ABSTRACT: Knowledge of the passive permeability coefficient for new drugs is useful for estimating the fraction absorbed across the gastrointestinal tract. The commonly used approximate formula for the passive permeability coefficient is based on the initial rate of permeation across cell monolayers, requires measurement during the linear phase of permeation, and is not applicable when there is significant back flux of compound or mass balance problem. To develop a rigorous equation that can be used at any time point, i.e., that is valid outside of the linear phase, the mass action equations were integrated for a standard single barrier model of passive permeability. The simple analytical solution found also allows correction for both loss of drug (e.g., due to binding and/or hydrolysis) and sampling volume loss for multiple time point experiments. To test this equation, we measured the passive permeation of three well characterized drugs (amprenavir, quinidine, and loperamide) across confluent monolayers of MDCKII-hMDR1 cells. The potent P-glycoprotein inhibitor GF120918 was used to inhibit P-glycoprotein activity, so only passive permeability was determined. Dramatically different time-dependent behavior was observed for the three compounds, with loperamide showing significant loss of compound, and loperamide and quinidine causing plasma membrane modifications over time. The simple and exact equation for the permeability coefficient developed here works from start of transport to equilibrium, being valid when the commonly used approximate equation may not be. Thus, the exact equation is safer to use in any context, even for single time point estimates in high-throughput permeability assays.

INTRODUCTION

The classic approximate solution for the initial passive permeability coefficient for a single barrier is

$$P_{apx} = \frac{V_R C_R(t_i)}{A t_i C_D(0)}$$

where $V_R$ is the volume of the receiver chamber, $C_R(t_i)$ is drug concentration in the receiver
chamber at the measurement time $t_i$, $A$ is the area of the permeability barrier, and $C_D(0)$ is the initial drug concentration in the donor chamber. This equation is widely used because tissues and cell monolayers can be modeled as single barriers for permeation, wherein it is the "rate limiting barrier" within the system that is being measured. More realistic models with two or more internal barriers require more complicated equations, more assumptions to implement them, and can be difficult to utilize in the drug discovery environment.

Equation (1) is based on the predicted initial rate of permeation and is accurate for an early time point, roughly defined by: linear drug transport with time, $<10\%$ of drug transport, negligible backflow, and no significant mass balance problems. This approximate equation depends only on the total mass transported to the receiver chamber relative to initial mass in the donor chamber, making the equation insensitive to mass balance problems; for example, drug stability/hydrolysis or binding to cells and/or apparatus. This is especially problematic when the donor side concentration is predicted from the addition, rather than measured directly. These limitations require preliminary or extra experiments to be done to validate the usage of eq. (1).

Corrective measures for this approximate equation have been published; see for a review. Although improvements can be made, usage of the approximate equation still requires the establishment of the linear phase of permeability because eq. (1) is just the initial slope of the transport curve. Demonstrating linear permeability is difficult to achieve in a drug discovery setting where compounds with diverse chemical properties are often being tested in a single permeability experiment. Therefore, the objective of this work was to derive an exact mathematical solution for the entire transport curve, which is more suitable for higher-throughput screening activities. Equation (2) is the outcome of this derivation (full derivation is given in Appendix A). The exact equation can be used for single or multiple time point measurements. It can be applied across a variety of situations; for example, when $>10\%$ of drug has been transported and when mass balance is poor. Because this exact solution is valid when the approximate equation may not be, it will provide a more accurate permeability value. Furthermore, the exact equation simplifies experimental protocols, by eliminating the need to establish the linear phase in the first place. The exact equation does not require maintenance of receiver sink conditions throughout the experiment, as required to maintain validity of the approximate equation.

MATERIALS AND METHODS

Materials

Amprenavir and GF120918 were from Glaxo SmithKline (United States). Loperamide was from Sigma, and quinidine from Fisher Scientific. $^3$H-Loperamide (10 Ci/mmol) and $^3$H-amprenavir (21 Ci/mmol) were custom synthesized by Amersham Pharmacia Biotech (United Kingdom). $^3$H-Quinidine (20 Ci/mmol) was from ICN Biomedical, Inc. (United States). $^{14}$C-Mannitol (53.7 mCi/mmol) was from PerkinElmer Life Sciences Inc. (United States). Dimethyl sulfoxide was from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) was from Mediatech VWR. Transport medium (DMEM with 25 mM HEPES buffer, high glucose, L-glutamine, pyridoxine hydrochloride, without sodium pyruvate, and without phenol red) was from Sigma. Transwell 12-well plates with polycarbonate inserts were obtained from Costar. Ultima Gold scintillation cocktail and Ultima-Flow M were from PerkinElmer Life Sciences. Unless otherwise stated, all other chemicals used in this study were reagent grade, or higher, and were obtained from commercial suppliers.

Cell Line and Culture Conditions

The Madin-Darby Canine Kidney cell line overexpressing human MDR1 (MDCKII-hMDR1) was purchased from the Netherlands Cancer Institute (Amsterdam, Netherlands). Cells were split twice a week and maintained in culture medium (DMEM supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin). Cells were kept at 37°C in 5% CO₂.

Transport Assays

Cells were seeded in 12-well plates at a density of 200,000 cells per insert and grown for 4 days in culture medium. Preliminary results using higher and lower plating densities suggested that this density was optimal with respect to obtaining transport stable confluent monolayers at 4 days. Cell passages 32 and 33 were used for amprena-
vir, 45 for quinidine, and 42 for loperamide. Cells were given fresh media 1 day after seeding.

