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# Biopharmaceutics Classification System: Validation and Learnings of an in Vitro Permeability Assay

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**Abstract:** The Biopharmaceutics Classification System (BCS) is the scientific basis for classifying drugs based on their aqueous solubility and intestinal permeability that supports in vivo bioavailability and bioequivalence waivers for immediate-release solid dosage form drugs. One requirement of the BCS is that the permeability method must be validated. In order to accommodate the variety of in vitro/in situ permeability models, the BCS Guidance gives a general framework for the validation requirements, necessitating implemented experimental details to be selected by the applicant laboratory. The objective of this work was to define the parameters for a cell based in vitro permeability method (e.g., cell type, pH, transport direction, time, and concentration) and validate the method to support formal BCS classification of drugs. Twenty reference drugs were selected and permeability values determined using the Madin-Darby canine kidney type II cell line heterologously expressing the human P-glycoprotein transporter (MDCKII-MDR1). A rank order relationship was established between the in vitro permeability value and human intestinal absorption values. This relationship was as predicted and validates the MDCKII-MDR1 permeability method as defined by the BCS Guidance. The final validated in vitro permeability method employs the MDCKII-MDR1 cell line incubated with the Pgp inhibitor GF120918. It is a unidirectional apical-to-basolateral transport assay performed at apical pH values of 5.5 and 7.4 and a basolateral pH of 7.4. Four reference standards (metoprolol, pindolol, labetalol and ranitidine) dosed and analyzed as a single cassette are included in each experiment. A strategy on selection of drug concentrations and on how to deal with problematic compounds (i.e., those suffering from poor mass balance) is discussed.

**Keywords:** In vitro; MDCKII-MDR1; BCS; BDDCS; passive permeability; biopharmaceutics classification system; intestinal absorption; Caco-2; P-glycoprotein; transporter

## Introduction

The Biopharmaceutics Classification System (BCS) is the scientific basis for classifying drug substances based on their aqueous solubility and intestinal permeability,[1−3] providing an opportunity for a waiver of the in vivo bioequivalence test for highly permeable, highly soluble drugs formulated as a rapidly dissolving solid oral dosage forms (e.g., BCS Class 1 drugs). The Guidance permits the use of human pharmacokinetic studies (absolute bioavailability or mass balance study) and intestinal permeability methods (e.g., in vivo human intestinal perfusion, in vivo or in situ animal intestinal perfusion, in vitro excised human or animal intestinal tissue, in vitro cultured epithelial cell monolayers) as methods to determine permeability classification. As in vivo studies such as human intestinal perfusion or absolute bioavailability are often difficult and costly to perform, the Guidance provides an alternative to extrapolate a drug's rate and extent of absorption using in vitro−in vivo correlations (IVIVC).

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According to the Guidance, to demonstrate suitability of a permeability method, a rank order relationship between drug substance permeability values and the extent of absorption in humans must be established using a set of 20 reference drugs. The method must be able to differentiate between low and high permeability drug substances. The Guidance provides this basic framework for the validation, along with a list of drugs for which reliable information is available on the mechanism and extent of absorption in humans and that can be considered in the validation of a permeability assay. Once validated, the permeability method can be used to classify drugs against a minimum of two reference standards. If the test drug's permeability is equal to or greater than that of the selected high permeability standard, the test drug can be classified as highly permeable.

Therefore, this study was designed to determine the in vitro permeability category of a set of 20 validation drugs according to the BCS utilizing MDCKII-MDR1 cells. The final validated in vitro permeability method is a unidirectional apical-to-basolateral transport assay completed at apical pH values of 5.5 and 7.4 employing the MDCKII-MDR1 cell line in the presence of the Pgp inhibitor GF120918. Four reference standards (metoprolol, pindolol, labetalol and ranitidine) dosed and analyzed as a single cassette are included in each experiment. A strategy on selection of drug concentrations and on how to deal with problematic compounds (i.e., those suffering from poor mass balance) is discussed.

