experiments were conducted twice in triplicate. Thus, 6 individual experimental values for each time point were used in data fitting. In the fitting procedure, various weighing schemes were tested (no weighing, 1/predicted and 1/predicted²). The weighing scheme of 1/predicted² was selected based on residual blots and estimated standard errors of parameter estimates. Other settings for minimization of the error were the default WinNonlin settings.

Model 1: Modelling the permeation and rapid cellular binding. The compartmental model published earlier (Korjamo et al., 2007) was used as the basis of the compartmental models (figure 1).

All the test compounds included in this study have relatively high transcellular permeability. Therefore, the contribution of paracellular flux of these compounds to the total transport is insignificant. Thus, paracellular space was omitted from the models.

The diffusion barrier at the apical side of the cell monolayer consists of unstirred water layer (UWL) and the cell membrane, whereas also the filter support is a part of the diffusion barrier at the basal side (figure 1). Further, the UWL is not likely to be evenly distributed to apical and basal sides because of the differences in compartment geometries (Ho et al., 1999; Korjamo et al., 2008). Additionally, the apical and basolateral membranes of epithelial cells have distinct lipid composition (Simons and van Meer, 1988) and, consequently, they may also have distinct permeation characteristics. Thus, it would be purely coincidental if the permeabilities through the apical and basal barriers of the cell monolayer (P_{api} and P_{baso}, respectively) would be equal. Therefore, it was assumed that permeabilities are equal to both directions and independent of the test compound concentration, but not necessarily equal for apical and basal barriers. Thus, P_{api} and P_{baso} were fitted separately.

The binding to the cell structures was concentration dependent (figure 2). Therefore, a simple sigmoid equation (equation 7) was employed to model the concentration dependence of the cell-buffer distribution coefficient (K). The derivation and theoretical background of the saturation
A model of cell-buffer distribution is presented in the supplemental material A. The differential equations for mass transfer in model 1 are presented in the appendix.

**Model 2: Modelling the lysosomal sequestration kinetics.** It was assumed that bafilomycin A1 affects the propranolol kinetics only by decreasing the pH gradient between cytosol and lysosomes. This is almost impossible to show experimentally, but the assumption is reasonable because the presence of bafilomycin A1 did not detectably affect the behaviour of ibuprofen or testosterone (data not shown). Therefore, bafilomycin A1 was assumed not to affect the cytosolic compartment. Further, fitting of too many parameters simultaneously results in obscure parameter estimates. Thus, the fitted permeability and cell-buffer distribution parameters ($P_{api}$, $P_{baso}$, $K_{max}$, $K_{min}$ and $EC_{50}$) were fixed to the values obtained from the data fitting of model 1 in the presence of bafilomycin A1.

Subsequently, the distribution between cytosolic and lysosomal compartments was described with $Q_{12}$ and $Q_{21}$, first order rate constants from cytosolic to lysosomal and lysosomal to cytosolic compartments, respectively. However, because of the possible concentration dependence of distribution kinetics, both, $Q_{12}$ and $Q_{21}$, were fitted separately for each concentration and direction. Schematic presentation of model 2 is shown in figure 1 and the differential equations for mass transfer in model 2 are presented in the appendix.

**Simulations.** Model 1 was used to simulate the transfer and cellular retention of virtual test compound in permeability experiment. In the simulations, $P_{api}$ and $P_{baso}$ were set to be 500 cm/s*10^{-6} and 200 cm/s*10^{-6}, respectively. The cell-buffer distribution coefficient was set to 4, 100 or 400. These values are in the range of the fitted parameter values (Tables 2 and 3).

**Estimation of permeability coefficients.** Several mathematical approaches have been suggested to estimate the apparent permeability of compounds through the cell monolayer. Typically, the cell monolayer is considered as a single barrier for solute transfer. Further, the data interpretation is usually based on assumption that the cell monolayer does not significantly retain the solute.
In addition to the experimental data, simulated data at the same time intervals than experimental data were used in permeability estimations. Because the simulated data does not include experimental variation, the variation in the permeability estimates of the simulated data is neither caused nor masked by experimental variation.

The apparent permeability through Caco-2 cell monolayer was estimated using the traditional equation derived from the Fick's first law.

\[
P_{\text{app,sink}} = \frac{M_R(t)}{A \cdot C_{D,0}} \tag{1}
\]

Where \(M_R(t)\) the solute amount in the receiver compartment at time \(t\), \(A\) is the area of the studied membrane and \(C_{D,0}\) is the initial donor concentration. This approach to estimate the permeability contains the assumption that the concentration gradient from donor to receiver compartment does not change significantly during experiment. This assumption is usually considered to hold true when so called sink conditions are maintained. Thus, this permeability estimate is referred in this report as \(P_{\text{app,sink}}\). However, the transfer rates of the solutes used in this study are relatively high and, consequently, the sink conditions are lost already at the early stage of the experiments. Therefore, the permeabilities were also estimated by iterative fitting of equation 2 (Palm et al., 1999), which does not require the sink conditions to hold true. This permeability estimate is referred in this report as \(P_{\text{app,nonsink}}\).