Before the experiment, culture medium was removed and cells were preincubated for 30 min with either transport medium alone (see above) or transport medium supplemented with 2 μM GF120918, a potent inhibitor of P-glycoprotein (P-gp).7–10 Transport of a range of concentrations of amprenavir, loperamide, and quinidine across the confluent monolayer of cells was measured in both directions, that is, apical to basolateral (A→B) and basolateral to apical (B→A) in the presence and absence of GF120918. For incubations in the presence of GF120918, the inhibitor was added to both chambers.3H-Amprenavir,3H-quinidine, or 3H-loperamide, 0.5 μCi/mL, was added to each respective drug concentration to allow quantitation of transport from donor to receiver chambers by liquid scintillation counter. 14C-mannitol (0.75 μCi/mL) was added to the donor chamber to monitor cell monolayer integrity. At the indicated time points, 25-μL samples were removed from the donor and receiver chambers, mixed with 10 mL of Ultima Gold scintillation cocktail, and analyzed by Packard TriCarb 3100-RT liquid scintillation counter.

One important change to common practice we have introduced is to measure the concentration of drug on the donor side at each time point, rather than assuming all drug added is “in solution.” Practically, this was accomplished by making the initial measurement at 6 min after addition and subsequent measurements at 15, 30, 60, 120, 180, 240, 300, and 360 min after addition for all studies. This means that the first passive permeability coefficient shown at 15 min covers the permeation from 6 to 15 min. Thus, extraneous rapid binding of the drugs to the apparatus does not affect our measurements for cell permeability. The fraction of drug recovered was calculated using the amount in the wells at 6 min for 100%.

**MDR1 Transport Stability during Six-Hour Incubations**

We considered the possibility that the stability of the cell monolayer and plasma membrane might be influenced by the prolonged exposure times used in these studies, that is, up to 6 h in the absence of serum. Cells were seeded as above. On the day of the experiment, half of the cell inserts were used in a B→A transport experiment with radiolabeled amprenavir (75 μM total concentration) in the presence and absence of GF120918. The other half of the cell inserts were incubated for 3 h with 75 μM cold amprenavir in the presence and absence of GF120918. After 3 h, these cells were washed twice with prewarmed fresh transport media, and radiolabeled amprenavir (75 μM total concentration) was added to the basolateral chamber. B→A transport was measured in the presence and absence of GF120918 for an additional 3 h. We found that preincubation of MDCK-MDR1 monolayers with amprenavir for 3 h did not alter the P-gp-mediated transport or passive permeability of [3H]-amprenavir or of [14C]-mannitol (data not shown) compared with control monolayers. Thus, transport is unaffected by up to 6 h incubation without serum. As described below, mannitol transport was always <7 nm/s, showing the stability of the monolayers over the entire 6-h experiment.

**Morphometric and Ultrastructural Analysis**

Cells prepared for transport assay (as described above) were incubated in test solutions consisting of 75 μM amprenavir and 2 μM GF120918, either alone or in combination, for 2, 4, or 6 h. Cells incubated in media alone were used as controls. Cells were fixed in situ with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer. Well insert membranes were cut into 1×2 mm strips, postfixed in 1% osmium tetroxide, dehydrated in serial alcohols, and embedded in epoxy resin. Thick sections (~0.4 μm) were collected on glass slides and stained with 1% toluidine blue in 1% aqueous sodium borate for light microscopic examination. Dead cells were discriminated from live cells by their greater staining intensity and quantified using Image-Pro Plus™ (Media Cybernetics, Silver Spring, MD) software. At least 200 cells were counted for each test solution. Thin sections (~80 nm) were stained with uranyl acetate and lead citrate and examined with a JEOL 1200EX transmission electron microscope at 80 kV. These measurements revealed minimal cell death after 6-h incubation with amprenavir or GF120918 alone. After 6 h of incubation, the longest time for any of the transport experiments, dead cells were <2% total (at least 200 cells sampled randomly) for amprenavir alone and GF120918 alone. The combination of amprenavir and GF120918 for 6 h showed 6% cell death, presumably due to the larger intracellular amprenavir concentrations, because P-gp efflux was inhibited.
Radio High-Performance Liquid Chromatography (HPLC)

The radiochemical purity of all three radiolabeled drugs was determined using radio HPLC analysis. The purities were analyzed on an Agilent 1100 HPLC system equipped with a Beta-Ram radiodetector (IN/US). Radio HPLC data captured on-line were processed using Win-Flow software (version 1.5, LabLogic). The respective manufacturers provided the methods used. \(^{3}\)H-Amprenavir was analyzed using a Luna C18(2), 250 × 4.6 mm, 5-μm column. Mobile phase solvents were (A) 0.05% trifluoroacetic acid in water and (B) 0.05% trifluoroacetic acid in acetonitrile. The gradient was from 0% B to 100% B over 20 min with a flow rate of 1 mL/min. \(^{3}\)H-Loperamide was analyzed using a Hypersil BDS-C18, 250 × 4.6 mm, 5-μm column. Mobile phase solvents were (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile. The gradient was 0% B to 100% B over 15 min with a flow rate of 1 mL/min. \(^{3}\)H-Quinidine was analyzed using a Prodigy ODS(2), 250 × 4.6 mm, 5-μm column at isocratic conditions with 50 mM potassium phosphate buffer pH 3/acetoniitrile (85:15). The same methods were used to examine the presence of metabolites in culture medium after 6 h of incubation. Three concentrations per drug were tested. Metabolism or decomposition of compounds was insignificant for all compounds, <1% for \(^{3}\)H-amprenavir [with \(\sim\)15% (B → A) and 50% (A → B) passively transported at 6 h] and \(^{3}\)H-loperamide [with \(\sim\)7% (B → A) and 20% (A → B) passively transported at 6 h], and <3% for \(^{3}\)H-quinidine [with \(\sim\)15% (B → A) and 50% (A → B) passively transported at 6 h].