## Material and Methods

**Materials.** Amoxicillin, atenolol, carbamazepine, cimetidine, hydrochlorothiazide, ketoprofen, lisinopril, metoprolol, minoxidil, nadolol, pindolol, propranolol, sulpiride and verapamil were purchased from Sigma-Aldrich (St Louis, MO). Acyclovir, amprenavir, [$^3$H]-amprenavir, labetalol, ranitidine, trimethoprim and GF120918 were supplied by GlaxoSmithKline Chemical Registry (Research Triangle Park, NC). Theophylline was purchased from Acros Organics (Morris Plans, NJ), and naproxen from Biomol International (Plymouth Meeting, PA). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO) or other commercial suppliers. The polarized Madin-Darby canine kidney (MDCKII) cells heterologously expressing human Pgp (MDCKII-MDR1 cell line) were obtained from The Netherlands Cancer Institute (Amsterdam, Netherlands). Transwells (24-well) were purchased from Corning Costar (Cambridge, MA).

**Cell Culture.** The MDCKII-MDR1 cells were cultured to confluency, trypsinized and seeded onto Becton Dickinson Falcon HTS 24-Multiwell Insert Systems with PET (polyethylene terephthalate) membranes (1 $\mu$m pore size and 0.30 cm$^2$ surface area) at a density of 170,000−300,000 cells/cm$^2$ in cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, 10% (v/v) fetal bovine serum and without 0.5% (v/v) penicillin/streptomycin 10,000 units/mL. The cell monolayers were fed with cell culture media 24 h prior to use and used for permeability studies 3 to 4 days post seeding.

**Preparation of Stock and Working Solutions.** Separate stock solutions (10 mM) of all of the validation drugs except lisinopril were prepared in dimethylsulfoxide (DMSO). Lisinopril (10 mM) was prepared in 50:50 acetonitrile/water. For experiments, donor working solutions of each test drug, with and without the P-glycoprotein inhibitor GF120918 (2 $\mu$M), were prepared at 10 $\mu$M by diluting the stock solutions in transport medium (DMEM supplemented with L-glutamine, 25 mM HEPES, pyridoxine HCl but without sodium pyruvate and phenol red). Donor working solutions were prepared at pH 5.5 and pH 7.4. Lucifer yellow, used as a paracellular marker, was also added to all donor working solutions at a concentration of 100 $\mu$M. Receiver working solutions were composed of transport medium, pH 7.4, with or without the addition of 2 $\mu$M GF120918. All working solutions were prepared such that the final concentration of DMSO was $\leq$1% (v/v).

**Permeability Studies for the 20 Validation Drugs.** The permeability of the 20 validation drugs was determined at apical pH values of pH 5.5 and pH 7.4 (basolateral pH 7.4) in two directions (apical to basolateral [A → B] and basolateral to apical [B → A]) in triplicate sets of wells, in the presence and absence of GF120918. MDCKII-MDR1 cell monolayers were preincubated at 37 °C in transport medium, with or without 2 $\mu$M GF120918, for 15 to 30 min prior to the initiation of the assay. For A → B directional transport, 0.45 mL of donor working solution was added to the A compartment and 1.3 mL of receiver working solution to the B compartment. For B → A directional transport, 1.3 mL of donor working solution was added to the B compartment and 0.45 mL of receiver working solution to the A compartment. For inhibition of P-glycoprotein, both receiver and donor compartments contained GF120918 (2 $\mu$M). The cells were incubated at 37 °C with shaking for up to 120 min. At 20, 45, 90 and 120 min, 50 $\mu$L samples were taken from both the donor and receiver compartments. In addition, a 50 $\mu$L aliquot was taken from the initial donor working solutions. All samples were diluted 1:1 with acetonitrile, and test drug concentration was determined using high-performance

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liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) analysis.

At the end of each experiment (120 min), the cell monolayer integrity was evaluated by measuring the Lucifer yellow concentration in the receiver compartment. A 50 $\mu$L sample was removed from donor and receiver compartments and transferred into 96-well clear bottomed black plates. Fluorescence was determined using a SpectraMax Gemini cytofluorimeter (Molecular Devices, Sunnyvale, CA) set to an excitation wavelength of 430 nm and an emission wavelength of 538 nm.

