

Kinetics of Cellular Retention during Caco-2 Permeation Experiments: Role of Lysosomal Sequestration and Impact on Permeability Estimates^[S]

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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 [(±)-1-isopropylamino-3-(1-naphthoxy)-2-propanol], weakly acidic ibuprofen [α -methyl-4-(isobutyl)phenylacetic acid], and neutral testosterone (17 β -hydroxy-4-androsten-3-

one), were determined. Furthermore, 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. Furthermore, the lysosomal sequestration was demonstrated to contribute to the extent and kinetics of the cellular retention of propranolol.

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).

In in vitro permeation experiments, typically, the permeability estimates are based on the appearance kinetics of the test compound to the receiver compartment. In addition, samples from both donor and receiver sides are often col-

lected 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); furthermore, 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 cellular retention of drug (Fisher et al., 1999; Östh et al., 2002; Palmgrén 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

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ABBREVIATIONS: A to B, apical to basal direction; B to A, basal to apical direction; 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); K , apparent cell-buffer distribution coefficient; K_{max} and K_{min} , higher and lower limits for apparent cell-buffer distribution coefficient, respectively; Q_{12} and Q_{21} , first-order rate coefficients for mass transfer from cytosol to lysosome and lysosome to cytosol, respectively; $P_{app,sink}$, estimated apparent permeability through cell monolayer assuming sink conditions; $P_{app,nonsink}$, estimated apparent permeability through cell monolayer without sink condition assumption; P_{app} , apparent permeability through cell monolayer.

recovery in permeation experiments cannot be applied. However, in some reported studies, the poor recovery has 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 the initial "cell loading" period in the beginning of the experiment (Tran et al., 2004; Korjamo et al., 2008).

In Caco-2 cells, drug metabolism is limited due to low expression levels of cytochrome P450-metabolizing enzymes (Prueksaritanont et al., 1996; Engman et al., 2001; Korjamo et al., 2006). Furthermore, 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 (Palmgrén et al., 2006). Therefore, retention into the cellular structures is likely to be the primary 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 electrostatic binding to the charged structures of the cell. Furthermore, pH gradient-driven sequestration of the ionizable 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 that 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 pK_a are sequestered into the acidic organelles, provided that the membrane permeability of the ionized and unionized forms differs substantially (Duvvuri et al., 2004). Furthermore, lysosomes are the most acidic organelles in the cells; 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, the term "lysosome" also is used to cover the other acidic vesicles than lysosomes.

In this study, we have explored and modeled the cellular retention kinetics of basic propranolol, acidic ibuprofen, and neutral testosterone during Caco-2 permeation experiments. Furthermore, we studied the role of lysosomal sequestration in cellular retention of propranolol. In addition, the effects of cellular retention kinetics on estimates of apparent permeability through the Caco-2 cell monolayer were evaluated.

Materials and Methods

Chemicals. Propranolol and ibuprofen were obtained from Sigma-Aldrich (St. Louis, MO), and testosterone was from Fluka (Buchs, Switzerland). [^3H]Ibuprofen and [^3H]testosterone were from American Radiolabeled Chemicals (St. Louis, MO), and [^3H]propranolol was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Bafilomycin A1 was from LC Laboratories (Woburn, MA). Fetal bovine serum was obtained from EuroClone Ltd. UK (Wetherby, West York, UK), and other cell culture reagents were from Lonza Verviers SPRL (Verviers, Belgium).

Cell Culture. Caco-2 cells (HTB-37; American Type Culture Collection, Manassas, VA) were cultured and grown for experiments as earlier described (Korjamo et al., 2005). In brief, 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, Acton, MA) and were grown for 21 to 24 days before 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 and 1.5 ml of transport buffer (Hanks' balanced salt solution buffered with HEPES, 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 min at 37°C in a humidified incubator. The integrity of the monolayers was checked by transepithelial electrical resistance measurement, and only monolayers with resistance higher than 300 Ωcm^2 were accepted for the experiments.

The donor solutions were spiked with the respective ^3H -labeled compound. The initial donor concentrations were 1, 50, and 300 μM for propranolol and ibuprofen and ~ 10 nM (blank transport buffer spiked with [^3H]testosterone) and 1 and 50 μM for testosterone. The stock solutions of unlabeled compounds were prepared in dimethyl sulfoxide, and the dimethyl sulfoxide 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 min), 100- μl samples were withdrawn from both chambers from three inserts. These three inserts were washed promptly with ice-cold transport buffer, and the cells were lysed with 300 μl of transport buffer supplemented with 1% Triton X-100 at least for 30 min at 37°C in a humidified incubator. The cell lysates were carefully

TABLE 1
Physicochemical properties of the model compounds used

Compound	pK_a^a	Log D at pH 7.4 ^a	Log D at 5.5 ^a	Log D at 5.0 ^a	Mol. Wt.
Propranolol	9.1 _{basic}	1.4	0.11	0.03	259
Ibuprofen	4.4 _{acidic}	0.8	2.6	3.0	206
Testosterone	N.A. ^b	3.5	3.5	3.5	288

^a Values were calculated by the ACDLABS ACD/ pK_a /log D program (version 6.0).

^b N.A., not available. Testosterone is a neutral compound.

mixed, and 100- μ l samples were withdrawn for liquid scintillation counting. For sample analysis, the samples were mixed with 500 μ l of OptiPhase HiSafe scintillation cocktail (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland), and the radioactivity was quantified using a MicroBeta liquid scintillation counter (PerkinElmer Life and Analytical Sciences-Wallac Oy).

Kinetic Modeling. The compartmental models were constructed, and data fitting and simulations were done with WinNonlin software (version 5.0.1; Pharsight, Mountain View, CA). In data fitting, six data sets (apical, basal, and cellular amounts at every sampled time point) of corresponding test compound were simultaneously used; three concentrations (1, 50, and 300 μ M for ibuprofen and propranolol and 0.01, 1, and 50 μ M for testosterone) in both directions. All the experiments were conducted twice in triplicate. Thus, six individual experimental values for each time point were used in data fitting. In the fitting procedure, various weighting schemes were tested (no weighting, 1/predicted, and 1/predicted²). The weighting scheme of 1/predicted² was selected based on residual blots and estimated S.E. of parameter estimates. Other settings for minimization of the error were the default WinNonlin settings.

Model 1: Modeling the Permeation and Rapid Cellular Binding. The compartmental model published earlier (Korjamo et al., 2007) was used as the basis of the compartmental models (Fig. 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 and the cell membrane, whereas the filter support is an additional part of the diffusion barrier at the basal side (Fig. 1). Furthermore, the unstirred water layer is not likely to be evenly distributed to the apical and basal sides because of the differences in compartment geometries (Ho et al., 1999; Kor-

jamo et al., 2008). In addition, the apical and basolateral membranes of epithelial cells have distinct lipid composition (Simons and van Meer, 1988); as a consequence, 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 (Fig. 2). Therefore, a simple sigmoid equation (eq. 7) was employed to model the concentration dependence of the cell-buffer distribution coefficient (K). The derivation and theoretical background of the saturation model of cell-buffer distribution are presented in Supplemental Material A. The differential equations for mass transfer in model 1 are presented under *Appendix*.

Model 2: Modeling 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 behavior of ibuprofen or testosterone (data not shown). Therefore, bafilomycin A1 was assumed not to affect the cytosolic compartment. Furthermore, 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.

