In vitro trans-monolayer permeability calculations: often forgotten assumptions

Kuresh A. Youdim, Alex Avdeef and N. Joan Abbott

In designing effective therapeutic strategies, novel drugs must exhibit favorable pharmacokinetic properties. The physicochemical characteristics of a drug, such as pK_a, molecular weight, solubility and lipophilicity, will influence the way the drug partitions from the aqueous phase into membranes, and thus, will influence its ability to cross cellular barriers, such as the lining of the gastrointestinal tract and the blood–brain barrier. Physicochemical characteristics also influence the degree to which a drug is able to cross a barrier layer, and the route by which it does this; whether transcellular (across the cells) - by diffusion, carrier-mediated transport or transcytosis - or paracellular - by diffusing through the tight junctions between the cells. The *in vitro* model systems that are currently employed to screen the permeation characteristics of a drug often represent a compromise between high throughput with low predictive potential and low throughput with high predictive potential. Here, we will examine the way in which *in vitro* cellular permeability assays are often performed and the assumptions that are implied but sometimes forgotten, and we will make simple suggestions for improving the methodological techniques and mathematical equations used to determine drug permeability.

Kuresh A. Youdim
Antioxidant Research Group
Wolfson Centre for
Age-Related Diseases
Guy's, King's and St Thomas'
School of Biomedical Sciences
King's College, London
UK SE1 1UL
Alex Avdeef*
*p*ION INC, 5 Constitution Way
Woburn, MA01801, USA
N. Joan Abbott
Blood-Brain Barrier Group
Centre for Neuroscience
Research, Guy's, King's and
St Thomas' School of
Biomedical Sciences, King's
College, London, UK SE1 1UL
*e-mail: joan.abbott@kcl.ac.uk

▼ Intestinal absorption of orally administered drugs from the gastrointestinal (GI) tract is essential for successful oral drug therapy [1]. Properties that influence the ease with which a drug can be absorbed include dissolution rate and solubility (determining how fast the drug reaches its maximum concentration (C) within the lumenal intestinal fluid), and the permeability coefficient (P) (determining the rate at which the drug will cross the intestinal wall to reach the portal blood circulation). Together, these factors comprise Fick's first law, describing the flux (J_{wall}) of a drug across the intestinal wall [2]:

$$J_{wall} = P_{wall} \times C \qquad \text{[Eqn 1]}$$

In designing effective therapeutic strategies, novel drugs must be proven to exhibit favorable pharmacokinetic properties. When screening for permeation characteristics, the choice of test system always represents a compromise between high throughput with low predictive potential and low throughput with high predictive potential. For pharmaceutical companies, the emphasis is placed on HTS, which provides an early understanding of the oral bioavailability of a drug, yielding important information to assess whether further development of lead compounds should continue. Among the *in vitro* systems employed to predict oral bioavailability in humans, Caco-2 monolayers have come to be regarded as the best model in terms of throughput and reliability [3–9]. As a result, this model is becoming the most widely used screen for oral absorption potential in drug discovery; MDCK cells are also widely used. One shortcoming of many *in vitro* studies examining drug permeability is that the experimental procedures employed do not mimic the physiological situation. In this regard, experimental attention to buffer pH, the use of serum proteins and cosolvents, and the influence of the unstirred water layer are sometimes overlooked. Figure 1 depicts the barriers across which drugs must pass to reach the apical surface of the epithelial or endothelial cell. From this, it is clear that when using Caco-2 cells to gain information about drug permeability across the small intestine, the experimental conditions should reflect the real *in vivo* conditions. Similar points arise from studies examining drug permeability across the blood–brain barrier (BBB) (Figure 2).