To determine the functional expression of human Pgp in the MDCKII-MDR1 cell line, the transport of [$^3$H]-amprenavir was evaluated several times during the validation studies; amprenavir is a well characterized Pgp substrate with high passive permeability.[4] Permeability studies were performed as outlined above. At the end of the 120 min incubation period, 100 $\mu$L samples were taken from both donor and receiver compartments. [$^3$H]-Amprenavir was quantified by liquid scintillation counting using a TriCarb 2900TR liquid scintillation counter (Perkin-Elmer, Boston, MA).

**LC/MS/MS Bioanalytical Analysis.** The concentrations of the 20 validation drugs were determined by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) using validated methods (GlaxoSmithKline, methods on file). Briefly, samples were diluted using solutions containing the corresponding stable isotopically labeled drugs as internal standards. HPLC was performed using either a HP1100 or Shimadzu LC-10A VP HPLC or a Waters Acquity UPLC. Chromatography was performed using appropriate mobile phases, HPLC, and UPLC columns as detailed in method reports. Samples were analyzed by positive or negative ion turbo ionspray LC/MS/MS with a PE/Sciex API 300 with Ionics EP10+ upgrade or a PE/Sciex API 5000 according to the requirements of the drug. The calibration ranges were 5 to 5000 nM for all the drugs evaluated with the exception of theophylline which had a calibration range of 25 to 25000 nM. Raw data were analyzed with PE/Sciex software Analyst 1.4.1. SMS2000 (version 1.6, GlaxoSmithKline) was used to calculate peak area ratios (i.e., analyte/internal standard peak area ratios versus analyte concentration were constructed and a weighted $1/x^2$ linear regression applied to the data) to construct the calibration lines from which concentrations of unknowns were interpolated.

**Data Analysis.** Data from the assay were used to calculate the following parameters.

(a) Rate of transport in nmol/h/cm$^2$:

$$\text{rate of transport} = \frac{\text{cumulative total nmol transported into receiver well at time } t}{\text{time (h)} \times \text{surface area (cm}^2\text{)}}$$

(b) Apical efflux ratio calculated in the absence and presence of the Pgp inhibitor GF120918:

$$\text{apical efflux ratio} = \frac{\text{drug transport B} \rightarrow \text{A (nmol/h/cm}^2\text{)}}{\text{drug transport A} \rightarrow \text{B (nmol/h/cm}^2\text{)}}$$

(c) Permeability at pH 5.5 ($P_{5.5}$) and pH 7.4 ($P_{7.4}$) at each time point.

Aliquots (50 $\mu$L) were removed from both the donor and receiver compartments at specified time points over the course of the incubation. The sample volume removed was not replaced back into the compartments. An automatic correction for this volume loss was encompassed within the analysis of the data by allowing each time interval to be calculated separately. In other words, the permeability at time $t$ would be calculated according to the initial conditions just after the previous sampling. The permeability of the test drug at each time point at both pH 5.5 and pH 7.4 was determined using the following equations as described by Tran et al.:[5]

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

$$P = -\left(\frac{1}{V_D(j)} + \frac{1}{V_R(j)}\right)^{-1} \frac{1}{A(t_j - t_{j-1})} \times \ln\left\{\frac{\gamma_R(t_j)}{\gamma_R(t_{j-1}{}^*)}\right\} \times 10^7 \text{ nm/s}$$

$$\gamma_R(t) = 1 - \frac{C_R(t_{j-1}{}^*)}{\langle C(t_{j-1}{}^*) \rangle}$$

where $P$ = permeability at the given time point (nm/s), $C\langle t \rangle$ = the average drug concentration over both aqueous chambers at time $t$, $C_D$ = the drug concentration in the donor chamber (nmol/mL), $C_R$ = the drug concentration in the receiver chamber (nmol/mL), $V_D$ = donor well volume (mL), $V_R$ = receiver well volume (mL), $A$ = membrane surface area (cm$^2$), $t_j$ = time (h) of the $j$th sampling, $t_j$* = time (h) just after the $j$th sampling, and $\gamma_r$ = a measure of the distance to equilibrium at time $t$.

An average permeability $P_x$ at pH 5.5 ($P_{5.5}$) and pH 7.4 ($P_{7.4}$) was calculated for three monolayers, derived from the average permeability value from two to three time points for each monolayer.