RESULTS

The Exact Equation for Passive Permeation through a Single Barrier

The standard mass action reactions for passive permeability for a single barrier are solved in Appendix A. When there is no loss of drug or drug loss is first order, an analytical integration yields a novel, compact, and rigorous formula for the passive permeability coefficient

\[
P = -\left(\frac{V_R V_D}{(V_R + V_D)At}\right) \ln \left(1 - \frac{C_R(t)}{C(t)}\right)
\]

where \(V_D\) and \(V_R\) are the donor and receiver chamber volumes (cm\(^3\)), \(A\) is the area of the permeability barrier (cm\(^2\)), and \(t\) is the time of measurement (s). This equation for computing the permeability coefficient is novel because it uses the average system concentration of drug, defined simply by

\[
\langle C(t) \rangle = \frac{V_D C_D(t) + V_R C_R(t)}{V_D + V_R}
\]

As before, \(C_R(t)\) and \(C_D(t)\) denote the drug concentrations in the receiver and donor chambers (mols/L) at time \(t\). The volume of the cell monolayer is neglected here, because it is small compared with the aqueous chambers. As time \(t \to 0\), eq. (2) becomes identical to eq. (1), as required because they are derived from the same mass action equations. The data needed to use eqs. (2) and (3) are the same as needed to use eq. (1), that is, the receiver and donor concentrations at each time point. When donor side concentrations are simply predicted from drug added initially, rather than being measured, then potential mass balance problems are ignored completely.

Equation (2) has replaced the concentration gradient between the chambers, \(C_D(t) - C_R(t)\), as the thermodynamic force term for permeation with the mathematically equivalent gradient between the receiver side concentration and the system average concentration, \(\langle C(t) \rangle\), which is calculated from the same data used to calculate the permeability coefficient. This makes the equation more compact and clear in how it works. Equilibrium is reached when receiver side and donor side concentrations equal the system average concentration. When there is no loss of compound, this exact equation is similar in form to an approximation derived by Ho et al.\(^{3}\)

\(\langle C(t) \rangle\) is a direct measure of loss of drug and is obtained from the experimental data at each time point. If there is no drug loss, then \(\langle C(t) \rangle = V_D C_D(0)/(V_D + V_R)\) is constant in time, which was seen for amprenavir and quinidine. If drug is lost from solution, for example, due to metabolism, binding to cells, and/or the experimental apparatus, then \(\langle C(t) \rangle\) will decrease in time. If that loss is first order, which is the case for loperamide, then \(\langle C(t) \rangle\) equals

\[
\langle C(t) \rangle = \left(\frac{C_D(0)V_D}{V_R + V_D}\right) \exp\{-k_v t\}
\]

as shown by eq. (A.4) in Appendix A.

Fitting eq. (4) to the experimental values of \(\langle C(t) \rangle\) from eq. (3) yields the first-order rate constant for loss, \(k_v\). In this way, loss of drug does
not affect the calculation of the passive permeability coefficient. If drug loss is not first order, due to more complex mechanisms of loss, then the analysis in Appendix A could be used to obtain the appropriate expression for the passive permeability coefficient, although an exact solution would probably have to be solved numerically.

It is not necessary to fit $k_v$ to use eq. (2), but eq. (3) was used in the derivation of eq. (2). We note that because the mechanisms of drug loss are many, the $k_v$ value for a $B \rightarrow A$ experiment need not equal that for an $A \rightarrow B$ experiment. This does not affect the validity of eq. (2), because loss for a single barrier model simply means that drug leaves the aqueous phase of the donor chamber and fails to arrive in a timely manner in the aqueous phase of the receiver chamber. Thus, the $k_v$ is the barrier average loss rate constant. For loperamide, we shall see that loss is greater when the apical chamber is the donor than when the basolateral chamber is the donor, which is represented properly by different $k_v$ values. This asymmetry is due to the fact that the cell monolayer responds differently depending on which chamber has the initial high concentration of drug. The site of loss is unknown, as is usually the case.

One final note on loss is very important. Loss of drug due to metabolism, hydrolysis, or binding to cells or apparatus is a common problem in drug permeability screening assays because it reduces the measured transport rate. However, the “lost” drug does not contribute to the concentration gradient across the barrier which drives drug flow. Of course, the drug is binding to something within the cell monolayer or the apparatus, but bound drug is not relevant to the driving force because its chemical potential is in equilibrium with the drug in transit, certainly over the hours of transport we monitor here. For the one barrier model, the force driving the flux of drug is the concentration gradient, either between the aqueous chambers or equivalently between the receiver chamber and the system average concentration [eq. (3)].

### Application of the Equation for Permeability Calculation

The passive and active transport of 90 $\mu$M amprenavir across MDCKII-MDR1 monolayers is shown in Figure 1. The passive permeability was determined in the presence of 2 $\mu$M GF120918, a potent inhibitor of P-gp.\textsuperscript{7–10} If the volume of a static passive permeability barrier, times the partition coefficient of the drug into the barrier, is negligible compared with the donor and receiver chambers, as it is for our experiments, then the passive permeability coefficient will be the same in both directions regardless of the inner structure of the static barrier.\textsuperscript{3} That is, flux is symmetric, because it depends only on the chamber concentration gradient. Figure 1 shows that the passive transport of amprenavir across the cell monolayer was symmetric. The effect of P-gp on transport of amprenavir across the cell monolayer, which occurs in the absence of GF120918, is shown by the matched open symbols, $B \rightarrow A$ (△) and $A \rightarrow B$ (■).