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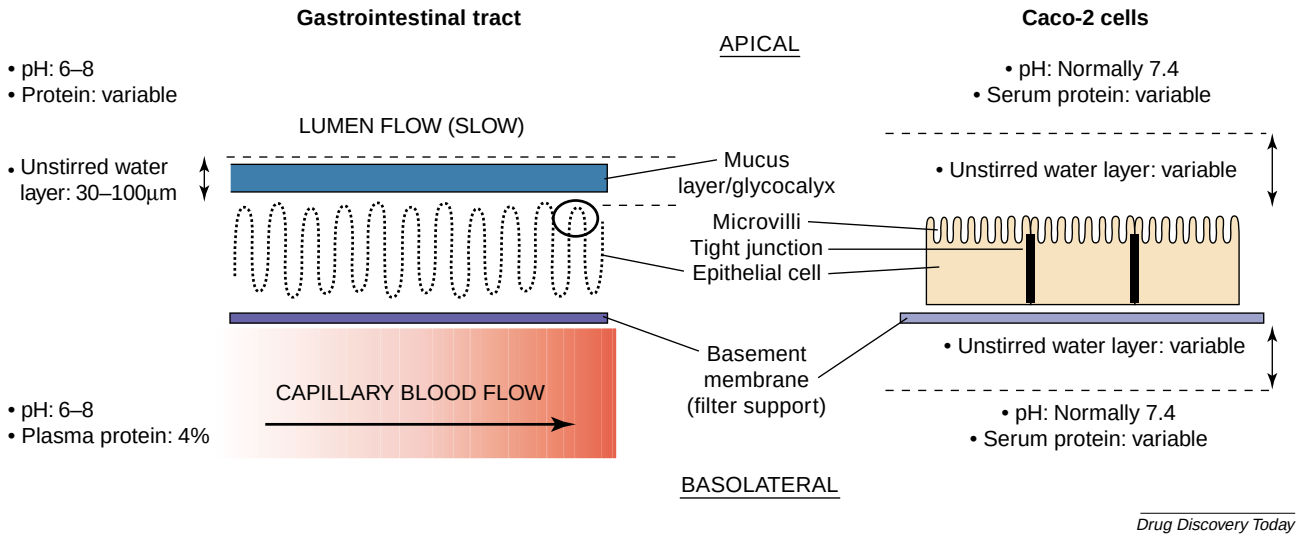

Figure 1. A comparison of the *in vivo* barrier, across which drugs must pass to reach the apical surface of the epithelial cell, with that generated by *in vitro* cell monolayer systems.

Important mathematical refinements are also often overlooked. Many of the permeability equations fail to take into account cellular retention. When examining drug permeability across *in vitro* models of the epithelial layer of the GI tract [3–14] and the brain endothelium forming the BBB [15–17], it is customary to use equation 2, derived from Fick's first law.

$$P_{app}\ (cm/s) = V_R / (A\ C_{D0}) \times (\Delta C_R / \Delta t) \qquad [Eqn\ 2]$$

where P_{app} = apparent permeability coefficient V_R = basolateral (receiver) volume (cm^3), A = membrane surface area (cm^2), C_{D0} = apical (donor) concentration at start of experiment, and $\Delta C_R/\Delta t$ = change in concentration of compound in receiver compartment over time (sec).

A mathematically equivalent version of equation 2 can be generated using mass terms instead of concentration (equation 3), which has the advantage that mass balance can be readily followed.

$$P_{app}\ (cm/s) = V_D / (A\ M_D) \times (\Delta M_R / \Delta t) \qquad [Eqn\ 3]$$