## Results and Discussion

**Drug Selection.** In order to validate the in vitro permeability method, twenty drugs were selected based on a range of previously reported human intestinal absorption (HIA) and

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***Table 1.*** Summary of Apical to Basolateral Membrane Permeability at pH 5.5 and pH 7.4 and Pgp Substrate Assessment in MDCKII-MDR1 Cell Monolayers for the 20 Validation Drugs

| drug | in vitro permeability class | human intestinal absorption (% HIA)[a] | $P_{7.4}$ (nm/s) (mean ± SD)[b] | $P_{5.5}$ (nm/s) (mean ± SD) | pH effect | Pgp substrate |
|---|---|---|---|---|---|---|
| carbamazepine[c] | high | 100 | 652 ± 125 | 734 ± 88 | no | no |
| ketoprofen[c] | high | 100 | 156 ± 15 | 805 ± 30 | yes | no |
| **labetalol** | high | 95 | 201 ± 7.0 | 42 ± 1.1 | yes | yes |
| **metoprolol**[c,d] | high | 95 | 410 ± 12 | 48 ± 22 | yes | no |
| naproxen[c] | high | 99 | 311 ± 72 | 1119 ± 154 | yes | no |
| **pindolol** | high | 90 | 266 ± 21 | 50 ± 9.7 | yes | no |
| propranolol[c] | high | 95 | 447 ± 190 | 56 ± 1.2 | yes | no |
| theophylline[c] | high | 100 | 303 ± 26 | 317 ± 23 | no | no |
| trimethoprim | high | 97 | 166 ± 24 | 61 ± 2.8 | yes | yes |
| verapamil[c] | high | 100 | 263 ± 23 | 53 ± 4.5 | yes | no |
| minoxidil | low | 95 | 26 ± 1.2 | 26 ± 0.25 | no | no |
| acyclovir | low | 20 | 20 ± 4.8 | 14 ± 2.7 | no | no |
| amoxicillin[c] | low | 94 | 9.7 ± 1.3 | 10 ± 2.6 | no | no |
| atenolol[c] | low | 50 | 12 ± 3.6 | 8.0 ± 4.3 | no | no |
| cimetidine | low | 85 | 13 ± 1.6 | 9.8 ± 3.5 | no | yes |
| hydrochlorothiazide[c] | low | 67 | 11 ± 2.1 | 7.6 ± 2.6 | no | no |
| lisinopril | low | 25 | 6.7 ± 2.1 | 5.0 ± 1.1 | no | no |
| nadolol | low | 35 | 11 ± 4.0 | 8.8 ± 3.0 | no | no |
| **ranitidine** | low | 50 | 7.5 ± 3.3 | 5.5 ± 1.7 | no | yes |
| sulpiride | low | 35 | 16 ± 3.8 | 8.9 ± 0.29 | no | no |

[a] Human intestinal absorption values were obtained from the literature.[2,6,16] [b] Data are the mean ± standard deviation from multiple (3 to 12) monolayers tested at a drug concentration of 10 $\mu$M. [c] Drugs listed in the BCS Guidance as possible reference drugs useful for validation.[1] [d] Drugs that are in bold were chosen as permeability reference standards.

in vitro permeability values (Table 1).[6−8] Half of the drugs were from the list provided in the FDA BCS Guidance, while the remaining drugs were chosen based on their HIA, in vitro permeability values and other characteristics (e.g., drug like properties, ease of analysis, and availability from commercial sources). The overall properties of the drug set include a wide range of human intestinal absorption values (20 to 100%), in vitro permeability values ($P_{7.4}$ range 6.7 to 652 nm/s), molecular weights (180 to 454 Daltons) and clog P values (−2.4 to 4.5) (Log P, version 8, Advanced Chemistry Development Inc., Toronto Canada).

**In Vitro Permeability Assay Development: Choice of Cell Line, Transport Direction, Drug Concentration, pH and Time Points.** The BCS Guidance does not mandate any single permeability method nor does it provide specific experimental details to guide a laboratory in establishment of an in vitro permeability assay for BCS classification. The purpose for this lack of detail is to provide flexibility and promote the implementation of BCS across laboratories, particularly those laboratories with established permeability methods.[9] Therefore, the selection and justification of the cell line, drug concentration, pH or time points is left to the laboratory establishing the assay. Caco-2 and MDCK are two of the most common cell lines used for in vitro permeability testing.[10−13] Permeability in both cell lines is well docu-

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mented to correlate with human intestinal absorption, and these are likely to be common choices for BCS permeability classification. We chose the MDCKII-MDR1 cell line based on our historical data, ease of use, acceptance in the literature as a permeability model,[10] and that we routinely culture these cells for use in other ADME studies. In our view, MDCKII and Caco-2 are both equally suited for use in BCS permeability classification.