![Figure 1. The passive and active transport of amprenavir across the monolayer of confluent MDCK cells is shown by nmol in the receiver chamber (corrected for loss due to sampling) over 6 h. For all cases, the donor side begins with 90 $\mu$M amprenavir. The filled symbols show passive permeability across the cell monolayer when the basolateral chamber was the donor side $B \rightarrow A$ (▲) and when the apical side was the donor, $A \rightarrow B$ (■). The active transport by P-gp was completely inhibited by the addition of 2 $\mu$M GF120918 to both chambers. The effect of P-gp on transport of amprenavir across the cell monolayer, which occurs in the absence of GF120918, is shown by the matched open symbols, $B \rightarrow A$ (△) and $A \rightarrow B$ (■).]
Although there was some variation between the concentrations, there was no correlation of this variability with concentration or cell passage number, thus we averaged the permeability coefficients over concentration (Fig. 2C). The largest variation for both directions was seen in the first hour, with the \( A \rightarrow B \) direction having the larger variability. After 1 h, both directions reached a steady-state permeability coefficient of around 200 nm/s, indicating that the passive barrier was stable over time. This is the same value as reported for a Caco-2 cell monolayer using the approximate equation over a 60-min time interval.\(^{10}\) The permeability coefficient of \(^{3}H\)-mannitol was <7 nm/s for all cases, showing high cell monolayer integrity.

For comparison, the approximate permeability coefficient (denoted \( P_{\text{apx}} \)) from eq. (1), is shown for comparison, \( B \rightarrow A \) (——) and \( A \rightarrow B \) (-----). The calculation assumed a single measurement at the time shown. Error bars for these data are not shown for clarity, but are less than ±25 nm/s. Clearly, the approximation always underestimates the permeability coefficient.
described in Appendix B. We compared $P$ [eq. (2)] with $P_{apx}$ [eq. (1)] using the same data. At each time point, $P_{apx}$ was calculated as though it were the only time point measured, because that is the standard approach. As expected, $P_{apx}$ and $P$ initially track closely, but $P_{apx}$ systematically underestimates the exact value predicted by eq. (2). Over time, the permeability value calculated with the $P_{apx}$ equations decreases because of significant back flux of amprenavir. Because the cell monolayer behaves like a stable barrier, the exact value $P$ is essentially constant over time whereas $P_{apx}$ declines because of backflow, eventually giving a twofold underestimate.

Quinidine passive permeability was similar to amprenavir (Fig. 3). The passive permeability was symmetric and there was no significant loss of drug during the experiment (data not shown). Figure 4A and B show the $P(B \rightarrow A)$ and $P(A \rightarrow B)$ passive permeability coefficients for quinidine at three concentrations in triplicate. Although there was some variation between the concentrations, there was no correlation of this variability with concentration, thus we averaged the permeability coefficient over concentration (Fig. 4C). Increase in $P$ was evident up to 30 min in both directions, then

Figure 3. Quinidine transport is shown in the same format as Figure 1, with 1 µM quinidine in the donor chamber. Passive transport, closed symbols with 2 µM GF120918 added, was symmetric, as expected for a stable and passive barrier. Quinidine showed no significant loss over 6 h for all concentrations used, like amprenavir.

Figure 4. Permeability coefficients for quinidine are shown using eq. (2). (A) The $P(B \rightarrow A)$ passive permeability coefficients, $P$ (nm/s), for 1 (×), 3 (□), and 10 (+) µM quinidine over 6 h. Mean of triplicates are shown without error bars for clarity. (B) The $P(A \rightarrow B)$ passive permeability coefficients, $P$ (nm/s) from eq. (1), for the same quinidine concentrations and symbols used in (A). Mean of triplicates are shown. (C) The average permeability coefficient, averaged over all concentrations, which represent nine individual experiments, that is, triplicates of each concentration times three concentrations. Error bars show the standard deviation over the concentration means. The approximate permeability coefficient, $P_{apx}$, from eq. (1), is shown for comparison, $B \rightarrow A$ (——) and $A \rightarrow B$ (-----). Error bars for these data are not shown for clarity, but are less than ±25 nm/s.
a 2–3 h decline occurred toward a steady state of $P \sim 300–350$ nm/s. The passive permeability coefficient was symmetric throughout the time course, that is, the same in both directions, but not constant in time. The increase in permeability coefficient $P$ in the first hour was not due to loading in the cells, for example, binding to intracellular compartments, because no quinidine was lost; that is, the percentage of quinidine recovered over time was 95–100% for the $B \rightarrow A$ experiments and 90–95% for the $A \rightarrow B$ experiments. These percentages were calculated from the amount of drug in both chambers at each time point and corrected for the amount of drug removed during the experiment for analysis (Appendix B). Some modification of the plasma membranes appears to occur, because they are the permeability barriers. As explained in Appendix A, given that the value of $P$, the permeability coefficient per unit area of insert, is changing over time, the time average value of $P$ is measured over each time interval. $P_{\text{apx}}$ from eq. (1) is close to the exact $P$ initially, but the changing permeation parameters causes $P_{\text{apx}}$ to underestimate the exact $P$ by as much as twofold (Fig. 4C). The approximate equation cannot track a time-dependent passive permeability coefficient with accuracy, even without mass balance problems. The permeability coefficient of mannitol was $<7$ nm/s for all cases.

Loperamide permeability in the presence of GF120918 was symmetric, similar to amprenavir and quinidine (Fig. 5); however, this symmetry turned out to be deceptive. This is because, unlike amprenavir and quinidine, there was substantial loss of loperamide over time. This loss was not due to metabolism, because no metabolites were found by radio HPLC, but rather due to binding to the cells and/or apparatus.