\[
C_{R,\text{end}} = \frac{M}{V_R + V_D} + \left(C_{R,\text{onset}} - \frac{M}{V_R + V_D}\right)e^{-P_{\text{app,nonsink}} \times \left(\frac{1}{V_R} + \frac{1}{V_D}\right) \Delta t} \tag{2}
\]

Where \(M\) is the total amount of the solute in the donor and receiver compartments at the onset of the time interval \(\Delta t\), \(C_{R,\text{end}}\) and \(C_{R,\text{onset}}\) refer to receiver concentrations at the end and at the onset of the time interval, respectively. \(V_R\) and \(V_D\) denote to receiver and donor compartment volumes, respectively.
Theoretically, the permeability is the reciprocal of the permeation resistance and the total permeation resistance of serial barriers is the sum of the individual resistances of barriers. Thus, the apparent permeability was also calculated from permeabilities through apical and basal barriers (Ho et al., 1999).

\[
P_{\text{app}} = \frac{1}{\frac{1}{P_{\text{api}}} + \frac{1}{P_{\text{baso}}}}
\]  

(3)
Results

Cellular retention during permeation experiments. Ibuprofen was not significantly retained in the cell monolayer whereas cellular retention of testosterone and propranolol was substantial (Figure 2). Further, in the presence of bafilomycin A1 the cellular retention of propranolol was significantly reduced (Figures 2A and 2B), whereas bafilomycin A1 did not affect the cellular retention of ibuprofen and testosterone (data not shown).

Although, the fraction of the test compound retained in the cell monolayer varied significantly between the test compounds, some similarities in cellular retention profiles were observed (Figure 2). Firstly, the fraction of the test compound retained in the cell monolayer was lower with the higher concentrations, resulting in apparently saturable cellular retention. Secondly, in A to B direction the test compounds accumulated into the cells fairly rapidly and after initial accumulation phase the amount retained in cells started to decline, whereas in B to A direction experiments the test compounds gradually accumulated into the cells for the whole duration of the experiments (90 minutes).

Kinetic modelling of the solute transfer and cellular retention. The transfer and cellular retention data were fitted to model 1 and, additionally, propranolol data were fitted to model 2. The fitted parameters are presented in tables 2 and 3.

Model 1 (cellular compartment modelled as a single kinetic compartment) was found to mimic the transfer and the cellular retention of ibuprofen and testosterone, and also of propranolol when bafilomycin A1 was present (Figure 3). However, the predicted cellular retention profile of propranolol without bafilomycin A1 did not follow the same profile than the observed data (Figure 4). Especially in A to B direction at 50 µM and 300 µM the amount retained into the cells did not decline as fast as model 1 predicted. Thus, the propranolol data were fitted also to model 2 which includes lysosomes as a distinct kinetic compartment. In the model 2 the cytosol-lysosome distribution rate coefficients were fitted individually for each experiment and the precision of rate
coefficient estimates was fairly poor, especially for B to A direction experiments (Table 3). However, the Q₁₂ to Q₂₁ ratios (estimated as secondary parameters in WinNonlin) could be estimated more precisely and they showed clear concentration dependency (figure 5). Further, the model 2 was better to mimic the cellular retention profile as well as disappearance from donor and appearance to receiver than model 1 (figure 4). In addition, the fitted values for Pₐₚᵢ were consistently significantly higher than the respective fitted values for Pₐ₉₀.

**Estimation of permeability coefficients.** Model 1 was used to simulate data for permeability estimation. The permeabilities estimated from simulated data using equation 1 (Pₐₚ₉ₛᵢₙ) increased initially and started to decline in the later phases (Figure 6A). Further, the Pₐₚ₉ₛᵢₙ values were lower with higher K, i.e. with higher cellular retention. However, the Pₐₚ₉ₛᵢₙ values in A to B and B to A directions at same cellular retention were identical. Further, Pₐₚ₉ₛᵢₙ was constantly lower than the theoretical Pₐₚ (calculated using equation 3) and also lower than permeabilities estimated using equation 2.

The permeabilities estimated using equation 2 (Pₐₚ₉ₙₒₙₛᵢₙ) increased initially and reached eventually a stable level (Figure 6B). However, the time to reach the stable level was longer with higher cellular retention. Pₐₚ₉ₙₒₙₛᵢₙ was consistently higher in A to B direction than in B to A direction. Further, Pₐₚ₉ₙₒₙₛᵢₙ at stable level in A to B direction was always higher and Pₐₚ₉ₙₒₙₛᵢₙ at stable level in B to A direction was always lower than the theoretical Pₐₚ. These discrepancies were higher with higher cellular retention.

Similar trends in permeability estimates were seen when data were simulated using model 2. However, the times to Pₐₚ₉ₛᵢₙ reach the maximum value and Pₐₚ₉ₙₒₙₛᵢₙ to reach the stable level were delayed when the sequestration to lysosomal compartment was slow (data not shown).