The distribution between cytosolic and lysosomal compartments was described subsequently 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 concentra-

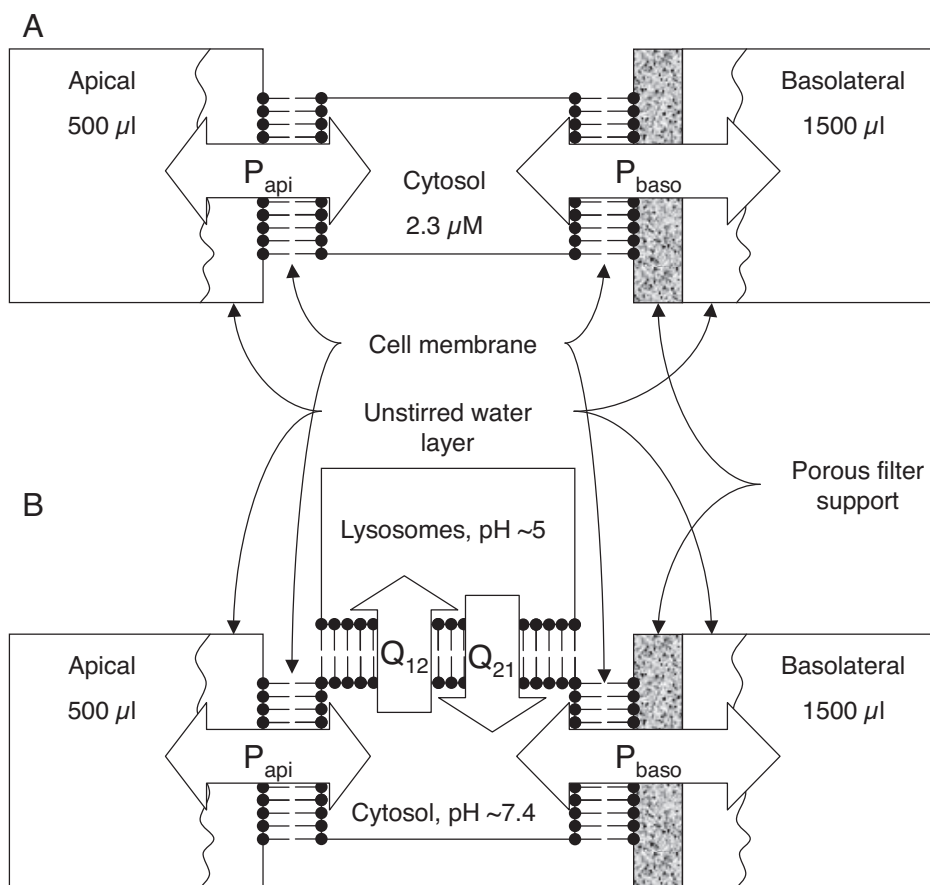


Fig. 1. Schematic presentation of models 1 (A) and 2 (B) describing the mass transfer in Caco-2 cell monolayer permeation experiments. The differential equations describing the mass transfer are presented under *Appendix*. The permeabilities through apical (P_{api}) and basal (P_{baso}) barriers consist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

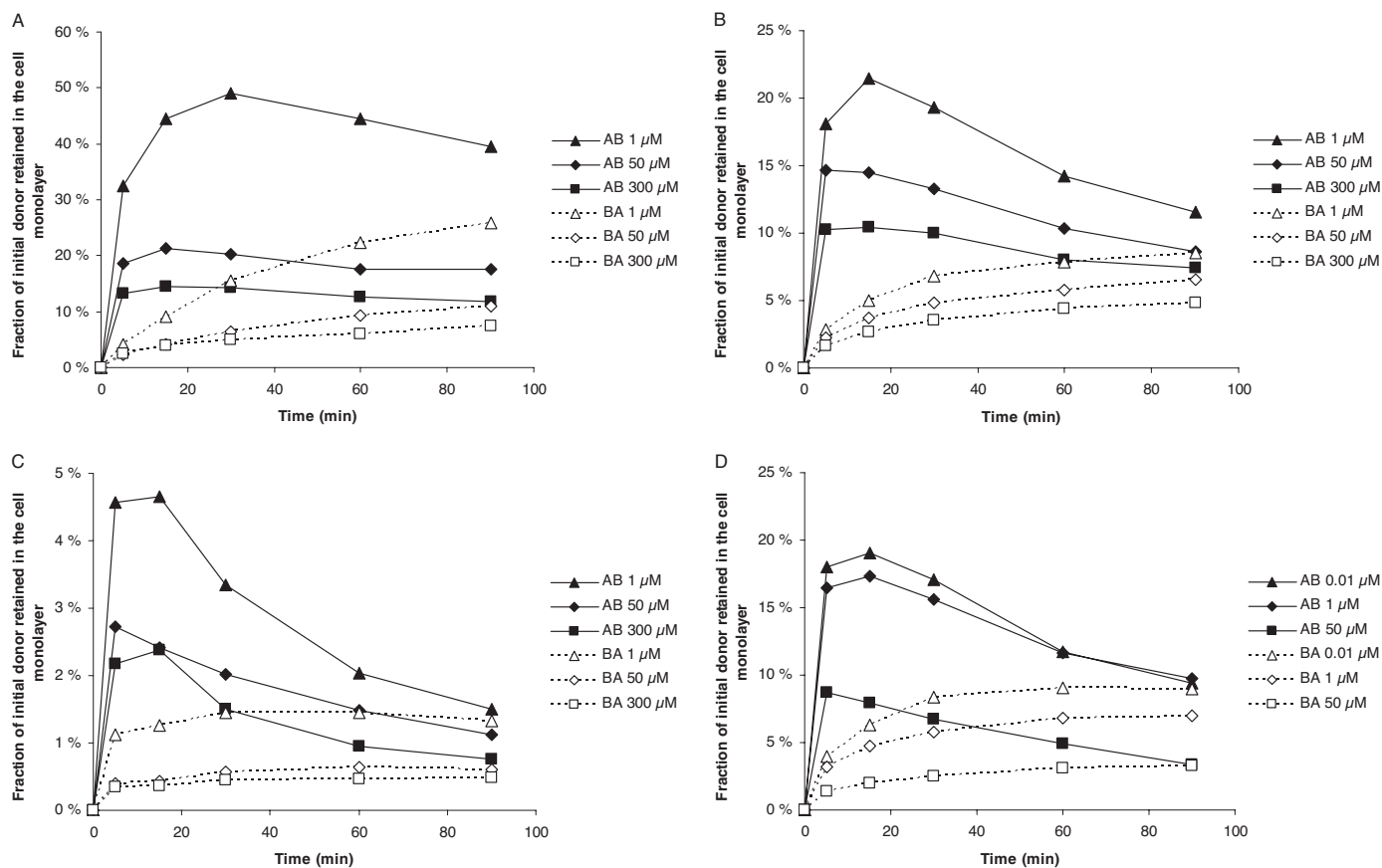


Fig. 2. The fraction of initial donor amount of propranolol (A), propranolol in the presence of 100 nM bafilomycin A1 (B), ibuprofen (C), and testosterone (D) retained in the cell monolayer during Caco-2 permeation experiments (note the different scales of the vertical axes). Closed and open symbols, apical to basal and basal to apical direction experiments, respectively. Triangles, diamonds, and squares, 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.

tion 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 Fig. 1, and the differential equations for mass transfer in model 2 are presented under 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 and 200 $\text{cm/s} \times 10^{-6}$, 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. The cell monolayer typically is considered as a single barrier for solute transfer. Furthermore, the data interpretation is usually based on the 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 do not include experimental variation, the variations in the permeability estimates of the simulated data are neither caused nor masked by experimental variation.

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

$$P_{app,sink} = \frac{M_R(t)/t}{A \cdot C_{D,0}} \quad (1)$$

where $M_R(t)$ is the solute amount in the receiver compartment at time t , A is the area of the studied membrane (area of the cell monolayer, 113.1 mm^2), 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 the experiment. This assumption is usually considered to hold true when so-called sink conditions

TABLE 2

The fitted parameters ($\pm$ estimated S.E.) for propranolol in the presence of 100 nM bafilomycin A1, ibuprofen, and testosterone in model 1

Compound	Propranolol + Bafilomycin A1	Ibuprofen	Testosterone
P_{api} ($\text{cm/s} \times 10^{-6}$)	530 ± 8	406 ± 16	523 ± 12
P_{baso} ($\text{cm/s} \times 10^{-6}$)	200 ± 2	183 ± 4	241 ± 3
P_{app} ($\text{cm/s} \times 10^{-6}$) ^a	145 ± 1	126 ± 2	165 ± 1
K_{max} (dimensionless)	100 ± 1	20 ± 1	109 ± 1
K_{min} (dimensionless)	44 ± 1	6.2 ± 0.2	30 ± 1
EC_{50} (μM)	11 ± 1	2.4 ± 0.5	0.8 ± 0.1

^a Apparent permeability was estimated as a secondary parameter in WinNonlin, $P_{app} = 1/(1/P_{api} + 1/P_{baso})$.