where V_D = apical (donor) volume (cm^3), M_D = apical (donor) amount (mol), $\Delta M_R/\Delta t$ = change in amount (mol) of compound in receiver compartment over time. ΔM_R, can be measured as: i) the amount of compound accumulating at the end of a single time point (t) or, ii) the amount of compound transferred to the receiver compartment (but removed to maintain sink conditions) over several smaller intervals of time (Δt). In the second case, $\Delta M_R/\Delta t$ is determined from the slope of ΣM_R versus time. In general, only incubations over longer periods of time, (>2 h), will have an effect on the P_{app} values. The approach used to denote the apical donor amount (M_D) will also influence the reported P_{app} values. Traditionally, P_{app} is calculated from the initial amount of compound in the donor well at time 0 ($M_D = M_{D0}$), referred to here, as the cumulative permeability, $P_{app}{}^{cum}$. However, because the amount of compound in the donor well will decrease over time under conditions of unidirectional flux (zero trans condition), a more accurate P_{app} can be calculated by using the actual amount present in the donor well at each sampling time point ($M_D = M_{D0} - M_R$, corrected for loss over time), thus giving the incremental permeability, $P_{app}{}^{incre}$. Employing these experimental and mathematical improvements when examining drug bioavailability will help to discriminate between the permeation properties of different drug candidates and provide a better understanding of their SAR.

Similar principles apply to the assessment of permeation across further specialized barriers, such as the endothelium that forms the BBB (Figure 2). Several *in vitro* models have been developed for assessing BBB permeability [18,19]. Some are derived from brain endothelial cells; in the more sophisticated models, the endothelium is cultured above a layer of astroglial cells to mimic the BBB-inducing effects of glia *in vivo*. Simpler models and 'surrogate BBB' models that show some, but not all, BBB features have also been employed – these include Caco-2 and MDCK epithelial monolayers, and the human ECV304 cell line (showing endothelial/epithelioid features) co-cultured with C6 glioma

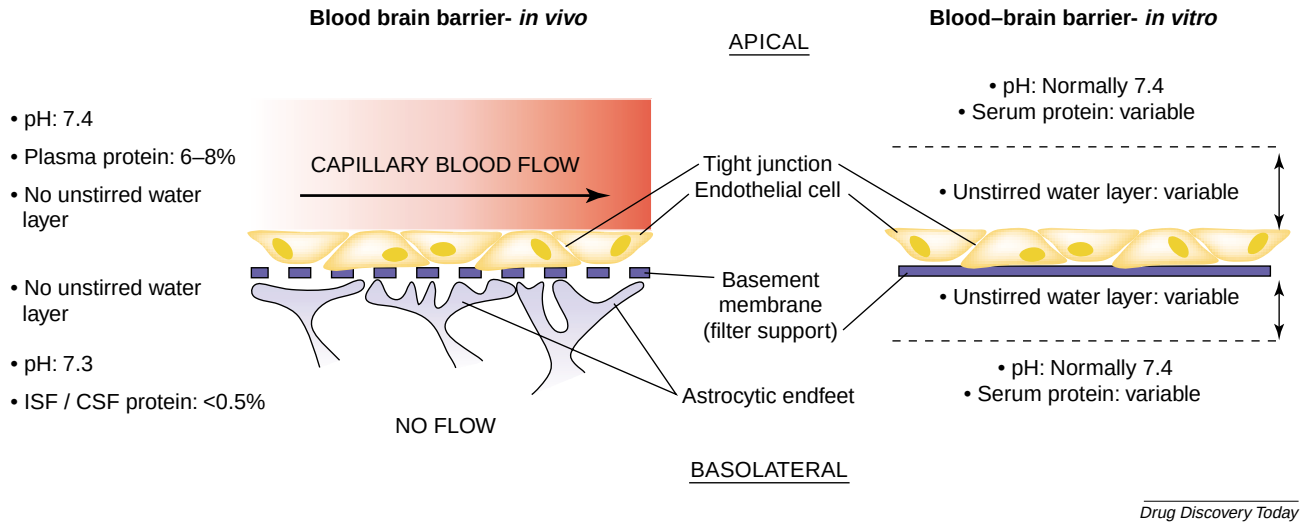

Figure 2. The differences between properties at the endothelial surface of blood-brain barrier capillaries *in vivo* and those generated by *in vitro* cell monolayer systems.

cells, and, again, Fick's first law applies. However, there are significant differences in the experimental design used for assessing permeability across the BBB and GI tract, reflecting the differences acting at apical and basal surfaces of these layers *in vivo* (Figures 1 and 2).