The permeability estimates using experimental data showed similar trends than permeability estimates of simulated data (Figure 7).
Discussion

This study demonstrates the errors caused by cellular retention in permeability estimates. Further, propranolol was observed to be significantly sequestered into the lysosomes during Caco-2 permeation experiments. The inhibition of lysosomal sequestration has recently been reported to increase the apparent Caco-2 permeability (estimated using equation 1) of amodiaquine (Hayeshi et al., 2008). These results suggest that the lysosomal sequestration of basic compounds may significantly contribute to the cellular retention of basic compounds and, thus, cause substantial errors in permeability estimates. However, it has to be noted that all weakly basic compounds may not be susceptible to lysosomal sequestration (Duvvuri et al 2004).

The data fittings to the model 1 suggest that cell monolayer behaves as a single kinetic compartment for testosterone and ibuprofen, whereas for propranolol also a slow intracellular compartment is needed to describe the cellular retention. However, in the presence of bafilomycin A1 a single intracellular compartment was adequate to describe also the cellular retention profile of propranolol, although it is possible that bafilomycin A1 does not neutralize the lysosomes completely (Yoshimori et al 1991).

The principal driving force to lysosomal sequestration of weak bases is pH gradient between cytosol and lysosomes (Kaufmann and Krise, 2007). However, lysosomal sequestration of bases has been reported to cause concentration dependent elevation of lysosomal pH (Poole and Ohkuma, 1981). Further, extensive accumulation of weak bases, including propranolol, causes osmotic swelling of lysosomes (Ohkuma and Poole, 1981), which may affect the surface area of the lysosomes. Thus, both the driving force and the area available for flux between cytosolic and lysosomal compartments are likely to be dynamic. Consequently, the distribution kinetics of weak bases between cytosolic and lysosomal compartments is variable, depending, among other factors, on the amount of the base in the system. Because of the complexity of the factors involved, it is not a trivial task to model rigorously the lysosomal sequestration kinetics.
Model 2 is an attempt to model the lysosomal sequestration kinetics during Caco-2 permeation experiments. Describing the flux rates between the cytosolic and lysosomal compartments with first order rate coefficients is a rough simplification of the underlying phenomena. The cellular data in this study was based on measurements of total cell lysates and, thus, we have no direct experimental knowledge of the test compounds intracellular localization, which would be needed to develop a more rigorous mechanistic model of the lysosomal sequestration kinetics. The methods to measure the intracellular localization are reviewed elsewhere (Kaufmann and Krise, 2007). However, with the currently available methodology it would be very challenging (if not impossible) to obtain rigorous intracellular localization data in permeation setting. Anyhow, two conclusions can be drawn from the current analysis. Firstly, the clear concentration dependence seen in Q_{12} to Q_{21} ratio (Figure 5), which describes the extent of lysosomal sequestration, suggests that the capacity of lysosomes to sequester propranolol is saturating within the concentration range used. Secondly, although the problems in identifying the lysosomal distribution rate coefficients, the model 2 was able to successfully mimic the total cellular retention profile of propranolol better than model 1. Also the AIC values (Table 3) favour the model 2 in describing the propranolol kinetics, suggesting that lysosomes may act as a kinetically slow intracellular compartment and the lysosomal distribution rate may be concentration dependent. Thus, the lysosomal sequestration of weak bases affects not only the extent but also the rate of cellular retention.

The cellular retention profile of test compound is dependent of the direction from where the compound enters the cell monolayer. Compartmental modelling was employed to clarify the factors causing this behaviour. The data fitting to the compartmental models suggest that the barrier at the basal side of the cell monolayer resist the flux of compounds more that the barrier at the apical side. This seems reasonable because the barrier at the apical side consists only of cell membrane and unstirred water layer, whereas the barrier at the basal side contains additionally the filter support. Further, it has been suggested that the resistance of unstirred water layer would lie predominantly at
the basal side because of the differential hydrodynamics (Ho et al., 1999). However, it has been difficult to obtain experimental evidence in favor of this (Korjamo et al., 2008).

As expected, using the equation that requires sink conditions to be fulfilled constantly underestimates the permeability because the concentrations in both compartments changes significantly during the experiments. Moreover, the estimated permeabilities lower with lower concentration due to higher cellular retention. Further, it is important to note that as the appearance kinetics to the receiver is irrespective of experimental direction and because $P_{app,\text{sink}}$ neglects the decline of donor concentration, there are no direction differences in $P_{app,\text{sink}}$ estimates at the same cellular retention level.