TABLE 3

The fitted parameters ($\pm$ estimated S.E.) for propranolol in models 1 and 2

	Model 1	Model 2
P_{api} (cm/s $\times 10^{-6}$)	598 ± 18	530^b
P_{baso} (cm/s $\times 10^{-6}$)	200 ± 3	200^b
P_{app} (cm/s $\times 10^{-6}$) ^a	150 ± 2	145^b
K_{max} (dimensionless)	414 ± 14	100^b
K_{min} (dimensionless)	70 ± 2	44^b
EC ₅₀ (μ M)	1.8 ± 0.2	11^b
Q_{12} , 1 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	300 ± 40
Q_{21} , 1 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	1.0 ± 0.2
Q_{12} , 50 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	140 ± 70
Q_{21} , 50 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	2.6 ± 1.4
Q_{12} , 300 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	2.1 ± 0.3
Q_{21} , 300 μ M AB (1/s $\times 10^{-2}$)	N.A. ^c	0.06 ± 0.01
Q_{12} , 1 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	300 ± 30
Q_{21} , 1 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	0.8 ± 0.1
Q_{12} , 50 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	320 ± 390
Q_{21} , 50 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	6 ± 7
Q_{12} , 300 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	50 ± 30
Q_{21} , 300 μ M BA (1/s $\times 10^{-2}$)	N.A. ^c	2 ± 1
AIC ^c	1307	1056

^a Apparent permeability was estimated as a secondary parameter in WinNonlin, $P_{app} = 1/(1/P_{api} + 1/P_{baso})$.

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

^c Akaike information criterion. N.A., not applicable.

are maintained. Thus, this permeability estimate is referred to in this report as $P_{app,sink}$. However, the transfer rates of the solutes used in this study are relatively high; as a consequence, the sink conditions are lost already at the early stage of the experiments. Therefore, the permeabilities were also estimated by iterative fitting of eq. 2 (Palm et al., 1999), which does not require the sink conditions to hold true. This permeability estimate is referred to in this report as $P_{app,nonsink}$.

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

where M is the total amount of the solute in the donor and receiver compartments at the onset of the time interval Δt , and $C_{R,end}$ and $C_{R,onset}$ refer to receiver concentrations at the end and at the onset of the time interval, respectively. V_R and V_D are 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).

$$P_{app} = \frac{1}{1/P_{api} + 1/P_{baso}} \quad (3)$$

Results

Cellular Retention during Permeation Experiments.

Ibuprofen was not significantly retained in the cell monolayer, whereas cellular retention of testosterone and propranolol was substantial (Fig. 2). Furthermore, in the presence of bafilomycin A1, the cellular retention of propranolol was significantly reduced (Fig. 2, A and B), 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 (Fig. 2). First, the fraction of the test compound retained in the cell monolayer was lower with the higher

concentrations, resulting in apparently saturable cellular retention. Second, in the A to B direction, the test compounds accumulated into the cells fairly rapidly, and after the initial accumulation phase, the amount retained in cells started to decline, whereas in the B to A direction experiments, the test compounds gradually accumulated into the cells for the whole duration of the experiments (90 min).

Kinetic Modeling of the Solute Transfer and Cellular Retention. The transfer and cellular retention data were fitted to model 1; additionally, propranolol data were fitted to model 2. The fitted parameters are presented in Tables 2 and 3.

Model 1 (cellular compartment modeled 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 (Fig. 3). However, the predicted cellular retention profile of propranolol without bafilomycin A1 did not follow the same profile as the observed data (Fig. 4). Especially in the A to B direction at 50 and 300 μ M, 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 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 Q_{12}/Q_{21} ratios (estimated as secondary parameters in WinNonlin) could be estimated more precisely, and they showed clear concentration dependence (Fig. 5). Furthermore, model 2 was better to mimic the cellular retention profile and disappearance from donor and appearance to receiver than model 1 (Fig. 4). In addition, the fitted values for P_{api} were consistently significantly higher than the respective fitted values for P_{baso} .

Estimation of Permeability Coefficients. Model 1 was used to simulate data for permeability estimation. The permeabilities estimated from simulated data using eq. 1 ($P_{app,sink}$) increased initially and started to decline in the later phases (Fig. 6A). Furthermore, the $P_{app,sink}$ values were lower with higher K , i.e., with higher cellular retention. However, the $P_{app,sink}$ values in A to B and B to A directions at same cellular retention were identical. Furthermore, $P_{app,sink}$ was constantly lower than the theoretical P_{app} (calculated using eq. 3) and also lower than permeabilities estimated using eq. 2.

The permeabilities estimated using eq. 2 ($P_{app,nonsink}$) increased initially and eventually reached a stable level (Fig. 6B). However, the time to reach the stable level was longer with higher cellular retention. $P_{app,nonsink}$ was consistently higher in the A to B direction than in the B to A direction. Furthermore, $P_{app,nonsink}$ at a stable level in the A to B direction was always higher, and $P_{app,nonsink}$ at a stable level in the B to A direction was always lower than the theoretical P_{app} . 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 $P_{app,sink}$ reach the maximum value and $P_{app,nonsink}$ 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