Here, we examine the way *in vitro* cellular assays on GI and BBB models are most often performed [3–22], what assumptions are implied but perhaps forgotten, and make suggestions for improving the methods in very simple ways. Some of the suggestions are based on the insights generated from artificial membrane models, based on the technique called PAMPA (parallel artificial membrane permeability assay) [2,23–25].

Physiologically-relevant *in vitro* conditions

pH

Many ionizable molecules coexist in both their charged and uncharged forms in solution. At physiological pH 7.4, most drug molecules are predominantly ionized. The pH partition hypothesis assumes that the uncharged form of a molecule might diffuse across lipid membranes, but that the charged form is impermeable. Lumenal pH values differ throughout the GI tract, therefore, the extent of permeation depends partly on where absorption takes place. The relationship between P_{app} and pH is an important consideration when designing *in vitro* permeability assays.

Said *et al.* [26] directly measured the 'acid microclimate' on the surface of intestinal epithelial cells (intact with mucus layer) in rats. The pH value on the apical (donor) side of the cells ranged from 6 to 8, whereas the value on the basolateral (receiver) side was ~7.4. Furthermore, the pH gradient between the donor and receiver sides varied along the longitudinal axis of the intestine. The surface pH value in the proximal duodenum is ~6.4, dropping to 6.0 in the proximal jejunum and then gradually rising to 6.9 in the distal ileum. Others have measured the intestinal microclimate pH value to be as low as 5.2 [27]. Surprisingly, the surface of the stomach is stable at pH 8, despite the contents being a possible pH <2 [28].

Measurements of permeability using cellular models are seldom performed at more than a single pH value. Adson *et al.* [4], Pade and Stavchansky [5], and Yamashita *et al.* [7] described Caco-2 measurements at two values of pH, whereas Palm *et al.* [15] thoroughly characterized the permeability–pH profiles of alfentanil and cimetidine at nine different pH values from pH 4.8–8.0. Yamashita and co-workers determined drug permeability by performing Caco-2 assays under the following pH conditions: pH 6.0 $_{apical}$ - 7.4 $_{basolateral}$ and pH 7.4 $_{apical}$ - 7.4 $_{basolateral}$ [7]. These values accurately reflect the microclimate range in the GI tract. Weak acids were more permeable under the gradient-pH condition, in comparison to the iso-pH condition; weak bases behaved in the opposite way; uncharged molecules showed the same permeability under the two conditions. The gradient-pH set of permeability measurements better predicted human absorption than the iso-pH set (r^2 = 0.85 versus 0.50, respectively).

At the BBB, the lumenal (apical) membrane of the endothelium faces a blood compartment of pH~7.40, whereas the brain side (basal) faces a pH value of ~7.33, hence, the effects of pH value are much less crucial here, than they are in the GI tract. Nevertheless, this small

difference in pH value might affect the ionization state and, thus, the permeation rate of drugs in subtle ways.

Serum proteins

The use of bovine serum albumin (BSA) in the receiver buffer is recommended for GI assays, because it has been shown to significantly improve permeability of more lipophilic drugs, by providing a more favorable 'sink condition' for partitioning of lipophilic compounds [2]. The inclusion of BSA (upto 4%) is particularly important for Caco-2 studies because it mimics the physiological presence of albumin within the capillary lumen [7,8]. Sawada and coworkers [12,13] characterized the iso-pH 7.4 permeabilities of very lipophilic molecules, including chlorpromazine, across MDCK cells. They used 3% wt/vol BSA on the apical side, and 0.1–3% BSA on the basolateral side, and found that plasma protein binding greatly affected the ability of lipophilic molecules to permeate cellular barriers. The group concluded that the rapid rate of disappearance of lipophilic compounds from the donor compartment was controlled by the unstirred water layer (UWL), a rate that was similar for most lipophilic compounds. However, the very slow appearance of the compounds in the receiving compartment depended on the rate of desorption from the basolateral side of the membrane, which was strongly influenced by the presence of serum proteins in the receiving compartment. They recommended the use of serum proteins in the receiving compartment, so as to better mimic *in vivo* conditions when using cultured-cell *in vitro* assays.