For high permeability compounds it is expected that $P_{app,\text{nonsink}}$ would be a better estimate of the permeability than $P_{app,\text{sink}}$, since the loss of sink conditions does not cause the $P_{app,\text{nonsink}}$ estimates to decline in the late phases. Also the actual concentration gradient at the onset of the sampling interval is taken into account. However, $P_{app,\text{nonsink}}$ underestimated the permeability in B to A direction for the whole time course whereas in A to B direction permeability is underestimated only in the very early stage and overestimated at the late stages. The errors made are higher when high amount of test compound is retained in the cells (figures 6 and 7). The appearance kinetics to the receiver is irrespective of experimental direction. Thus, this discrepancy must be caused by the difference in disappearance kinetics from the donor. The permeabilities through apical barrier are estimated to be 2 to 3 times higher than the permeabilities through basal barrier (Table 2), for the compounds used in this study. Consequently, at the apparent steady state permeation (when the $P_{app,\text{nonsink}}$ has reached the stable level), the free cellular concentration as well as amount retained in the cell monolayer follows closely the concentration of the apical compartment irrespective of the direction of the experiment. Therefore, at the apparent steady state permeation in A to B experiments the test compound appears to the receiver side faster than disappears from the donor side, whereas the opposite is seen in B to A experiments, ultimately causing the errors seen in
Further, it has to be noted that with compounds which are significantly retained in the cell monolayer the time to reach the apparent steady state permeation may be delayed significantly. Therefore, at the steady state permeation the concentrations at the donor and receiver may already be near to equilibrium and, thus, the concentration change within a sampling interval is small. Consequently, already minor experimental variation causes significant variation in \( P_{\text{app,nonsink}} \) causing the behaviour of estimated permeabilities to be erratic.

Usually in cell permeation experiments only the appearance rate is measured whereas disappearance rate is not observed in detail. Such data does not contain information about the cellular retention kinetics and, thus, it is impossible to take the cellular retention rigorously into account in permeability estimates. Anyhow, it has been suggested that poor recovery could be taken into account in \( P_{\text{app,sink}} \) estimate based on recovery calculation at a single time point, in practice at the end of the experiment (Youdim et al., 2003). However, because the cellular retention time profiles are dependent on the experimental direction (Figure 2), correcting the permeability estimates with a single time point recovery may lead into apparent directionality although the transfer of the test compound were solely due to passive diffusion.

Sampling of both receiver and donor compartments has been suggested for more rigor correction for poor recovery (Tran et al. 2004). Similarly, the \( P_{\text{app,nonsink}} \) approach uses the actual concentration gradient at each sampling time. However, both of these single barrier models seems to show systematic errors when significant cellular retention is involved (see supplemental material B for further analysis of the approach proposed by Tran et al. 2004).

Often in cell monolayer permeation studies the conclusions are drawn based on the assumption that the cell monolayer is a single barrier which does not significantly retain the compound studied. This approach has been shown to be applicable for screening for transporter interactions (Polli et al., 2001) and prediction of intestinal absorption of passively absorbed compounds (Artursson et al., 2001). However, it has been suggested that as good predictions of intestinal absorption of passively
absorbed compounds can be obtained even by computational methods (Linnankoski et al., 2008). The use of the cell monolayer permeability studies (alongside protein binding and intrinsic clearance measurements) to predict the in vivo pharmacokinetics have been justified with the presence of relevant transfer routes, which are absent in artificial membranes and are not necessarily taken into account in computational approaches, such as paracellular space and active transporters (Artursson et al., 2001). However, the relative role of different transfer routes in in vitro cell models tend to differ from the in vivo setting. Therefore, successful scaling from in vitro data to in vivo setting is likely to require detailed mechanistic insight. The data shown in this report suggests that considering the cell monolayer as a single barrier is in some cases an over simplified view even when studying passive transfer of the solutes through the cell monolayer. Further, flaws of applying single barrier view in mechanistic studies, such as studies to elucidate active transporter function, have recently been reported (Bentz et al., 2005; Korjamo et al., 2007; Sun and Pang, 2008). Thus, it seems evident that more sophisticated data analysis and interpretation approaches than traditional single barrier view should be employed when detailed mechanistic insight of solute transfer is to be obtained.
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References


Footnotes

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Legends for figures

Figure 1. Schematic presentation of models 1 (a) and 2 (b) describing the mass transfer in Caco-2 cell monolayer permeation experiments. The differential equations describing the mass transfer are presented in the appendix. The permeabilities through apical (P_{api}) and basal (P_{baso}) barriers consist of the total permeability through unstirred water layer, filter support (only basal side) and cell membrane. Q_{12} and Q_{21} are the cytosol to lysosome and lysosome to cytosol distribution rate coefficients, respectively.

Figure 2. The fraction of initial donor amount of (A) propranolol, (B) propranolol in the presence of 100 nM bafilomycin A1, (C) ibuprofen and (D) testosterone retained in the cell monolayer during Caco-2 permeation experiments (note the different scales of the vertical axes). Closed and open symbols represent the apical to basal and basal to apical direction experiments, respectively. Triangles, diamonds and squares denote to low, intermediate and high concentration, respectively. The symbols represent the average of six measurements. The error bars are omitted for clarity, but the coefficient of variation was generally 10% or less.

Figure 3. The time course of 1 µM (A, B) propranolol, (C, D) propranolol in the presence of 100 nM bafilomycin A1, (E, F) ibuprofen and (G, H) testosterone transfer in Caco-2 permeation experiments. The symbols (solid squares and triangles for apical and basal compartments, respectively, and open squares for cell monolayer) represent the average of 6 measurements (± standard deviation) and the solid and dashed lines represent the fitted results of model 1 (C to H) and model 2 (A and B). The fitted curves mimic the observed data equally well also with the other concentrations used (data not shown).
Figure 4. The time course of cellular retention of (A, B) 1 µM, (C, D) 50 µM and (E, F) 300 µM propranolol in Caco-2 permeation experiments (note the different scales of the vertical axes). The symbols represent the average of 6 measurements (± standard deviation). The thin and thick lines represent the fitted results of model 1 and model 2, respectively. The dashed and dotted lines represent the predicted time course of propranolol in cytosolic and lysosomal compartments in model 2, respectively.