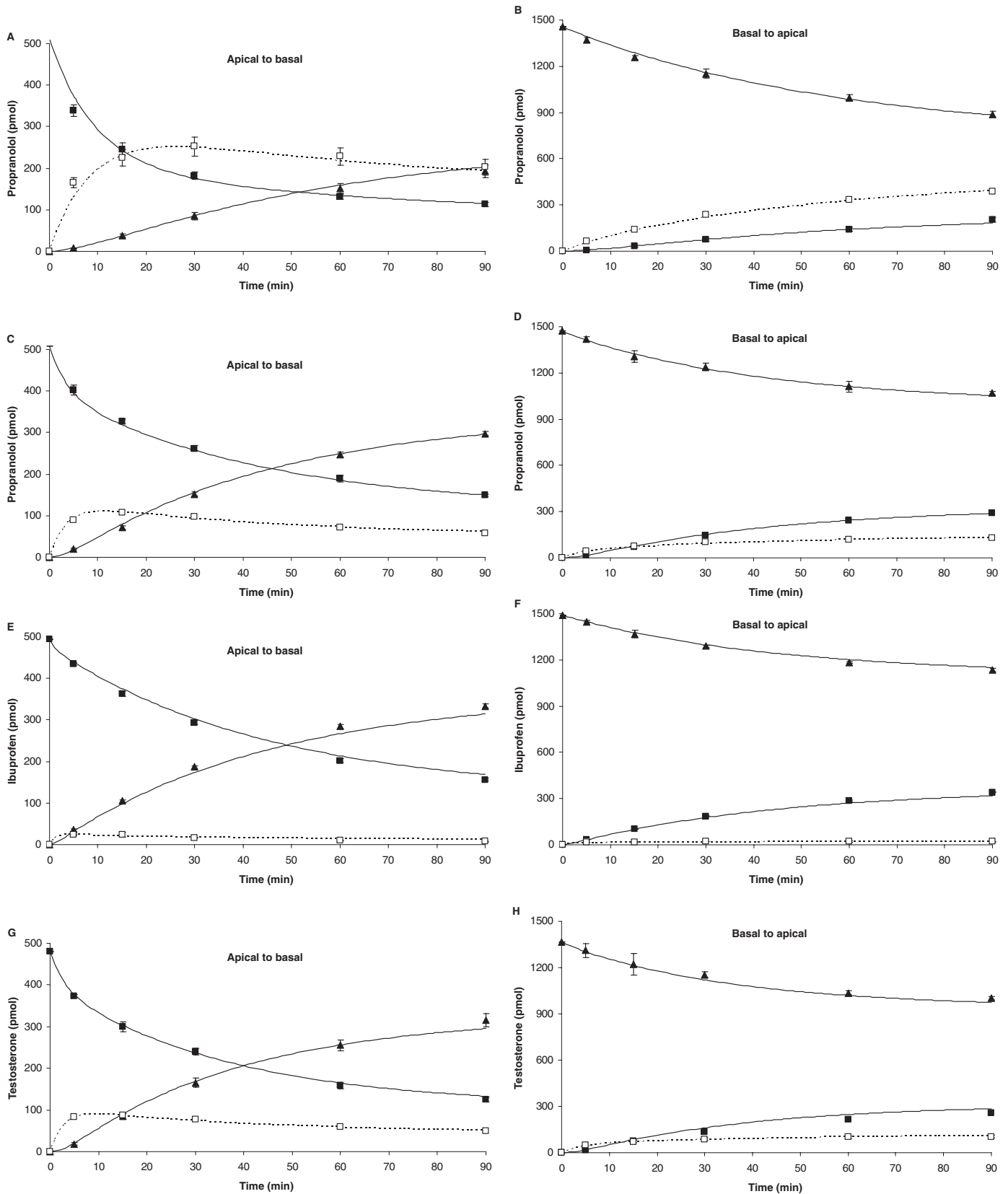


Fig. 3. The time course of 1 μ M propranolol (A and B), propranolol in the presence of 100 nM bafilomycin A1 (C and D), ibuprofen (E and F), and testosterone (G and H) transfer in Caco-2 permeation experiments. The symbols (solid squares and triangles for apical and basal compartments, respectively, and open squares for cell monolayer) represent the average of six measurements ($\pm$ S.D.), and the solid and dashed lines represent the fitted results of models 1 (C–H) and 2 (A and B). The fitted curves mimic the observed data equally well also with the other concentrations used (data not shown).

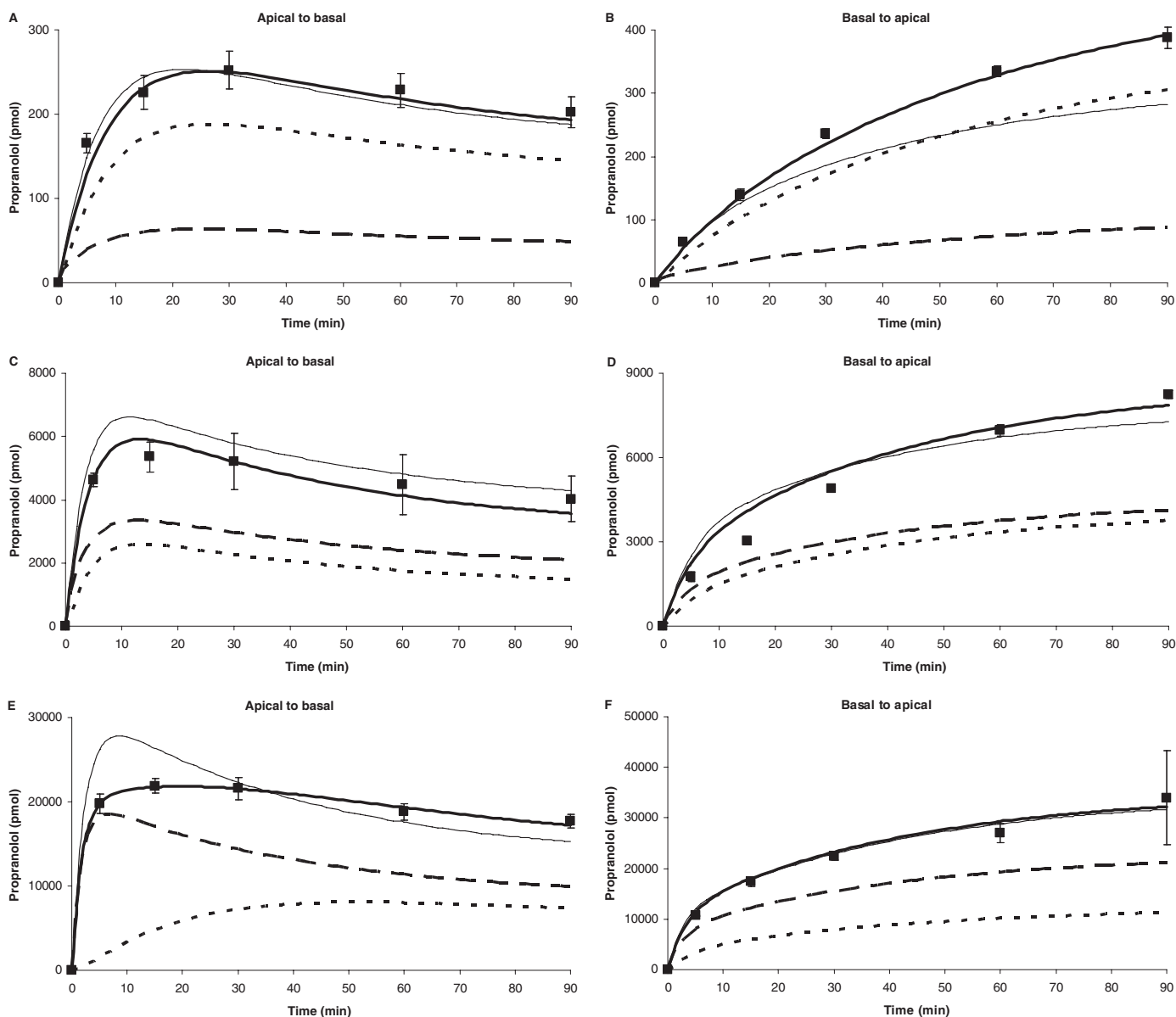


Fig. 4. The time course of cellular retention of 1 (A and B), 50 (C and D), and 300 (E and F) μM propranolol in Caco-2 permeation experiments (note the different scales of the vertical axes). The symbols represent the average of six measurements ($\pm\text{S.D.}$). The thin and thick lines represent the fitted results of models 1 and 2, respectively. The dashed and dotted lines represent the predicted time course of propranolol in cytosolic and lysosomal compartments in model 2, respectively.

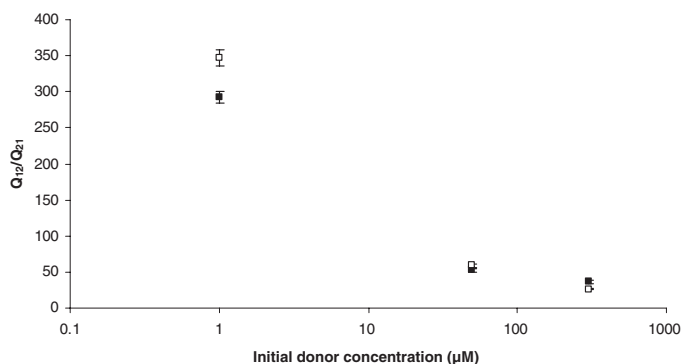


Fig. 5. The concentration dependence of lysosomal sequestration of propranolol. The closed and open symbols refer to the estimated ratio of flux rates between cytosolic and lysosomal compartments ($\pm\text{S.E.}$) in A to B and B to A direction experiments, respectively.

trends than permeability estimates of simulated data (Fig. 7).