Yamashita *et al.* [7] also studied the effect of BSA on transport properties in Caco-2 assays. They observed that the permeability of highly lipophilic molecules could be rate-limited by the process of desorption from the cell surface into the receiving solution, due to high membrane retention and very low water solubility. They recommended the use of serum proteins in the receiver compartment when lipophilic molecules are assayed (a common circumstance in discovery settings). However, their advice is not usually followed in HTS applications.

The use of BSA in BBB permeability studies is less crucial than other considerations but inclusion of 0.1% BSA helps to stabilize tight junctions and is close to the normal CSF level. At the BBB, the blood compartment contains high circulating levels of plasma proteins (~6–8% of plasma, w/v), whereas, the brain interstitial fluid (ISF) and cerebrospinal fluid (CSF) are low protein solutions (≤1/200 of the concentration in plasma). Hence, it could be argued that this kind of protein gradient should be mimicked *in vitro*; adding protein to the ablumenal compartment could artificially elevate P_{app} by increasing desorption from the cell surface.

Cosolvents, bile acids and other surfactants

Yamashita *et al.* [7] added upto 10 mM taurocholic acid, cholic acid, or sodium lauryl sulfate (SLS) to the donating solutions in Caco-2 assays, to mimic the GI contents and drug excipients. The two bile acids did not affect the transport of dexamethasone. However, SLS caused the Caco-2 cell junctions to become leakier. Also, the permeability of dexamethasone decreased at 10mM SLS. The researchers also tested the effect of the solubilising agents PEG400, DMSO and ethanol, with upto 10% added to solutions in Caco-2 assays. PEG400 caused a dramatic decrease (75%) in the permeability of dexamethasone at 10% cosolvent concentration, DMSO caused a 50% decrease, but ethanol had only a slight decreasing effect. For the BBB, cosolvents are generally absent from the blood or brain side *in vivo*, and so do not generally need to be considered under normal conditions *in vivo*. Exceptions might include cases where a drug is designed for intravascular administration (for example, i.v.) in a cosolvent.

Unstirred water layer (UWL)

Passive transport across a membrane barrier is a combination of diffusion through the membrane and diffusion through the unstirred water layers (UWLs) either side of the membrane. When the solute is introduced into the bulk aqueous phase, convective mixing, resulting from applied stirring, quickly positions the drug molecule next to the UWL. At this point, the passage through the UWL is governed by the laws of diffusion. In simple hydrodynamic models, the UWL is thought to have a distinct boundary with the rest of the bulk water; it can be 'thinned' by more vigorous stirring, but cannot be made to vanish.

Owing to the efficient mixing near to the surface of the GI epithelium, the *in vivo* UWL is estimated to be 30–100μm thick [1]. In the case of Caco-2 studies (Figure 1), in the absence of stirring, the thickness of the UWL can be >1000μm. Studies investigating drug permeability across the BBB *in vitro* should also take in account the influence of the UWL (Figure 2). Physiologically, the UWL in the endothelial microcapillaries of the brain is nil, considering that the diameter of the capillaries is ~6μm and the tight fit of the distorted erythrocytes gives efficient mixing [29]; the dynamics of blood-flow through structures this narrow, result in virtually no UWL at the apical (lumenal) surface of the endothelial cells. A recently introduced 'dynamic *in vitro* BBB' (DIV-BBB) model, in which endothelial cells are grown, lining porous tubes with flowing lumenal solutions [37], reduces UWL on the lumenal side, but the perfused lumen format makes flux calculations more complex than those for flat cultures. To reflect the behaviour of a drug at the *in vivo* epithelial and endothelial surfaces, it is necessary to minimize the UWL

produced in the *in vitro* model. If the cellular assays ignore the UWL effect with lipophilic test compounds, the resulting permeability values will not correctly indicate the *in vivo* conditions of permeability, and will merely reveal properties of water, rather then membrane permeation. For horizontal filters, in the normal HTS mode, this is best achieved by performing permeability studies on a rocking plate or shaker, which reduces the influence of the UWLs (normally present under static conditions), thereby increasing drug permeability. Optimum speeds have been determined to range from 25–150 RPM [4,6,8].