Figure 5. The concentration dependence of lysosomal sequestration of propranolol. The closed and open symbols refer to the estimated ratio of flux rates between cytosolic and lysosomal compartments (± standard error) in A to B and B to A direction experiments, respectively.

Figure 6. The apparent permeabilities estimated from the simulated data using model 1 in A to B direction (closed symbols, solid line) and B to A direction (open symbols, dash line). $P_{app,sink}$ was estimated using equation 1 (A) and $P_{app,nonsink}$ was estimated using equation 2 (B). Grey dash line refers to the theoretical $P_{app}$ calculated from $P_{api}$ and $P_{baso}$ using equation 3. In the simulation $P_{api}$ and $P_{baso}$ were set to 500 and 200 cm/s*10^{-6}, respectively and K was set to 4 (squares), 100 (diamonds) or 400 (triangles). There were no direction difference in $P_{app,sink}$ at the same cellular retention, thus, only A to B direction is shown.

Figure 7. The apparent permeabilities of (A) 50 µM propranolol, (B) 50 µM propranolol in the presence of 100 nM bafilomycin A1, (C) 50 µM ibuprofen and (D) 1 µM testosterone estimated from the experimental data in A to B direction (closed symbols, solid line) and B to A direction (open symbols, dash line). $P_{app,sink}$ (triangles) was estimated using equation 1 and $P_{app,nonsink}$ (squares) was estimated using equation 2. The symbols represent the average.
permeabilities and the error bars are omitted for clarity. At late time points, the testosterone (D) concentrations at the donor and receiver side are close to equilibrium. Consequently, the experimental errors cause significant variation in $P_{\text{app,nonsink}}$. Therefore, $P_{\text{app,nonsink}}$ is presented only up to 60 minutes for testosterone. Grey dashed line refers to the theoretical $P_{\text{app}}$ calculated from the fitted values of $P_{\text{ap}}$ and $P_{\text{baso}}$ (table 2) using equation 3.
Table 1. Physicochemical properties of the model compounds used.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa&lt;sup&gt;a&lt;/sup&gt;</th>
<th>logD at pH 7.4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>logD at 5.5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>logD at 5.0&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>9.1&lt;sub&gt;basic&lt;/sub&gt;</td>
<td>1.4</td>
<td>0.11</td>
<td>0.03</td>
<td>259</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>4.4&lt;sub&gt;acidic&lt;/sub&gt;</td>
<td>0.8</td>
<td>2.6</td>
<td>3.0</td>
<td>206</td>
</tr>
<tr>
<td>Testosterone</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>288</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values were calculated by the ACDLABS ACD/pKa/LogD program (version 6.0)

<sup>b</sup>Testosterone is a neutral compound
Table 2. The fitted parameters (±estimated standard errors) for propranolol in the presence of 100 nM bafilomycin A1, ibuprofen and testosterone in model 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Propranolol + bafilomycin A1</th>
<th>Ibuprofen</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{api}$ (cm/s*10^{-6})</td>
<td>530 ±8</td>
<td>406 ±16</td>
<td>523 ±12</td>
</tr>
<tr>
<td>$P_{baso}$ (cm/s*10^{-6})</td>
<td>200 ±2</td>
<td>183 ±4</td>
<td>241 ±3</td>
</tr>
<tr>
<td>$P_{app}$ (cm/s*10^{-6})$^a$</td>
<td>145 ±1</td>
<td>126 ±2</td>
<td>165 ±1</td>
</tr>
<tr>
<td>$K_{max}$ (dimensionless)</td>
<td>100 ±1</td>
<td>20 ±1</td>
<td>109 ±1</td>
</tr>
<tr>
<td>$K_{min}$ (dimensionless)</td>
<td>44 ±1</td>
<td>6.2 ±0.2</td>
<td>30 ±1</td>
</tr>
<tr>
<td>EC50 (µM)</td>
<td>11 ±1</td>
<td>2.4 ±0.5</td>
<td>0.8 ±0.1</td>
</tr>
</tbody>
</table>