Discussion

This study demonstrates the errors caused by cellular retention in permeability estimates. Furthermore, propranolol was observed to be significantly sequestered into the lysosomes during Caco-2 permeation experiments. The inhibition of lysosomal sequestration has been reported recently to increase the apparent Caco-2 permeability (estimated using eq. 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

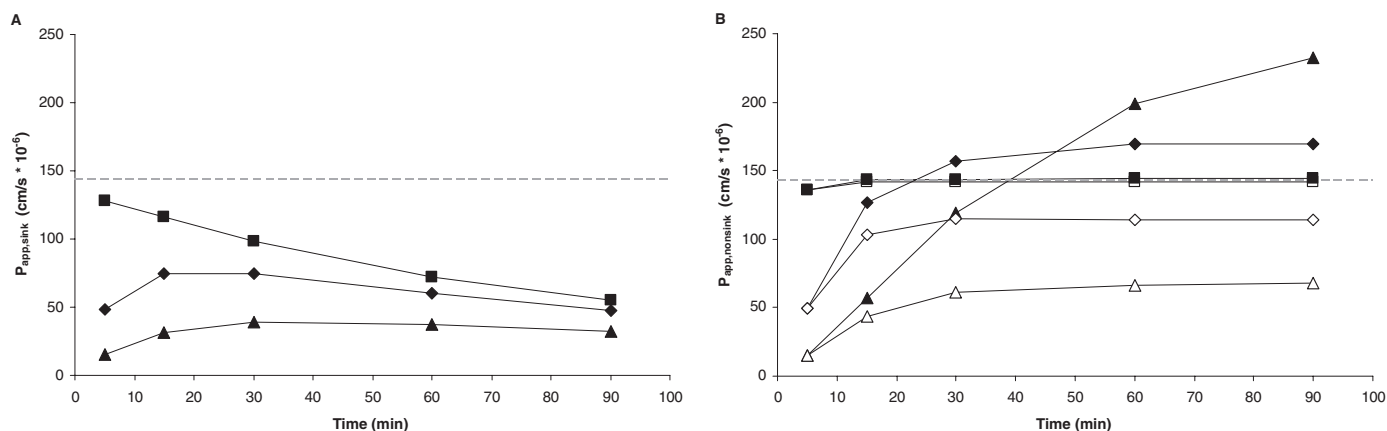


Fig. 6. The apparent permeabilities estimated from the simulated data using model 1 in the A to B direction (closed symbols) and the B to A direction (open symbols). $P_{app,sink}$ was estimated using eq. 1 (A), and $P_{app,nonsink}$ was estimated using eq. 2 (B). Gray dashed line, theoretical P_{app}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

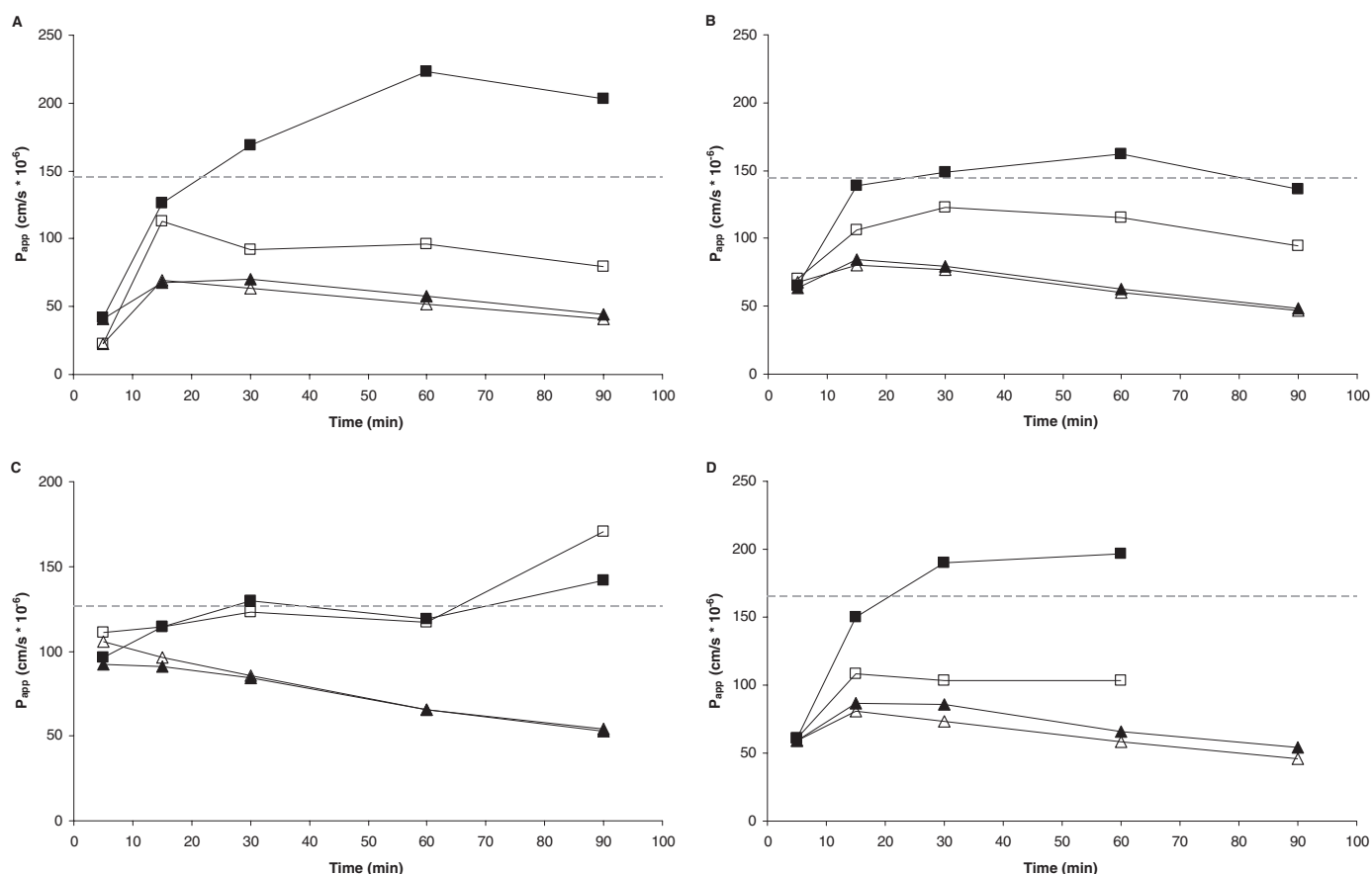


Fig. 7. The apparent permeabilities of 50 μ M propranolol (A), 50 μ M propranolol in the presence of 100 nM bafilomycin A1 (B), 50 μ M ibuprofen (C), and 1 μ M testosterone (D) estimated from the experimental data in the A to B direction (closed symbols) and the B to A direction (open symbols). $P_{app,sink}$ (triangles) was estimated using eq. 1, and $P_{app,nonsink}$ (squares) was estimated using eq. 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. As a consequence, the experimental errors cause significant variation in $P_{app,nonsink}$. Therefore, $P_{app,nonsink}$ is presented only up to 60 min for testosterone. Gray dashed line, theoretical P_{app} calculated from the fitted values of P_{api} and P_{baso} (Table 2) using eq. 3.

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, a slow intracellular compartment also 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 the 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). Furthermore, 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. As a consequence, 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 were based on measurements of total cell lysates; 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 the permeation setting. Anyhow, two conclusions can be drawn from the current analysis. First, the clear concentration dependence seen in the Q_{12}/Q_{21} ratio (Fig. 5), which describes the extent of lysosomal sequestration, suggests that the capacity of lysosomes to sequester propranolol is saturating within the concentration range used. Second, 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. In addition, the Akaike information criterion values (Table 3) favor 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 modeling was employed to clarify the factors causing this behavior. The data fitting to the compartmental models suggest that the barrier at the basal side of the cell monolayer resists the flux of compounds more than 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 additionally contains the filter support. Furthermore, it has been suggested that the resistance of the 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 be-

cause the concentrations in both compartments changes significantly during the experiments. Moreover, the estimated permeabilities lower with lower concentration due to higher cellular retention. Furthermore, it is important to note that because the appearance kinetics to the receiver are irrespective of experimental direction and because $P_{app,sink}$ neglects the decline of donor concentration, there are no direction differences in $P_{app,sink}$ estimates at the same cellular retention level.

For high permeability compounds, it is expected that $P_{app,nonsink}$ would be a better estimate of the permeability than $P_{app,sink}$ because the loss of sink conditions does not cause the $P_{app,nonsink}$ estimates to decline in the late phases. In addition, the actual concentration gradient at the onset of the sampling interval is taken into account. However, $P_{app,nonsink}$ underestimated the permeability in B to A direction for the whole time course, whereas in the 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 a high amount of test compound is retained in the cells (Figs. 6 and 7). The appearance kinetics to the receiver are irrespective of experimental direction. Thus, this discrepancy must be caused by the difference in disappearance kinetics from the donor. The permeabilities through the apical barrier are estimated to be 2 to 3 times higher than the permeabilities through the basal barrier (Table 2) for the compounds used in this study. As a consequence, at the apparent steady-state permeation (when the $P_{app,nonsink}$ has reached the stable level), the free cellular concentration and the amount retained in the cell monolayer follow 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 it disappears from the donor side, whereas the opposite is seen in B to A experiments, ultimately causing the errors seen in $P_{app,nonsink}$. Furthermore, it has to be noted that with compounds that 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; thus, the concentration change within a sampling interval is small. As a consequence, already minor experimental variation causes significant variation in $P_{app,nonsink}$, causing the behavior of estimated permeabilities to be erratic.