For the validation, each of the 20 drugs was tested for efflux by measuring transport in both apical (A) to basolateral (B) and B → A directions in the absence and presence of GF120918. This was done to provide a context for P-glycoprotein efflux for drugs tested using this system in the future. Selecting the test concentration was one of the more challenging decisions, which included considerations of analytical sensitivity, cellular toxicity, historical experience and experimental efficiency. The BCS Guidance does not provide any specific framework on how best to select the concentrations for the reference standards. One key question was whether the 20 validation drugs needed to be tested at 1, 0.1, and 0.01 times (0.01×) the highest dose strength dissolved in 250 mL of water. Although technically feasible, the effort and resource requirements was determined to be impractical and unnecessary based on the wealth of information available for these drugs in the literature, as many are common standards used in permeability assays. Therefore, we selected 10 $\mu$M as the test concentration as it afforded analytical and experimental efficiencies. Based on our analysis, 14/20 compounds are within ~3-fold of the 0.01× concentration and 2/20 at the 0.1× concentration. Four compounds (amoxicillin, cimetidine, naproxen, theophyline) were tested at ~0.001× concentration (10-fold lower than the calculated 0.01× concentration). One recommendation resulting from this validation would be for others who may validate their in vitro system for BCS permeability to consider a test concentration between 50 and 100 $\mu$M. This concentration allows the validation to be run most efficiently from a bioanalytical viewpoint, results in most compounds being tested in the 0.1× to 0.01× range, and will yield little effect on the monolayer tight junctions (see further discussion below).

The absorption and permeability of a drug can be influenced by pH. For example, it is well established that acid drugs have higher permeability values at lower pH.[14,15] Further, there is increased interest in determining in vitro permeability rates at acidic as well as neutral pH to more closely mimic the in vivo conditions in the intestine and to support physiologically based pharmacokinetic modeling. Therefore, the permeability of each drug was determined at apical pH values of 5.5 and 7.4, while the basolateral pH was held constant at 7.4.[14,15] Finally, each drug was sampled at 20, 45, 90 and 120 min, and permeability values calculated over the 20 to 45, 45 to 90 and 90 to 120 min intervals as described in the Data Analysis section.

**Validation of the in Vitro Permeability Method.** According to the Guidance, to demonstrate suitability of a permeability method, a rank order relationship between drug substance permeability values and human extent of absorption must be established using a set of 20 reference drugs. The method must be able to differentiate between low and high permeability drug substances. A summary of the permeability coefficients at pH 5.5 and pH 7.4 for the 20 validation drugs determined using the MDCKII-MDR1 permeability method is shown in Table 1. Monolayer integrity of all wells was acceptable (Lucifer yellow $P_{7.4}$ values <50 nm/s), and the average mass balance of the validation drugs was between 80−120%.

A rank order relationship for the set was established between the passive permeability value calculated at (a) pH 5.5, (b) pH 7.4 or (c) highest passive permeability value at either pH 5.5 or pH 7.4 and the HIA value (Figure 1). This relationship was as predicted[11,12] and validates the MDCKII-MDR1 permeability method for BCS categorization purposes as defined by the FDA Guidance. Of the twenty drugs, ten were classified as having high permeability as their permeability values were equal to (±20%) or greater than labetalol, a drug suggested by the FDA as a high permeability reference standard.[13,16] All of these drugs have an HIA of >90%, which is consistent with the current criteria in the BCS Guidance defining a drug as "highly permeable" based on the extent of absorption in humans. Further analysis reveals that a number of drugs in the high permeability group were notably sensitive to pH, with permeability values changing greater than 3-fold for eight of ten drugs. As many of these drugs were basic drugs, the permeability values were higher at pH 7.4 than at pH 5.5. In contrast, the two acidic drugs, naproxen and ketoprofen, had higher permeability at pH 5.5. Of the ten drugs in the high permeability group, only two (labetalol and trimethoprim) were Pgp substrates. This is of note, as both labetalol and trimethoprim are well absorbed drugs, and further supports that Pgp often does not have a significant impact on the intestinal absorption of drugs[9] (see further discussion below). However, for the purpose of BCS categorization, drugs will be classified against the intrinsic passive permeability of labetalol determined in the presence of inhibition of Pgp by GF120918.