Figure 6A and B show that the loss was not dependent on drug concentration. Thus, the kinetics of drug loss was first order, the key assumption used in the derivation of the exact permeability coefficient when there is loss of drug (see Appendix A). Figure 6C shows the average fraction recovery across the three test concentrations. The average rate constant for loss ($k_v$) as a function of time highlights that $k_v$ is about twice as large in the $A \rightarrow B$ direction, where the apical chamber is donor, than in the $B \rightarrow A$ direction during the first 2 h (Fig. 6D). After 3 h, the calculated rate constants approached zero, mirroring the cessation of loperamide loss. That is, the first-order mechanism of loperamide loss persisted for 2–3 h and then stopped. The small negative value for $k_v$ at 4 h was basically a fluctuation around zero.

Figure 7A and B show the $P(B \rightarrow A)$ and $P(A \rightarrow B)$ passive permeability coefficients (nm/s) for loperamide at three concentrations. Although there was some variation between the concentrations, there was no correlation of this variability with concentration, thus we averaged the permeability coefficients over concentration (Fig. 7C). Up to 1 h, $P(B \rightarrow A)$ and $P(A \rightarrow B)$ were the same and increased with time, then $P(B \rightarrow A)$ reached a steady state of about 100 nm/s. $P(A \rightarrow B)$ continued to increase for 2–3 h, then reached a steady state of about 250 nm/s. So after the first hour, the passive permeability coefficients diverged. Because $P_{\text{apx}}$ [eq. (1)] is simply proportional to the amount transported, it misses this deviation of passive permeability coefficients. This is because the loss of drug compensates, perhaps serendipitously, for the increase in the permeability coefficient. The permeability coefficient of mannitol was $<7$ nm/s for all cases.

Figures 2C, 4C, and 7C show very distinct patterns in the establishment of steady-state conditions for passive permeability. Amprenavir achieves steady state within 15 min, quinidine has a relatively rapid rise in $P$ followed by a decline to steady state after 2 h, whereas loperamide shows an asymmetric rise, achieving steady state after 1 h for $B \rightarrow A$ and after 3 h for $A \rightarrow B$. The data were averaged over drug concentrations because no concentration dependence was apparent; however, the ranges of concentrations used were different for the three drugs. We measured the passive permeability of 1 µM amprenavir and found that the permeability coefficients were the
same as measured for the higher concentrations (data not shown), proving that the differences between the drugs were not due to the concentrations tested.

DISCUSSION

In high-throughput discovery drug screening studies, there will be low (<10 nm/s) and high (>300 nm/s) permeability compounds run at the same time, while the test concentrations must be balanced with the analytical detection. Likewise, mass balance problems are not usually predictable. We have found and tested an exact equation for the passive permeability coefficient through a single barrier, eq. (2), which provides the flexibility needed to solve these two problems. First, because the equation is valid throughout the transport curve, from onset to equilibrium, a high-throughput screen can use a time point appropriate for compounds with low permeability, which would certainly yield >10% transport when high permeability compounds are applied. The exact equation would be valid in both cases. Second, it is often the case that discovery drugs...
show substantial mass balance problems, which must be dealt with in order to reliably measure passive permeability coefficients. Although there are several likely mechanisms of drug loss, for example, metabolism, binding to cell proteins, and/or the experimental apparatus, the real mechanism(s) will seldom be known. Equation (2) automatically corrects for loss of drug using the same data required to correct the approximate equation.

To obtain the exact solution for passive permeability, we assumed that when there is loss of compound, it can be modeled as a first-order process. In addition to the fact that the loss was first order for loperamide, there are four good general reasons to model loss as a first-order process:

1) It introduces only one new parameter, which can be independently fitted from the data.

**Figure 7.** Permeability coefficients for loperamide are shown using eq. (1). (A) The \( P(B \rightarrow A) \) passive permeability coefficients, \( P \) (nm/s), for 0.15 \((\times)\), 0.35 \((\square)\), and 1.05 \((\circ)\) \(\mu\)M loperamide over 6 h. Mean of triplicates are shown without error bars for clarity. (B) The \( P(A \rightarrow B) \) passive permeability coefficients, \( P \) (nm/s) from eq. (1), for the same loperamide concentrations and symbols used in (A). Mean of triplicates are shown. Like amprenavir and quinidine, there was variation between the different loperamide concentrations, but there was no correlation with concentration, thus averaging for the permeability coefficient over concentration was allowed. (C) The average values, which also represent nine individual experiments, that is, triplicates of each concentration times three concentrations. Error bars show the standard deviation over the concentration mean values. The approximate permeability coefficient, \( P_{\text{apx}} \), is shown for comparison, \( B \rightarrow A \) (---) and \( A \rightarrow B \) (-----). Error bars are not shown for clarity. Clearly, the approximation does not show the asymmetry in passive permeability and estimates are twofold too small at 1 h and for \( B \rightarrow A \) are fivefold too small at 6 h.
Invoking a second-order process requires fitting two new parameters, that is, the concentration and binding constant for "the" receptor.

2) It allows a simple, analytical solution for the permeability coefficient. More complex assumptions yield solutions that will not analytically invert, which means that they would have to be solved numerically and cannot be incorporated into a spreadsheet format.

3) It is accurate for weak binding to a large number of sites; for example, the membranes and/or chamber walls.

4) If drug disappearance from solution is by a higher-order process, then eq. (2) would still be a good approximation, because the fitting of $k_v$ would best fit the loss between data points, regardless of mechanism. It can accurately interpolate for the "correct" curve over time, where $k_v$ would change between data points.