In cell permeation experiments, usually only the appearance rate is measured, whereas disappearance rate is not observed in detail. Such data do not contain information about the cellular retention kinetics; 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 $P_{app,sink}$ 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 (Fig. 2), correcting the permeability estimates with a single time point recovery may lead into apparent directionality, although the transfer of the test compound was solely due to passive diffusion.

Sampling of both receiver and donor compartments has been suggested for more rigorous correction for poor recovery (Tran et al., 2004). Likewise, the $P_{app,nonsink}$ approach uses

the actual concentration gradient at each sampling time. However, both of these single-barrier models seem to show systematic errors when significant cellular retention is involved (for further analysis of the approach proposed by Tran et al., 2004, see Supplemental Data B).

Often in cell monolayer permeation studies, the conclusions are drawn based on the assumption that the cell monolayer is a single barrier that 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 has 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 tends 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 oversimplified view, even when studying passive transfer of the solutes through the cell monolayer. Furthermore, flaws of applying the single-barrier view in mechanistic studies, such as studies to elucidate active transporter function, have been reported recently (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 the traditional single-barrier view should be employed when detailed mechanistic insight of solute transfer is to be obtained.

Appendix

Differential Equations for Mass Transfer in Model 1

Apical Compartment.

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

Basal Compartment.

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

Cellular "Cytosolic" Compartment.

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

where the apparent cell-buffer distribution coefficient K is defined as:

$$K = K_{\text{max}} - (K_{\text{max}} - K_{\text{min}}) \frac{M_{\text{cyto}}/V_{\text{cell}}K}{M_{\text{cyto}}/V_{\text{cell}}K + \text{EC}_{50}} \quad (7)$$

M_{api} , M_{baso} , M_{cyto} , and M_{lyso} are amounts in apical, basal, cytosolic, and lysosomal compartments, respectively.

Differential Equations for Mass Transfer in Model 2

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

Cellular "Cytosolic" Compartment.

$$\begin{aligned} \frac{dM_{\text{cyto}}}{dt} = & P_{\text{api}}A \left(\frac{M_{\text{api}}}{V_{\text{api}}} - \frac{M_{\text{cyto}}}{V_{\text{cell}}K} \right) - P_{\text{baso}}A \left(\frac{M_{\text{cyto}}}{V_{\text{cell}}K} - \frac{M_{\text{baso}}}{V_{\text{baso}}} \right) \\ & - Q_{12} \frac{M_{\text{cyto}}}{K} + Q_{21}M_{\text{lyso}} \quad (8) \end{aligned}$$

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

Cellular "Lysosomal" Compartment.

$$\frac{dM_{\text{lyso}}}{dt} = Q_{12} \frac{M_{\text{cyto}}}{K} - Q_{21}M_{\text{lyso}} \quad (9)$$

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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

$$K = \frac{M_{cyto}}{M_{cyto, free}}, \quad (SA1)$$

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

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

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

$$M_{cyto} = K_0 * M_{cyto, free} + B_{max} \frac{C_{cyto, free}}{C_{cyto, free} + EC_{50}}, \quad (SA3)$$

where K_0 is the distribution coefficient representing the unsaturable binding, $B_{\max}$ 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

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

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 $K_{\max}$ and $K_{\min}$, apparent higher and lower limits for K, respectively.

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

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

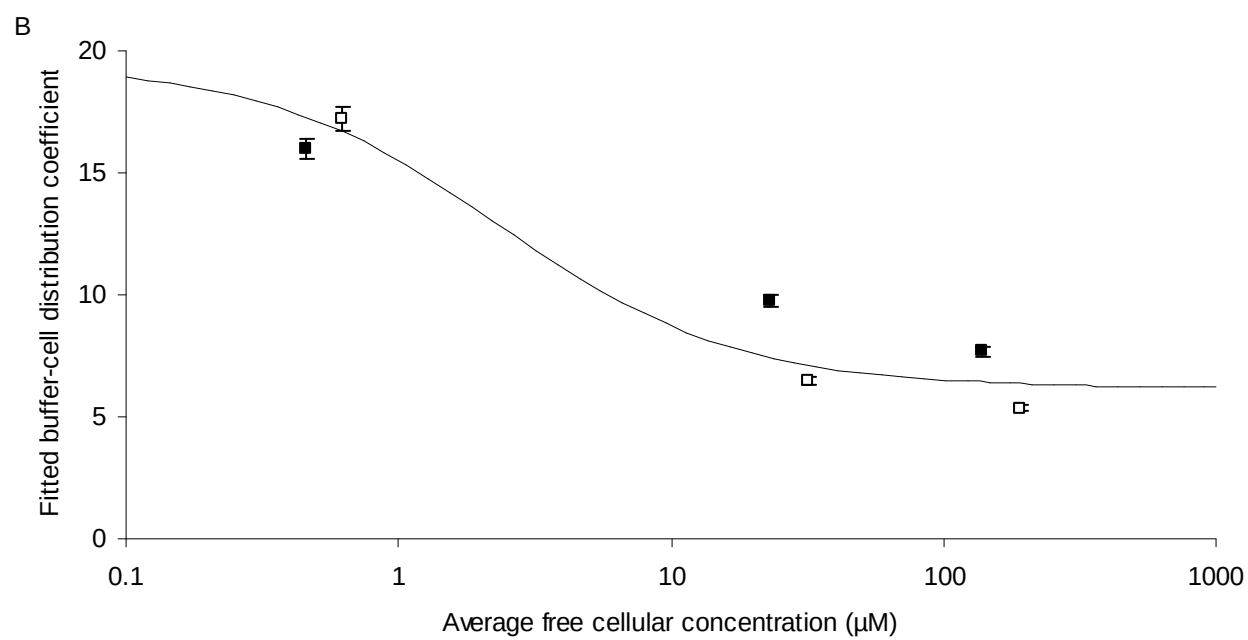
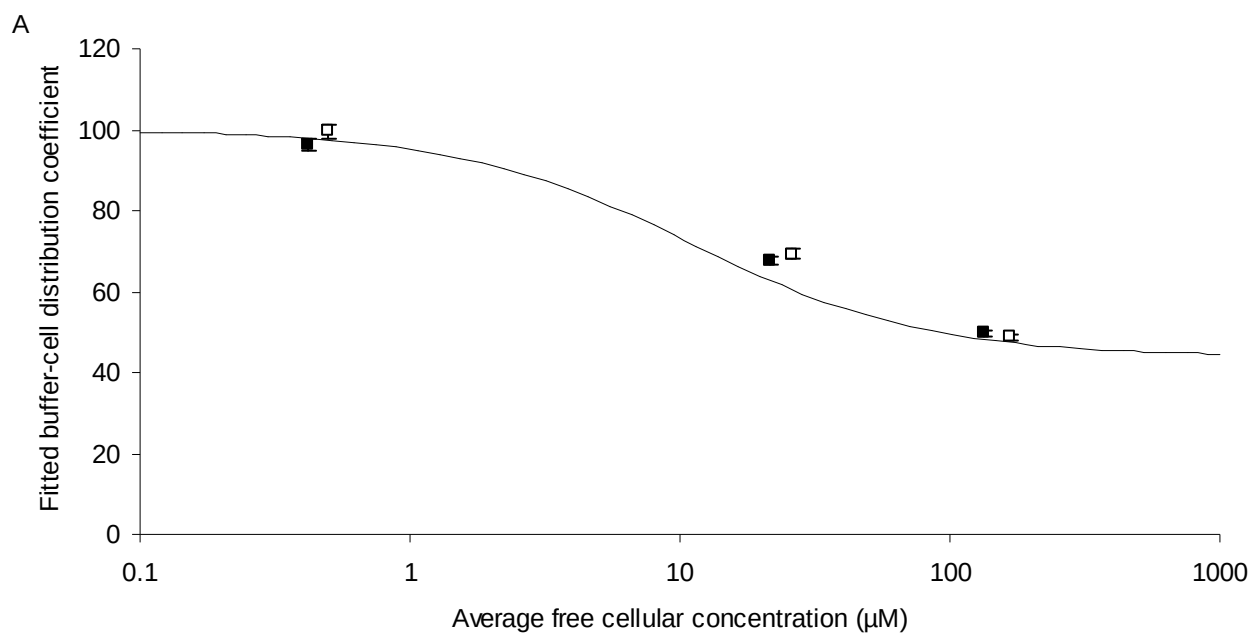
whereas with high concentrations K approaches to $K_{\min}$

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

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

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

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 $K_{\max}$, $K_{\min}$ 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).