The remaining ten drugs had permeability values considerably lower (~5-fold) than that observed for labetalol. The permeability values for these drugs were independent of pH and only two drugs (cimetidine and ranitidine) were Pgp

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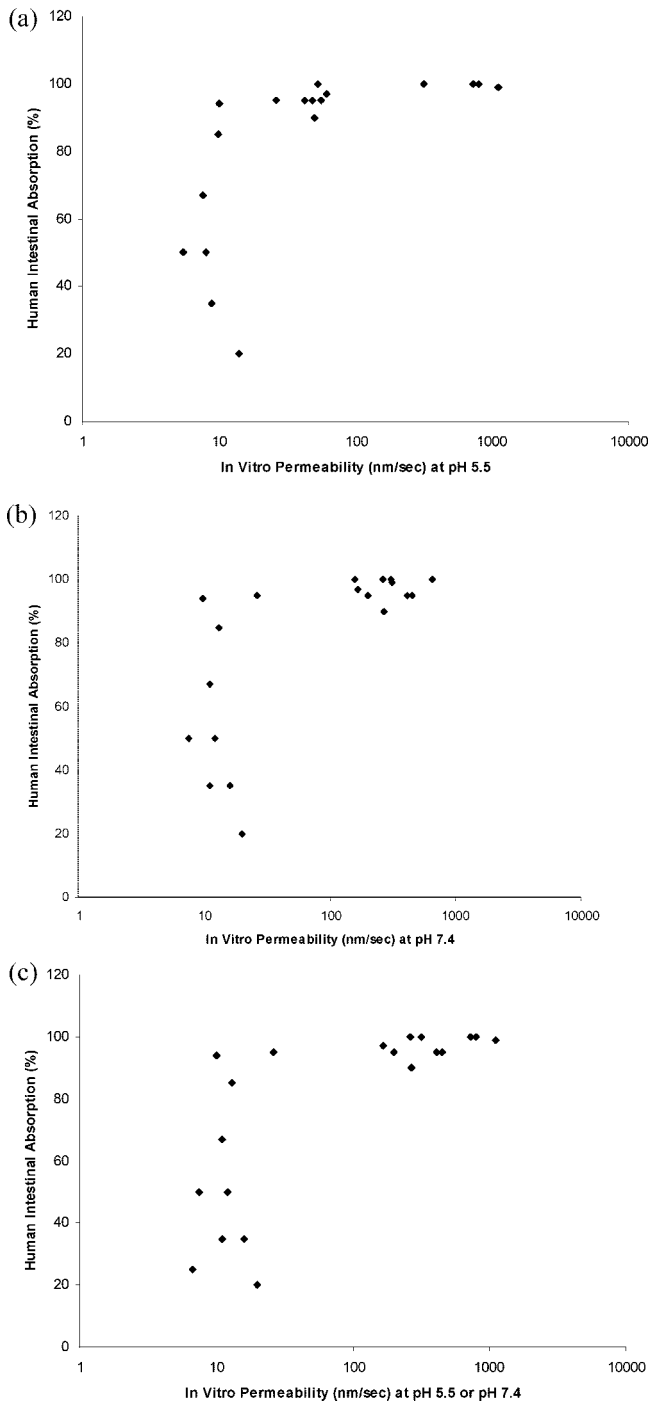


***Figure 1.*** In vitro permeability values from MDCKII-MDR1 cell monolayers versus human intestinal absorption (HIA) of the 20 drug validation set at (a) pH 5.5, (b) pH 7.4 and (c) highest permeability value at pH 5.5 or 7.4.

substrates. In contrast to the high HIA (>90%) observed for the ten highly permeable drugs above, the HIA values for this second group of drugs varied widely (25 to 95%), yielding little relationship between the HIA and permeability values. For example, minoxidil, amoxicillin and cimetidine all have reported HIA values that are between 85 to 95%. Their in vitro permeability values ranged from 9.7 to 26 nm/s. This correlation has been observed previously and was as expected for the selected validation set.[11,13] Thus, the poor relationship makes it difficult to estimate the fraction absorbed from an in vitro permeability value when the permeability value is far below that of labetalol. Therefore, labetalol is currently being used as a reference standard to classify drugs as high or low permeants.