Cellular retention

Cellular measurements of permeability usually focus on appearance or disappearance kinetics, carefully characterizing the concentration of the solute only on one side of the membrane barrier. As lipophilic compounds permeate across a cellular barrier, a fraction of the solute is retained by the barrier. Sawada *et al.* [17] studied the transport of chlorpromazine across MDCK cell monolayers and observed 65–85% retention of the drug molecule by the MDCK cells. Wils *et al.* [14] reported retentions as high as 44% in Caco-2 cells. In a later publication, Sawada *et al.* [13] cited values as high as 89% for a homologous series of lipophilic molecules. More recently, Krishna *et al.* [11] reported Caco-2 permeability results for lipophilic molecules, including progesterone and propranolol, finding retentions to be as high as 54%. This is clearly a common phenomenon with research compounds, which are often very lipophilic. Yet, in most reported assays, the effect is ignored. Ho *et al.* [8] have suggested an equation to describe the phenomenon in cultured cells, but its application in cultured-cell assays is, thus far, scarce. However, it has been considered in a recent *in vitro* BBB study [30].

We recommend a refined approach, by which P_{app} is calculated by (a) taking into account the real driving force acting on molecules moving across the monolayer and (b) allowing for retention in the cells; but these refinements are not generally used in standard methods of calculation [20,21]. Equation 3 assumes that there is no mass loss to the membrane (or cell monolayer). However, as suggested previously, membrane retention can be substantial with some lipophilic compounds [11,14]. The use of equation 4 (derived from equation 3), takes into account mass balance, correcting for any compound retention in the cell monolayer.

$$P_{app} = V_D / [A \times (M_D - M_{cells})] \times (\Delta M_R / \Delta t) \qquad \text{[Eqn 4]}$$

where M_{cells} is the amount (mol) of compound retained by the membrane/cells. By correcting M_D for the amount lost to the cells, $P_{app}^{\ cum(corrected)}$ and $P_{app}^{\ incre(corrected)}$ values will take into account the disposition of the entire compound mass, a feature that many permeability studies overlook. For stable compounds, the mass lost to the cells/membrane (retention) is calculated from the difference between the total starting amount and the amounts in donor and receiver compartments at the end of the experiment (time=t) ($M_{cells}=M_{D0}-(M_{Dt} + \Sigma M_R)$. If any loss is due to compound breakdown or metabolism, this will affect the accuracy of the P_{app} calculation; analytical techniques that detect metabolites should be used to reduce these errors, and, experimentally, the use of plastics and filters should be consistent

Use of artificial membranes to identify factors important in permeabililty

Those aspects of *in vitro* cellular models that relate to passive diffusion of drug molecules can be studied using a simpler model, called PAMPA, first described by Kansy *et al.* [23]. This method uses a phospholipid-coated filter, separating two aqueous compartments, to mimic the passive transport of small molecules. Because of its speed and versatility, it is a particularly helpful complement to cellular permeability models; readily providing information about passive-transport permeability that is not complicated by other mechanisms. Most PAMPA research has focused on identifying ideal experimental conditions for *in vivo–in vitro* correlations. Modifications of the phospholipid components, solvent constituents/additives/pH, and thermodynamics (e.g. chemical scavanger in the acceptor compartment) have all been tested [2,25]. Interestingly, different compositions of artificial or reconstituted lipids have to be used to mimic *in vivo* GI and BBB permeability [31]. This is likely to reflect differences in the *in vivo* membrane composition of the rate-limiting barrier layer (GI epithelium and BBB endothelium) [32,33].