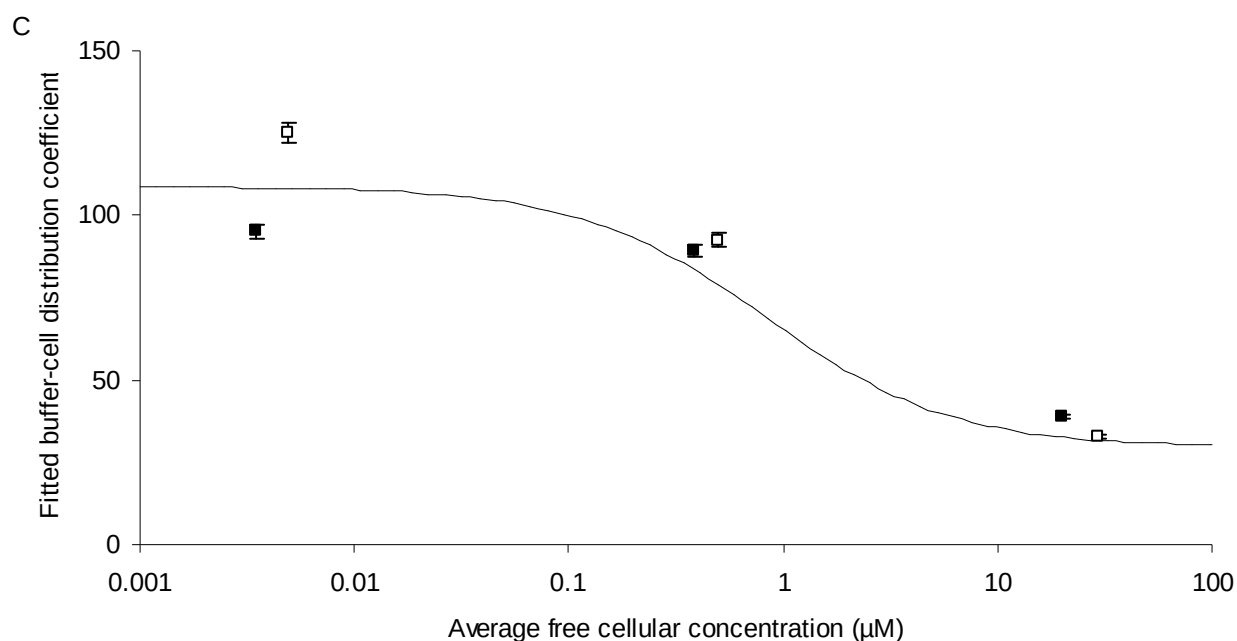


Figure SA1. The concentration dependence of cellular retention of (A) propranolol in the presence of 100 nM bafilomycin A, (B) ibuprofen and (C) testosterone. The symbols (closed for apical to basal and open for basal to apical experiments) represent the individually estimated apparent cell-buffer distribution coefficients ($\pm$ 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" ($\langle C \rangle$) 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, $\langle C \rangle$ does not need to be 'predicted' by using a rate coefficient because $\langle C \rangle$ 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)

$$\langle C(t) \rangle = \frac{V_d C_d(t) + V_r C_r(t)}{V_d + V_r} \quad (\text{SB1})$$

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

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

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

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

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

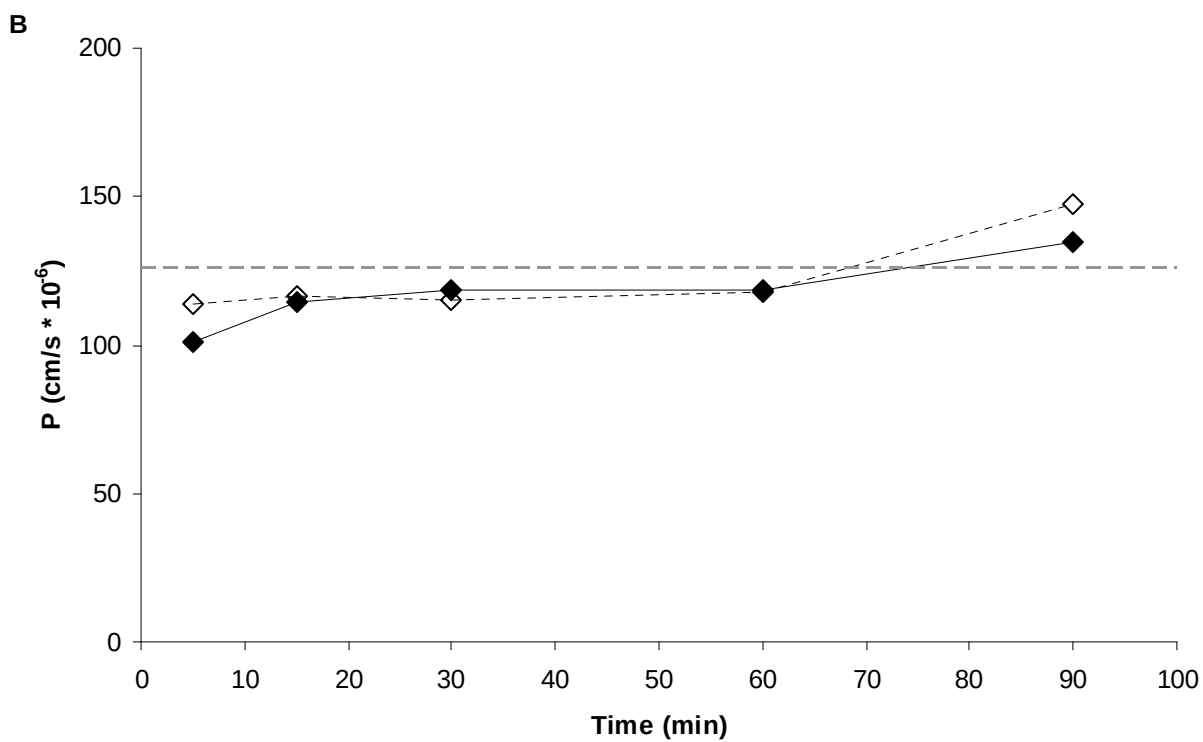
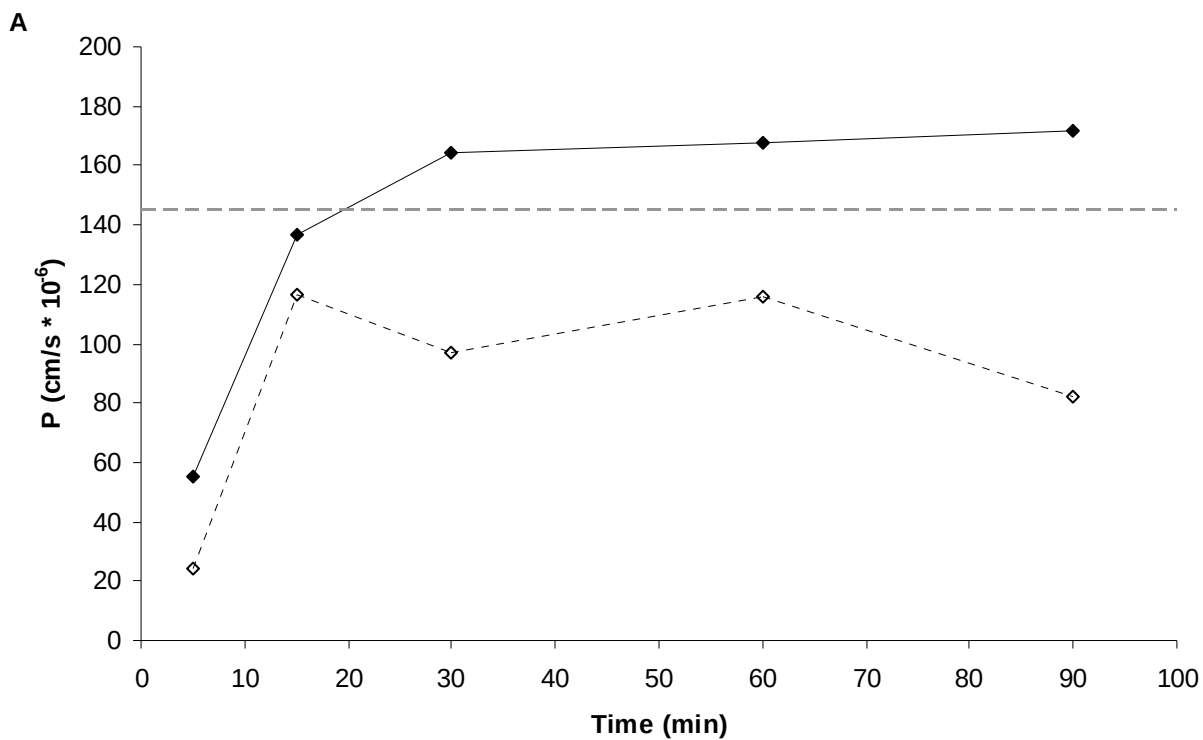
The permeabilities for each sampling interval were calculated from experimental and simulated data using equation SB3. The representative results are shown in figure SB1. When there is no significant cellular retention involved (ibuprofen and simulation K=4), this approach works perfectly in estimating 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 $P_{\text{app, nonsink}}$. 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 $\langle C \rangle$. However, taking the cellular compartment into account in $\langle C \rangle$ 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.