The experiments described here use a polarized monolayer of MDCKII cells which have two plasma membranes that drugs need to cross, basolateral and apical. Despite the fact that a monolayer of cells is far more complex than a single bilayer of lipids, it is common to fit the passive permeability data as a single barrier. Two barrier models for passive transport are much more complex. If intracellular concentrations can be measured or estimated, then two barrier models could be used to assess differences in apical and basolateral passive permeabilities.

When the cell monolayer is treated as a single permeability barrier and it is static, that is, unchanging in time, then the passive permeability coefficient will be the same in both directions regardless of the inner structure of the barrier, provided that the volume of a static passive permeability barrier times the partition coefficient of the drug into the barrier, is negligible compared with the donor and receiver chamber volumes; this is the case in our system. That is, flux is symmetric because it depends only on the concentration gradient across the barrier. Static permeability across two membranes is like parallel resistance in electricity, that is, the reciprocal of the sum of the reciprocals.

For amprenavir, the passive permeability coefficient $P$ was symmetric and constant, implying that, in its presence, the cell monolayer was a static permeability barrier. With quinidine, there was an early and symmetric increase in $P$ and then a symmetric decrease in $P$. There was no significant loss of either drug. Thus, in the presence of quinidine, the change in permeability coefficient over time must reflect a structural change in one or both of the polarized cell membranes. With loperamide, for the first hour, $P$ increased symmetrically, followed asymmetric permeability coefficients, because $P(B \rightarrow A)$ reached a steady state in 1 h, whereas $P(A \rightarrow B)$ continued to increase until steady state at about 3 h. Early on, loperamide induced a structural change in one or both of the polarized cell membranes, like quinidine. However, when the permeability coefficients became asymmetric, another explanation is required. We will discuss these changes in turn.

For amprenavir and quinidine, our measurements at a given time point are similar to recent reports in which the approximate equation was used. There are also some interesting differences.

Aungst et al. reported a $P_{apx}$ for amprenavir across a Caco-2 monolayer that was essentially the same as the passive permeability coefficient we report here for the hMDR1-MDCKII cell line in the presence of the Pgp inhibitor GF120918, that is, about 200 nm/s, using the exact equation for $P$.

For quinidine, Troutman and Thakker also reported that for some drugs (ritonavir, saquinavir, taxol, and vinblastine), passive permeability measured by $P_{apx}$ was greater $B \rightarrow A$ than $A \rightarrow B$. For loperamide, we also found that $P_{apx}$ is greater $B \rightarrow A$ than $A \rightarrow B$ (Fig. 7C), which is precisely the opposite result we obtained using the exact equation. For loperamide with these cells, $P_{apx}$ cannot reliably measure permeability because there are significant mass balance problems. What is actually the case with ritonavir, saquinavir, taxol, and vinblastine is not known. Suffice it to say that a clear explanation of these asymmetries in passive transport for other drugs will require the same sort of experiment and analysis performed with loperamide, that is, usage of the exact equation at a few time points to sort out these exceptions to "expected" symmetric passive permeability behavior.

The time-dependent changes we monitored for amprenavir and quinidine are true changes in the
cell’s permeability over time, and not attributable to simple binding to cells or drug metabolism. Cellular changes to the challenge of a drug could be expected and for a cell to change its passive permeability requires modification of apical and/or basolateral membranes, because those are the primary barriers to drug permeation. This could happen in several ways, because a time scale of change of 15 min is consistent with membrane recycling.\(^\text{14}\) An increased/decreased permeability coefficient could be explained by an increase/decrease in the total area of cell membrane, on either or both of the apical and basolateral faces, or if the specific permeability per unit area was increased/decreased, due to a change in membrane bilayer composition. Both a change in membrane area and a change in membrane bilayer composition require membrane recycling. In addition, cytoskeletal changes could increase the area of membrane presentation to the aqueous compartments, without necessarily changing the total area or the composition of the plasma membrane. Understanding the molecular basis for the observed changes will be greatly facilitated by the usage of the exact equation provided here to monitor the process.

The asymmetry of the permeability coefficients for loperamide after an hour suggests more than just membrane area or accessibility changes, because those should still yield symmetric changes, that is, the same rates for B → A and A → B. The greater loss of loperamide from the apical chamber is unlikely to be solely an extraneous physical effect, for example, binding to the apparatus or collecting at the air–water interface. Something caused the cells to enhance their permeability when loperamide was added to the apical chamber.

Perhaps the simplest hypothesis for the asymmetry of loperamide passive permeation is that there is a “receptor” that binds loperamide in or near the apical membrane, whose activation or inhibition can increase the permeability of the apical membrane and the loss of drug on the apical side. When the apical chamber is donor, a relatively high loperamide concentration is available to bind to a “receptor.” However, when the basolateral chamber is the donor, then the concentration of loperamide on the apical side is relatively small for an hour or so, by which time the “receptor” may be desensitized somehow. The rate of loss of loperamide from the donor chamber, corrected for what actually reaches the receiver chamber, starts off about five times faster from the apical donor than from the basolateral donor. It is possible that the mechanism of loss for loperamide is connected to the mechanism of increased permeation. The compensation could also be serendipitous. Further experiments will be required. Clearly though, the approximate equation was insensitive to this unexpected behavior by loperamide.