$^a$ Apparent permeability was estimated as a secondary parameter in WinNonlin, $P_{app}=1/(1/P_a+1/P_b)$
Table 3. The fitted parameters (±estimated standard errors) for propranolol in model 1 and model 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>P^n (cm/s*10^-6)</td>
<td>598 ± 18</td>
<td>530^b</td>
</tr>
<tr>
<td>P^b (cm/s*10^-6)</td>
<td>200 ± 3</td>
<td>200^b</td>
</tr>
<tr>
<td>P^app (cm/s*10^-6)^a</td>
<td>150 ± 2</td>
<td>145^b</td>
</tr>
<tr>
<td>K_max (dimensionless)</td>
<td>414 ± 14</td>
<td>100^b</td>
</tr>
<tr>
<td>K_min (dimensionless)</td>
<td>70 ± 2</td>
<td>44^b</td>
</tr>
<tr>
<td>EC50 (µM)</td>
<td>1.8 ± 0.2</td>
<td>11^b</td>
</tr>
<tr>
<td>Q12, 1 µM AB (1/s*10^-2)</td>
<td></td>
<td>300 ± 40</td>
</tr>
<tr>
<td>Q21, 1 µM AB (1/s*10^-2)</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Q12, 50 µM AB (1/s*10^-2)</td>
<td>140 ± 70</td>
<td></td>
</tr>
<tr>
<td>Q21, 50 µM AB (1/s*10^-2)</td>
<td>2.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Q12, 300 µM AB (1/s*10^-2)</td>
<td>2.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Q21, 300 µM AB (1/s*10^-2)</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Q12, 1 µM BA (1/s*10^-2)</td>
<td>300 ± 30</td>
<td></td>
</tr>
<tr>
<td>Q21, 1 µM BA (1/s*10^-2)</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Q12, 50 µM BA (1/s*10^-2)</td>
<td>320 ± 390</td>
<td></td>
</tr>
<tr>
<td>Q21, 50 µM BA (1/s*10^-2)</td>
<td>6 ± 7</td>
<td></td>
</tr>
<tr>
<td>Q12, 300 µM BA (1/s*10^-2)</td>
<td>50 ± 30</td>
<td></td>
</tr>
<tr>
<td>Q21, 300 µM BA (1/s*10^-2)</td>
<td>2 ± 1</td>
<td></td>
</tr>
<tr>
<td>AIC^c</td>
<td>1307</td>
<td>1056</td>
</tr>
</tbody>
</table>

^a Apparent permeability was estimated as a secondary parameter in WinNonlin, \( P_{app}=1/(1/P_a+1/P_b) \)

^b Values taken from Model 1 in the presence of 100 nM bafilomycin A1 (Table 2)

^c Akaike information criterion
Figure 1
Figure 2A
Figure 2B
Figure 2C

The graph shows the fraction of initial donor retained in the cell monolayer over time for different concentrations of AB (1 µM, 50 µM, 300 µM) and BA (1 µM, 50 µM, 300 µM). The x-axis represents time in minutes, while the y-axis represents the fraction of the initial donor retained. The data indicates a decrease in the fraction retained as time progresses, with different slopes for each concentration level.
Figure 2D

The graph shows the fraction of initial donor retained in the cell monolayer over time for different concentrations of AB (0.01 µM, 1 µM, 50 µM) and BA (0.01 µM, 1 µM, 50 µM). The x-axis represents time in minutes, ranging from 0 to 100, and the y-axis represents the fraction of initial donor retained, ranging from 0% to 25%. The data points are plotted for each concentration, with distinct symbols and line styles for each condition.
Figure 3A
Figure 3B

Basal to apical

Propranolol (pmol)

Time (min)
Figure 3C
Figure 3D
Figure 3E
Figure 3F
Figure 3G
Figure 3H
Figure 4A
Figure 4B
Figure 4C

C

Apical to basal

Propranolol (pmol)

Time (min)

0 10 20 30 40 50 60 70 80 90

0 2000 4000 6000 8000

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Figure 4D
Figure 4E
Figure 4F
Figure 5
Figure 6A
Figure 6B
Figure 7A
Figure 7B
Figure 7C
Figure 7D
Appendix

Differential equations for mass transfer in model 1

Apical compartment

\[
\frac{dM_{\text{api}}}{dt} = -P_{\text{api}} A \left( \frac{M_{\text{api}}}{V_{\text{api}}} - \frac{M_{\text{cyto}}}{V_{\text{cell}} K} \right)
\]  (4)

Basal compartment

\[
\frac{dM_{\text{baso}}}{dt} = P_{\text{baso}} A \left( \frac{M_{\text{cyto}}}{V_{\text{cell}} K} - \frac{M_{\text{baso}}}{V_{\text{api}}} \right)
\]  (5)

Cellular "cytosolic" compartment

\[
\frac{dM_{\text{cyto}}}{dt} = P_{\text{api}} A \left( \frac{M_{\text{api}}}{V_{\text{api}}} - \frac{M_{\text{cyto}}}{V_{\text{cell}} K} \right) - P_{\text{baso}} A \left( \frac{M_{\text{cyto}}}{V_{\text{cell}} K} - \frac{M_{\text{baso}}}{V_{\text{api}}} \right)
\]  (6)

where apparent cell-buffer distribution coefficient K is defined as

\[
K = K_{\text{max}} - (K_{\text{max}} - K_{\text{min}}) \frac{M_{\text{cyto}}}{V_{\text{cell}} K + EC_{50}}.
\]  (7)

Differential equations for mass transfer in model 2. Mass transfer in apical and basal compartments was described identically in models 1 and 2. Thus, equations 4 and 5 describe the mass transfer in apical and basal compartments, respectively, in model 2.