Recommendations

Study of the literature suggests that the ideal intestinal *in vitro* permeability assay would have pH 6.0 and 7.4 in the donor wells, with pH 7.4 in the receiver wells (a two-pH combination could differentiate acids from bases and non-ionizables by the differences between the two P_{app} values) [2]. Furthermore, the receiver side would have 3% wt/vol BSA (or some sink-forming equivalent) to maintain a sink condition. The donor side may benefit from having a bile acid (i.e. taurocholic or glycocholic, 5–15mM), to solubilize the most lipophilic sample molecules. This might be important in HTS applications, when screening very lipophilic molecules. Excessive DMSO (>10% w/v) or other cosolvents should be avoided, due to their multi-mechanistic

Table 1. Experimental and mathematical recommendations for monolayer permeability studies

General recommendations	Industry-HTS	Mechanistic
Use Eqn 3 to include mass terms	X	X
Use Eqn 4 to account for mass loss the cell monolayer (retention)	X	X
Perform permeability studies at multiple time points (ΔM_R / Δt determined from the slope of the plot ΣM_R versus time)		X
Minimize influence of UWL by conducting studies on shaker or rocking platform	X	X
Introduce BSA (upto 4%) in receiver medium		
Caco-2	X	X
BBB		X

effects [2]. In *in vitro* assays, where permeabilities of lipophilic molecules are diffusion-limited, the role of the UWL should be taken into account, because under *in vivo* conditions, the UWL is almost absent, especially in the BBB. It is further proposed that drug permeability, whether across Caco-2 or BBB monolayers, should be calculated using mass terms, taking into account membrane retention (Eqn 4). When possible, it is recommended that experimental conditions are employed to mimic physiological conditions; for example, performing studies on a shaker to reduce the effects of the UWL and including BSA in the receiver buffer (Caco-2) for highly lipophilic drugs. Where possible, the kinetic transport of a drug into the receiver well should be studied at several, rather than at a single time point (using fresh wells for each time point). This will reduce back flux and maintain conditions closer to unidirectional flux throughout the experiment, mimicking the physiological clearance of drug in the capillary. For HTS robotic mode, the donor solution is sampled at t=0 and at the end of the experiment, whereas, receiver wells can be sampled at several time points (e.g. 30, 60, 90, 120 min). Under these conditions, aliquots are removed from the receiver well and replaced with an equal volume of fresh buffer. Although employing this procedure will reduce back flux, it fails to fully reproduce the physiological situation.

To validate the importance of each of these recommendations in establishing robust *in vitro* models that represent the physiological scenario, a series of detailed *in vitro–in vivo* comparisons are required. In addition to experimental and mathematical considerations, *in vitro* models must also demonstrate the physiological influence of active transporters and metabolizing enzymes [38]. For example, the efflux transporter, P-glycoprotein (P-gp) might be the single most important factor that limits the permeability of certain drugs. Hence, a properly optimized permeation assay will take in account the transporter profile and the influence of metabolizing enzymes, relevant to the model used, GI or BBB.

Conclusion

The choice of a test system always represents a compromise between high throughput with low predictive potential and low throughput with high predictive potential. Table 1 highlights mathematical and experimental techniques that should be taken into account when performing HTS and/or mechanistic studies. The introduction of radical changes to permeability studies is not the intention here; the intention is to make researchers, working within the industrial sector or academia, aware of how their drug permeability data reflects what might occur physiologically. The recommendations are directed towards obtaining more consistent data that will enable reliable comparisons between permeability values obtained from different researchers, helping to discriminate between the permeation properties of different drug candidates, providing a better understanding of SAR and helping to produce accurate data of value in producing the next generation of *in silico* modelling and predictive tools for barrier-layer permeation of drugs.

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