***Table 2.*** Summary of Membrane Permeability from a Single Run or Cassetted Assay at pH 5.5 and pH 7.4 in MDCKII-MDR1 Cell Monolayers for the Four Reference Standards (Ranitidine, Labetalol, Pindolol and Metoprolol)

| drug | | $P_{7.4}$ (nm/s) (mean ± SD) | $P_{5.5}$ (nm/s) (mean ± SD) |
|---|---|---|---|
| ranitidine | single[a] | 7.5 ± 3.3 | 5.5 ± 1.7 |
| | cassette[b] | 14 ± 4.9 | 18 ± 7.0 |
| labetalol | single | 201 ± 7.0 | 42 ± 1.1 |
| | cassette | 179 ± 33 | 40 ± 9.7 |
| pindolol | single | 266 ± 21 | 56 ± 9.7 |
| | cassette | 290 ± 79 | 39 ± 13 |
| metoprolol | single | 410 ± 12 | 48 ± 22 |
| | cassette | 439 ± 120 | 60 ± 25 |

[a] Data are the mean ± standard deviation from $n = 3$ monolayers. [b] Data are the mean ± standard deviation from $n = 5-6$ monolayers.

As part of this validation exercise, the reproducibility and ability to cassette dose and analyze four reference standards (ranitidine, pindolol, labetalol and metoprolol; 10 $\mu$M of each drug) was completed. The permeability values and reproducibility of the four reference standards are summarized in Table 2. The rank order of the drugs was identical and the absolute permeability values did not differ statistically, except for ranitidine. The statistical difference for ranitidine is the result of its low permeability; however, this difference would not alter the BCS classification. Therefore, a cassette approach for the multiple reference standards has been adopted to support classification of future test drugs.

**Considerations on Drug Concentration, pH, Mass Balance and Pgp Efflux.** For in vitro permeability, the Guidance suggests that permeability studies be done at 1, 0.1 and 0.01 times the dose strength of the final product in 250 mL aqueous media. This yields high in vitro test concentrations in many cases. For instance, a 500 Da drug with a dose strength of 100 mg would have test concentrations of 800, 80 and 8 $\mu$M respectively. These concentrations will likely be difficult to achieve for many drugs in development due to their limited solubility in the transport medium or an effect on monolayer tight junctions at high concentrations. Therefore, a preliminary solubility and monolayer integrity experiment is conducted prior to the formal in vitro BCS permeability experiment. This preliminary study generally has a minimum of six test concentrations (1.0, 0.3, 0.1, 0.03, 0.01, and 0.003 times the dose strength dissolved in 250 mL of transport medium) and is rapid and facile to perform, as the end point for solubility is a visual inspection of the drug solution and the end point for monolayer integrity is determination of Lucifer yellow permeability using a fluorescence readout. If all these concentrations pass the solubility and monolayer integrity

criteria, then the standard 1, 0.1 and 0.01 concentrations are used for the BCS in vitro permeability study. However, if certain concentrations fail (drug visible precipitate or LY values fail the <50 nm/s pass criterion), then the highest concentrations that passes along with two other concentrations (selected based on the lower limit of quantification of the analytical method) are used.