Cellular "cytosolic" compartment:

\[
\frac{dM_{\text{cyto}}}{dt} = P_{\text{api}} A \left( \frac{M_{\text{api}}}{V_{\text{api}}} - \frac{M_{\text{cyto}}}{V_{\text{cell}} K} \right) - P_{\text{baso}} A \left( \frac{M_{\text{cyto}}}{V_{\text{cell}} K} - \frac{M_{\text{baso}}}{V_{\text{api}}} \right) - Q_{12} \frac{M_{\text{cyto}}}{K} + Q_{21} M_{\text{lyso}}
\]  (8)

where apparent cell-buffer distribution coefficient K is defined by equation 7.

Cellular "lysosomal" compartment:

\[
\frac{dM_{\text{lyso}}}{dt} = Q_{12} \frac{M_{\text{cyto}}}{K} - Q_{21} M_{\text{lyso}}
\]  (9)
Supplemental material A for

Kinetics of cellular retention during Caco-2 permeation experiments: Role of lysosomal sequestration and impact on permeability estimates

Aki T. Heikkinen, Jukka Mönkkönen and Timo Korjamo

Development and derivation of the saturation model of cellular retention

In Caco-2 permeation experiments the fraction of model compounds (testosterone, ibuprofen and propranolol) retained in the cell monolayer decreased with higher donor compartments, i.e. the cellular retention of these compounds was apparently saturable.

To mathematically derive the saturation model of the cellular retention the apparent cell-buffer distribution coefficient $K$ is first defined as

$$K = \frac{M_{cyto}}{M_{cyto,free}},$$  \hspace{1cm} (SA1)

where $M_{cyto}$ and $M_{cyto,free}$ are the total and free amount in cytosolic compartments, respectively.

Subsequently, using the cellular volume $V_{cell}$ the free cytosolic concentration $C_{cyto,free}$ is defined as

$$C_{cyto,free} = \frac{M_{cyto,free}}{V_{cell}} = \frac{M_{cyto}}{V_{cell} K}.$$  \hspace{1cm} (SA2)

The apparent cellular binding is assumed to consist of unsaturable (e.g. distribution into lipids) and saturable (e.g. specific protein binding) components. More than one saturable binding site may be involved. However, it would require much more experimental data at various concentrations to identify them. Therefore, saturable binding is assumed to follow one site binding. Thus, total cytosolic amount is defined as

$$M_{cyto} = K_0 * M_{cyto,free} + B_{max} \frac{C_{cyto,free}}{C_{cyto,free} + EC_{50}},$$  \hspace{1cm} (SA3)
where $K_0$ is the distribution coefficient representing the unsaturable binding, $B_{\text{max}}$ is the maximum amount bound to the saturable component and $EC_{50}$ is the free cytosolic concentration needed to for half maximum binding to the saturable component.

By combining the equations SA2 and SA3, we obtain the equation for saturation of $K$

$$K = K_{\text{nonspecific}} + V_{cell} \frac{B_{\text{max}}}{C_{\text{cyto,free}} + EC_{50}}.$$ (SA4)

However, because it is not known in detail what are these unsaturable and saturable components, we decided to represent the apparent saturation of cellular binding in terms of $K_{\text{max}}$ and $K_{\text{min}}$, apparent higher and lower limits for $K$, respectively.

With low concentrations ($C_{\text{cell,free}}$ approaches to zero) $K$ approaches to $K_{\text{max}}$

$$K_{\text{max}} = K_{\text{nonspecific}} + V_{cell} \frac{B_{\text{max}}}{EC_{50}}.$$ (SA5)

whereas with high concentrations $K$ approaches to $K_{\text{min}}$

$$K_{\text{min}} = K_{\text{nonspecific}}.$$ (SA6)

The equation used in modelling to describe the apparent saturation of cellular binding is obtained by combining the equations SA4, SA5 and SA6.

$$K = K_{\text{max}} - (K_{\text{max}} - K_{\text{min}}) \frac{M_{\text{cyto}} / V_{cell} K}{M_{\text{cyto}} / V_{cell} K + EC_{50}}.$$ (SA7)

In the preliminary fitting round of the model 1, the cell-buffer distribution coefficient ($K$) was individually fitted for each data set. Subsequently, the fitted $K$'s were blotted against the average intracellular free solute concentration to see the trend of concentration dependency (Figure SA1). This blot was used to estimate the initial values for $K_{\text{max}}$, $K_{\text{min}}$ and $EC_{50}$. Further, the concentration dependency of the apparent cell-buffer distribution coefficient predicted by the saturation model was blotted in the same figure with the individually fitted $K$ to visualize the appropriateness of the saturation model (Figure SA1).
**Figure SA1.** The concentration dependence of cellular retention of (A) propranolol in the presence of 100 nM bafilomycin A, (B) ibuprofen and (C) testosterone. The symbols (closed for apical to basal and open for basal to apical experiments) represent the individually estimated apparent cell-buffer distribution coefficients (± estimated standard error) from preliminary data fitting of model 1 and the solid line is the fitted curve of the saturation model.
Supplemental material B for

Kinetics of cellular retention during Caco-2 permeation experiments: Role of lysosomal sequestration and impact on permeability estimates

Aki T. Heikkinen, Jukka Mönkkönen and Timo Korjamo

Evaluation of single barrier approach to estimate permeability through cell monolayer proposed Tran et al. (2004)

Tran et al (2004) have suggested an approach for exact kinetic analysis of passive transport across cell monolayer modelled as a single barrier. This approach incorporates the measured recovery at each sampling time and it was suggested that this kind of analysis would correct for possible problems with poor recovery. Additionally, the loss of sink conditions is taken into account. The performance of this approach was evaluated using the experimental and simulated data presented in the actual article.