Although a kinetic analysis of P-gp activity is a future goal, it is clear that passive permeability for the typical P-gp substrates studied here was substantial and not simple. This must be taken into account quantitatively in order to measure the true active transport. The common practice is to simply subtract passive transport curves from the active transport curves. Although this might be adequate for semiquantitative work, it neglects the reversibility of transport, which is substantial over the longer time courses required to model the active transport of P-gp. For example, with B → A transport, at any time point, active transport yields a higher concentration of drug in the apical chamber than does passive transport. Thus, at any given time, the passive backflow of drug from the apical chamber will be greater with active transport than with passive transport. For A → B transport, the subtraction is just as inaccurate because the passive backflow is less with active transport than with passive transport. It is more accurate to measure the passive permeability coefficients and use them directly in the mass action kinetics of active transport.

The data needed to use the approximate eq. (1) correctly or the exact eq. (2) are the same, that is, the donor and receiver concentrations at whatever time points are needed. In the past, inadequate mass balance meant that the permeability coefficient could not be obtained with any rigor, because the simple approximate equation, eq. (1), does not separate mass balance problems from passive transport, although corrections are possible.\(^\text{9}\) Our exact equation used for fitting the passive permeability yields a simple and compact equation for the calculation of a passive permeability coefficient which quantitatively accounts for poor mass balance and can be used in situations in which significant backflux may occur. The same data are used to calculate first the rate of drug loss and then the correct passive permeability coefficient. Because eq. (2) is valid when the approximate equation may not be, it is simply safer to use across a wide range of situations, even for single time point estimates in high-throughput assays.
APPENDIX A: DERIVATION OF THE PASSIVE PERMEABILITY COEFFICIENT

The instantaneous permeability coefficient \( P \) (cm/s) across a single barrier is defined as\(^{1-3}\)

\[
\frac{dC_R}{dt} = \frac{PA}{V_R} (C_D - C_R)
\]

\[
\frac{dC_D}{dt} = -\frac{PA}{V_D} (C_D - C_R)
\]

where \( A \) is the area of the cell monolayer (cm\(^2\)) available for permeation, \( V_D \) and \( V_R \) are the donor and receiver chamber volumes (cm\(^3\)) and \( C_D \) and \( C_R \) denote the molar concentrations of drug in the donor and receiver chambers. If the barrier does not change in time, \( P \) (cm/s) would be constant and the same value irrespective of which side is chosen as the donor. \( PA/V \) has the units of s\(^{-1}\), appropriate for a first-order rate constant. We found for quinidine and loperamide that the rate of passive permeation changes in time, beyond the simple dependence on the concentration gradient, due to changes in specific permeability or area of the apical and/or basolateral membranes. The simplest way to quantify this effect is to fit the value of \( PA/V \) for each time interval used, that is, between 0.1–0.25 h, 0.25–0.5 h, etc. The alternative would be to assume a functional form for the time dependence of \( PA/V \), which might become reasonable when more drugs have been tested and there is enough data to establish an appropriate functional form.

Although there are several permeability barriers across the cell monolayer,\(^3\) including the apical and basolateral membranes, our focus is to obtain an accurate fit for the exact overall average value. If drug is not sequestered substantially, then the volume of the cell monolayer, including partitioning of these compounds into the bilayers is <1% of the aqueous chambers and can be neglected.

We know that some compounds have mass balance problems, that is, loss of drug from the aqueous chambers, due to many possible reasons, including metabolism, binding to cellular sites, and/or binding to the apparatus. As explained in the Discussion section, we need to account for this loss in the simplest way and this turns out to be the assumption that loss is first order. For loperamide, this assumption was shown to be correct. For quinidine and amprenavir, there was no loss of compound, so the equation is exact for them also.

So eq. (A.1) will be expanded to

\[
\frac{dC_R}{dt} = \frac{PA}{V_R} (C_D - C_R) - k_v C_R
\]

\[
\frac{dC_D}{dt} = -\frac{PA}{V_D} (C_D - C_R) - k_v C_D
\]

where \( k_v \) is the rate constant for the vanishing of compound, by whatever mechanism. This addition is quite useful, in that it allows us to quantitate the importance of the other processes competing with pure transport, using the same data. Then, we can normalize the data for permeability by eliminating the competing processes from its analysis. If \( k_v \) is found to be near zero, that is, essentially all drug can be transported, then sequestration of drug by cells is also negligible. If a given drug shows loss by a higher order than first, then the protocol used here can give the appropriate equations, but it is likely that numerical methods will be needed to solve for the passive permeability coefficient.

To quantitate the loss of compound, we define the average concentration of drug over both aqueous chambers as

\[
\langle C(t) \rangle = \frac{C_D V_D + C_R V_R}{V_D + V_R}
\]

This average concentration is unaffected by transport and changes only when drug vanishes from the chambers. Taking the time derivative of the average concentration and using eqs. (A.2) and (A.3) yields

\[
\frac{d\langle C \rangle}{dt} = \frac{V_D dC_D}{dt} + V_R \frac{dC_R}{dt}/(V_D + V_R)
\]

\[
= -k_v \frac{(V_D C_D + V_R C_R)}{V_D + V_R} = -k_v \langle C \rangle
\]

so that,

\[
\langle C(t) \rangle = \langle C(0) \rangle \exp \{-k_v t\}
\]

By fitting the average concentration, \( \langle C(t) \rangle \), from the data using eq. (A.3), the value of \( k_v \) can be easily fitted. A process such as cellular metabolism of drug could be first order, and so be part of \( k_v \), or second order and not fit eq. (A.4) exactly, suggesting the need for a more sophisticated analysis. For example, if drug binding to the chamber wall is rapid and limited, then the average concentration will show a sharp decrease and then remain constant. This offset in the data can be normalized to permit a proper estimate of the permeability coefficient between
time points by fitting \( k_v \) between time points, as was shown for loperamide.