Another important variable is the selection of relevant pH conditions to classify a drug's permeability, in particular for acidic drugs. This has been reviewed in the literature with a recommendation to provide waivers to class III acidic drugs (low permeability, high solubility).[17−19] In the current validation exercise, two pH conditions were tested for each drug. One useful and simple predictor of a pH effect was the change in clogP across the pH range. Having this in silico information a priori can help with the experimental design and in the determination of the lower limit of quantitation for the analytical method. The marked pH-dependent permeability for eight of the ten highly permeably drugs (Table 1) highlights the importance of collecting permeability values at multiple pH conditions. For example, the acidic drug ketoprofen has a $P_{7.4}$ of 156 nm/s, which is a rate just below that of labetalol, and a $P_{5.5}$ of 805 nm/s, which is a rate almost 20-fold higher than that of labetalol at this pH. On the other hand, the five basic test drugs (three are structurally related to labetalol) had much lower permeability values at pH 5.5 compared to pH 7.4. However, their permeability values were equal to or higher than labetalol when tested at either pH, resulting in no change in the test drugs' permeability classification. The caution to using pH 5.5 permeability values for basic drugs is that the dynamic range between the high and low permeability drugs is lost, as there is only a 16 nm/s difference between labetalol (the slowest "high" permeable drug) and minoxidil (the fastest "low" permeable drug). Even with this limitation, generating permeability values at multiple pH conditions is essential and physiologically relevant,[14] especially in conjunction with other preclinical and clinical data to support a test drug's permeability classification.

Although not specified in the Guidance, there is general agreement among in vitro permeability scientists that mass balance should be determined in order to assess the quality of the in vitro permeability value.[9] However, there is no consensus on acceptable minimum mass balance, in part as low mass balance only presents a risk of under estimating the true permeability of a test drug. Most investigators use an approximate solution to calculate permeability[5] and are thereby limited to sink conditions and compounds that do not suffer from a mass balance issue. However, this is often not the case for new test drugs in development. Therefore, we have developed an exact solution to the permeability equation, which is valid when sink conditions are not maintained and that corrects for loss of mass between time points;[5] it is of note that this equation collapses to the approximate equation when sink conditions are maintained and there are no mass balance issues. Passive permeability is proportional to flux divided by concentration difference and is calculated from donor and receiver concentrations over time. Both flux and concentration difference change in time, but the ratio (the permeability) remains constant. When mass balance is poor, the concentration difference as a function of time needs to be corrected for loss of compound. Assuming loss is first order (infinite supply of weak binding sites and loss is independent of compound concentration) allows an analytical solution to the permeability equation (see Data Analysis and reference by Tran[5]). The potential error introduced by the assumption of first order loss is minimized by using multiple time points. Since the permeability is calculated between two time points, the only loss that is relevant is the loss between these time points, not the loss over the entire time course.

The current Guidance, which has not been changed since its original implementation in 2000, suggests that drugs exhibiting active efflux in vitro should continue to be excluded from consideration for a biowaiver and debate on this topic has been discussed in the literature.[9,20−22] In the current in vitro validation, the test drug's permeability was determined both in the presence and absence of drug efflux and in two directions (A → B and B → A). However, future studies will classify a test drug's permeability only in absence of drug efflux (e.g., GF120918 present in the media) and in the "absorptive" permeability direction" (e.g., A → B direction). The rationale for this approach is that there will be separate studies evaluating if a test drug is a substrate for efflux transporters. This information, along with other preclinical and clinical information such as exhibition of dose linearity in humans and drug product performance (e.g., the other BCS testing required), should drive the decision for a wavier, not a single in vitro test for drug efflux or permeability. Further, the overall literature data supports a limited role of efflux transporters in limiting the absorption of

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drugs.[23] In addition, there is a lack of clinical evidence supporting exposure differences between formulations of the same drug due to efflux transporters.[9,23] Drug transporters are an important area of research in ADME; however, the impact of these proteins on drug absorption requires further research in order to improve current understanding of in vitro to in vivo correlations.

In conclusion, the in vitro permeability assay has been validated for use to classify drugs according to the FDA Guidance on BCS. The final validated in vitro permeability method is a unidirectional transport (A → B) assay performed at apical pH values of 5.5 and 7.4, using the MDCKII-MDR1 cell line in the presence of the Pgp inhibitor GF120918. Four reference standards (metoprolol, pindolol, labetalol and ranitidine) dosed and analyzed as a single cassette will be included in each experiment. The assay will include multiple time points and test article concentrations, and be completed in the absence of drug efflux. A test drug will be classified as highly permeable if the passive permeability equals (±20%) or exceeds that of labetalol, the reference drug defining the low/high permeability classification boundary.

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**Supporting Information Available:** Table of data from individual experiments for the 20 validation drugs. This material is available free of charge via the Internet at http://pubs.acs.org.

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