This novel approach to estimate permeability proposed by Tran et al. (2004) uses the difference of the "average system concentration" ($<C>$) and the receiver concentration as the thermodynamic force term for transfer and requires the measurement of both donor and receiver concentrations. It was suggested that the apparent loss of the compound from the buffer compartments could be described by a first order rate coefficient (additionally the possibility to use some more complex mathematics for this purpose were speculated). However, as Tran et al. themselves state, this first order rate coefficient has been used in the derivation of the permeability equation and it is not necessary to fit rate coefficient for compound loss to use the permeability equation. Thus, $<C>$ does not need to be 'predicted' by using a rate coefficient because $<C>$ at each sampling time can be directly calculated from the donor and receiver concentrations (for derivation of the equations shown, see the original article by Tran et al., 2004)
\[
\langle C(t) \rangle = \frac{V_d C_d(t) + V_r C_r(t)}{V_d + V_r}
\]  
(SB1)

Where \( V \) and \( C(t) \) refers to volume and concentration at time \( t \), respectively, and the sub indexes \( d \) and \( r \) refer to the donor and receiver compartments, respectively. Using the difference of the "average system concentration" \( \langle C \rangle \) and the receiver concentration as the thermodynamic force term for transfer the permeability through a single barrier can be defined as

\[
P = -\left( \frac{V_r V_d}{(V_r V_d) A t} \right) \ln \left\{ 1 - \frac{C_r(t)}{\langle C(t) \rangle} \right\}
\]  
(SB2)

where \( A \) is the cross sectional area of the cell monolayer. This would estimate the permeability within time interval from 0 to \( t \). In practice, in the report by Tran et al. (2004) the permeabilities were calculated for each sampling interval separately. In this case the permeability equation is reformed to

\[
P = -\left( \frac{V_r V_d}{(V_r V_d) A (t_{end} - t_{onset})} \right) \ln \left\{ \frac{1 - C_r(t)_{end}}{\langle C(t) \rangle_{end}} \right\} \left\{ 1 - \frac{C_r(t)_{onset}}{\langle C(t) \rangle_{onset}} \right\}
\]  
(SB3)

where the sub indexes \( end \) and \( onset \) refer to the end and onset of the sampling interval, respectively.

The permeabilities for each sampling interval were calculated from experimental and simulated data using equation SB3. The representative results are shown in figure SB1. When there is no significant cellular retention involved (ibuprofen and simulation \( K=4 \)), this approach works perfectly in estimating the passive permeability. However, when significant cellular retention is involved (propranolol, testosterone and simulations \( K=100 \) and \( K=400 \)), this approach seems to encounter similar type of errors than another single barrier approach \( P_{app,nonsink} \). More the compound is retained in the cells larger is the apparent direction dependence of permeability.

The approach suggested by Tran et al. is based on assumption of a single barrier and the amount of compound lost (due to any mechanism) from the solutions is assumed to 'vanish' from the system.
and, thus, assumed not to contribute to the transfer by any means. If this assumption would strictly hold true, it would mean that if the solution at the both apical and basal compartments would be replaced with fresh buffer, none of the compound retained in the cells would flux out from the cells. There are several examples in the literature showing that this is not usually true if metabolism or other degradation of the compound is not involved (see Ho et al 1999 for example). When the amount of compound retained in the cells is affecting the transfer, the cellular 'volume of distribution' and the amount of compound in the cell monolayer should be taken into account to obtain correct \( <C> \). However, taking the cellular compartment into account in \( <C> \) calculation would not be applicable to single barrier model the rest of the calculations are based on. Taken together, this provides further support to the main conclusion that considering cell monolayer as a single barrier is in some cases an over simplified view when studying transfer through cell monolayer.

References


Figure SB1. The estimated permeabilities of 50 μM propranolol (A), 50 μM ibuprofen (B), 1 μM Testosterone (C) and simulated data using model 1 (D) in A to B direction (closed symbols, solid line) and B to A direction (open symbols, dash line). Grey dash line refers to the theoretical $P_{\text{app}}$ calculated from $P_{\text{api}}$ and $P_{\text{baso}}$. In the simulations $P_{\text{api}}$ and $P_{\text{baso}}$ were set to 500 and 200 cm/s*10^{-6}, respectively and $K$ was set to 4 (squares), 100 (diamonds) or 400 (triangles).