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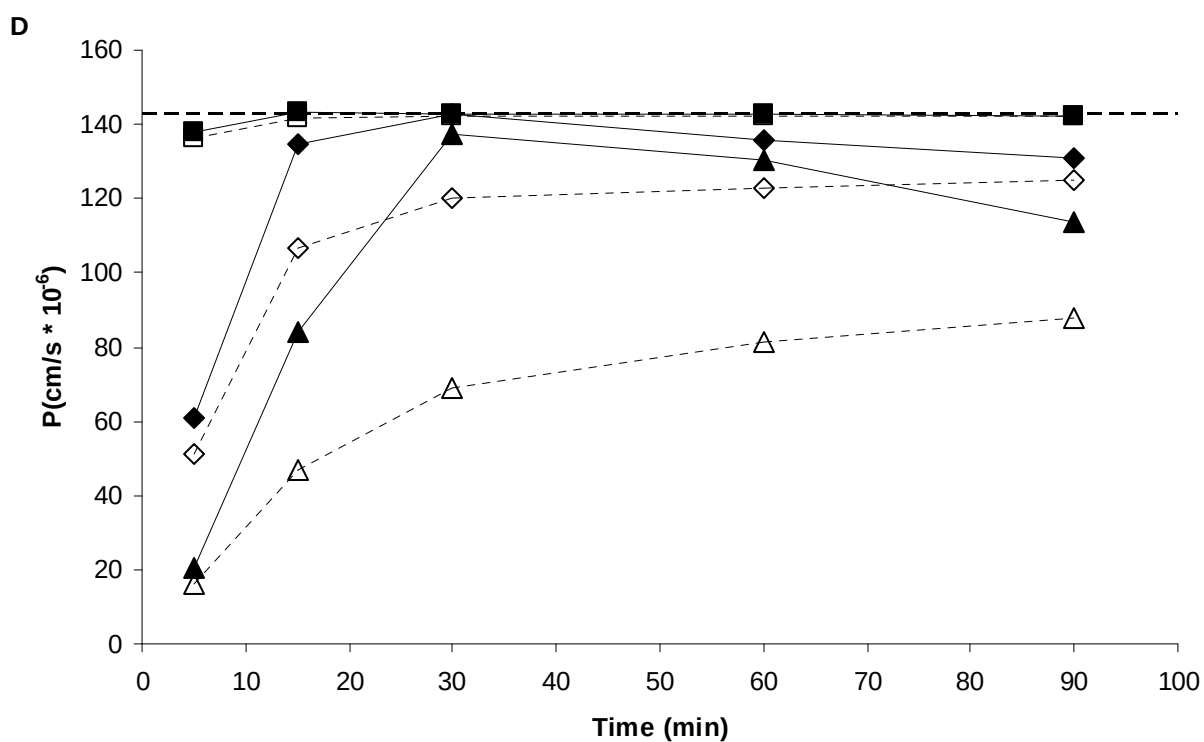
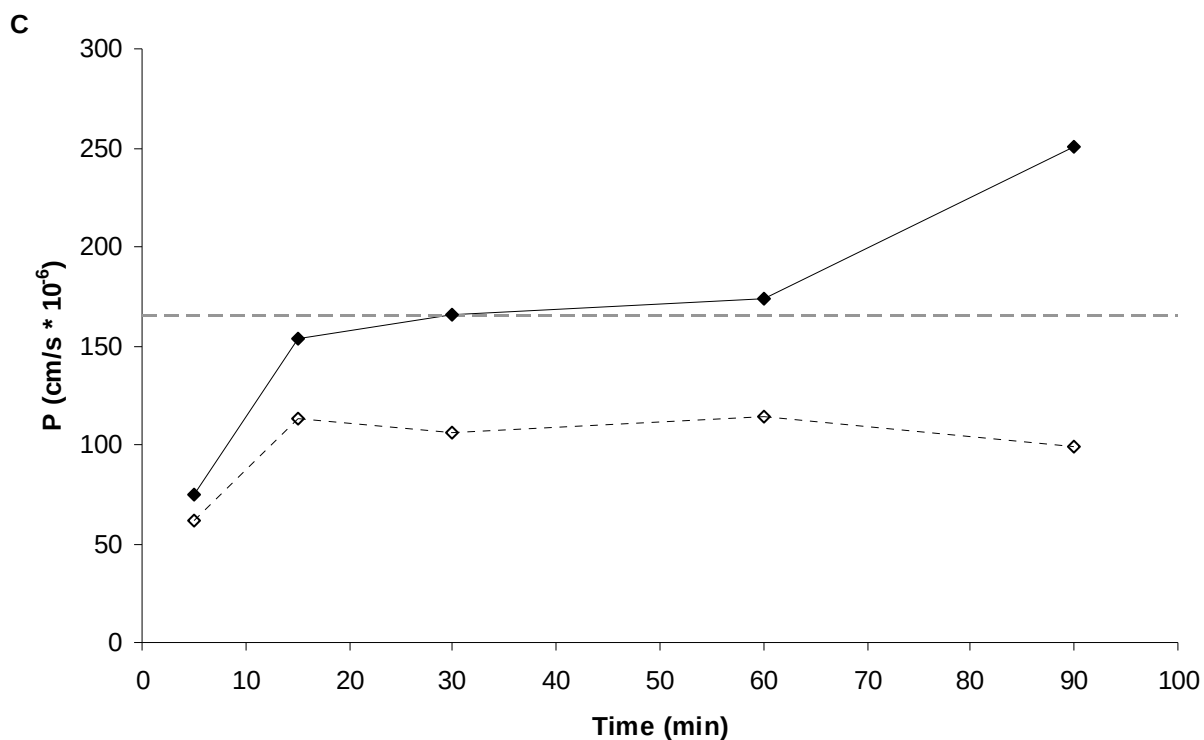


Figure SB1. The estimated permeabilities of 50 μ M propranolol (A), 50 μ M ibuprofen (B), 1 μ M Testosterone (C) and simulated data using model 1 (D) in A to B direction (closed symbols, solid line) and B to A direction (open symbols, dash line). Grey dash line refers to the theoretical P_{app} calculated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