Now, using eq. (A.4), eqs. (A.2) and (A.3) become

\[
\frac{dC_R}{dt} = k_P(C) - (k_P + k_v)C_R
\]

where

\[
k_P = \frac{(V_D + V_R)}{V_DV_R}PA
\]

\( k_P \) is the first-order rate constant for passive transport of compound, normalized over compartment volumes. Assuming that drug concentration on the receiver side is initially zero, that is, \( C_R(t = 0) = 0 \)

\[
C_R(t) = k_P \exp\left\{- (k_P + k_v)t\right\} \int_0^t \exp\left\{+ (k_P + k_v)s\right\} ds
\]

By the same integration scheme

\[
C_D(t) = \langle C(t) \rangle\left(1 + \frac{V_R}{V_D} \exp\{-k_Pt\}\right)
\]

Solving for \( P \) in eq. (A.6) yields

\[
P = -\left(\frac{V_RV_D}{(V_R + V_D)At}\right) \ln\left\{1 - \frac{C_R(t)}{\langle C(t) \rangle}\right\}
\]

This equation is a novel formulation for computing the permeability coefficient, because it uses the average system concentration of compound, \( \langle C(t) \rangle \), obtained from the same experimental data, to compensate for drug vanishing.

The classic approximate solution is defined in eq. (1). In the limit of \( t \rightarrow 0 \), eq. (A.8) and eq. (1) are equal. In time, the approximate equation will progressively underestimate the permeability coefficient. When there is drug loss, the estimate will become worse, as shown in Figures 2C, 4C and 7C.

**APPENDIX B: AUTOMATIC CORRECTION FOR THE MULTIPLE TIME POINT SAMPLING VOLUME**

The equations derived thus far assume that there is no significant loss of drug due to sampling, which need not be the case. Based on our previous experience with the kinetics of membrane fusion, \(^{15}\) to fit active transport for P-gp will require eight or more time points, for both active and passive transport. If sampling volumes are 0.025 mL, one would remove >40% of the drug in the apical chamber, which initially holds 0.5 mL. To add these volumes back experimentally doubles data acquisition times and proper mixing cannot be guaranteed without turbulence which could affect cell integrity and transport. We have chosen to correct for this volume loss within the analysis of the data, which makes the experimental protocol far simpler.

Permeation can be understood as being driven by the distance to equilibrium, which is measured by the difference between the chamber concentrations, \( C_D(t) \) and \( C_R(t) \), and the system average concentration, \( \langle C(t) \rangle \). Equilibrium is reached when all are equal. Sampling leaves the chamber concentrations unchanged, but not the system average concentration, because the apical and basolateral chambers have different volumes. \( \langle C(t) \rangle \) will either “instantly” increase, when \( V_D > V_R \), or decrease, when \( V_D < V_R \). Mass action kinetic equations require the initial conditions of the system at each step, which changes discontinuously at each of the sample times.

There are two ways to do this correction, which would be identical if there were no experimental error. The first way is to correct the measured concentrations at each time point for the volume loss and use the equations as written above. In other words, the permeability at time \( t \) would be calculated according to the initial conditions at \( t = 0 \). The advantage is that the equations used are the simplest to write down. The disadvantage is that an error in sampling at some intermediate time point would be propagated through all subsequent time points. This is a serious flaw.

The second way to introduce the sampling correction is to treat each time interval separately. In other words, the permeability at time \( t \) would be calculated according to the initial conditions just after the previous sampling. The disadvantage is that the equations appear more complex. The advantage is that sampling error within the interval is contained within the interval. This seems the better course to take.
Thus yields for use the initial condition that which is correct within any time interval, and denoted the time of the time period. Because that is the initial condition for the next time period.

To keep these separate needs straight, within a reasonably convenient notation, we will let \( t_j \) denote the time of the \( j \)th sampling and \( t_{j-1}^* \) denote the time just after the \( j \)th sampling, when the concentrations and volumes just after the sampling, because that is the initial condition for the next time period.

We assume that sampling is rapid compared with the permeation kinetics, so that \( t_{j-1}^* - t_j \leq 1 - 2 \text{ s} \), and can be considered negligible because permeation occurs over hours. This means that \( t_{j-1}^* - t_{j-1} \approx t_j - t_{j-1} \), which is used in eq. (B.2). Thus

\[
k_v = -\ln\left(\frac{(C(t_j))}{(C(t_{j-1}))}\right) / (t_j - t_{j-1})
\]

To calculate \( C_R(t_j) \), we can start with eqs. (A.6), which is correct within any time interval, and use the initial condition that \( C_R(t_{j-1}) > 0 \) for \( j \geq 1 \). This yields for \( t_{j-1} \leq t \leq t_j \)

\[
C_R(t) = \frac{(C(t))(1 - \gamma_R(t_{j-1}) \exp(-k_p(t)(t - t_{j-1}))}{C_D(t) = \frac{V_R(j)\gamma_R(t_{j-1}) \exp(-k_p(j)(t - t_{j-1}))}{Y_R(j)}}
\]

where

\[
k_p(j) = \left( \frac{1}{V_D(j)} + \frac{1}{V_R(j)} \right) PA
\]

\[
\gamma_R(t) = 1 - \frac{C_R(t_{j-1})}{(C(t_{j-1}))}\n\]

Solving for \( P \), for the time range of \( t_{j-1} \geq t \geq t_j \), yields

\[
P = -\left( \frac{1}{V_D(j)} + \frac{1}{V_R(j)} \right)^{-1} \frac{1}{A(t_j - t_{j-1})} \ln\left( \frac{\gamma_R(t_j)}{\gamma_R(t_{j-1})} \right)
\]

REFERENCES


