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The Suitability of an in Situ Perfusion Model for Permeability Determinations: Utility for BCS Class I Biowaiver Requests

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Abstract: The FDA has published recommendations for sponsors who wish to request a waiver of in vivo bioavailability (BA) or bioequivalence (BE) studies for immediate release (IR) solid oral dosage forms based on the Biopharmaceutics Classification System (BCS). Biowaivers can be requested for IR formulations in which the active ingredient is shown to be a BCS class I drug: that is, a drug showing high permeability and high solubility over a pH range of 1−7.5. For permeability determinations, a variety of experimental methods can be used, such as the rat in situ single pass perfusion or Caco-2 cell culture models, once the suitability of the particular method is established. Following the recommended procedure for assessing the suitability of permeability determinations, we determined the permeability of 20 test drugs using the in situ single pass perfusion model in rats. The test compounds were coperfused through jejunal intestinal segments with an internal permeability reference standard (metoprolol) over a 90 min time period. Sample analysis was performed by HPLC, and the ratio of the effective permeability, P_{eff} (cm/s), of test compound to that of metoprolol was determined. To address the question of test drug permeabilities that approach that of the internal standard, we propose that a statistical analysis such as the "0.8−1.25 rule" used for in vivo or in vitro bioequivalence studies provide guidance for permeability classification using the in situ single pass perfusion model. We developed a method using the 90% confidence interval of the permeability ratio of the test to internal reference standard in order to differentiate between high and low permeability compounds. This analysis allowed for the proper permeability classification of all of the test compounds and suggests a robust means for assessing drug permeability classification.

Keywords: Biopharmaceutics Classification System; bioavailability; in situ rat perfusion; permeability; confidence interval

Introduction

In August 2000, the FDA published a guidance for industry titled "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate Release Dosage Forms Containing Certain Active Moieties/Active Ingredients based on a Biopharmaceutics Classification System".[1] Through the use of data obtained from solubility and permeability experiments, an active moiety can be classified into one of four biopharmaceutical classes: class I (high solubility, high permeability), class II (low solubility, high permeability), class III (high solubility, low permeability), class IV (low solubility, low permeability). The guidance describes alternative ways to demonstrate bioequivalence for rapidly dissolv-

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(1) Center for Drug Evaluation and Research. Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate Release Dosage Forms Containing Certain Active Moieties/Active Ingredients based on a Biopharmaceutics Classification System. FDA: 1999. http://www.fda.gov/cder/guidance/3618fnl.pdf (accessed August 2005).

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ing, immediate-release (IR) solid oral dosage forms that contain highly soluble and highly permeable (class I) active ingredients. For this type of drug product, in vivo demonstration of bioequivalence may not be necessary because, once ingested, the drug product behaves like a solution of the drug substance, meaning that the rate and extent of drug absorption are mainly determined by gastric emptying and largely independent of drug dissolution or gastrointestinal transit time. Therefore, provided that the inactive ingredients do not significantly affect absorption of the active moiety, rapidly dissolving IR drug products containing the same BCS class I active moiety will be bioequivalent.

A drug substance is considered to be highly permeable when the extent of absorption in humans, the fraction of dose absorbed, is 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose. Typical means of assessing permeability in clinical studies include determination of mass balance, systemic (absolute) BA, or intestinal perfusion.[2−4] Nonclinical methods include in vivo or in situ intestinal perfusion studies in a suitable animal model (e.g., rats), or in vitro permeability methods using excised intestinal tissues or monolayers of suitable epithelial cells.[5−9] The suitability of a given permeability method should be established whereby a rank-order relationship exists between the test permeability values and the extent of drug absorption data in human subjects using a sufficient number of model drugs. For human intestinal perfusion studies,6 model drugs are recommended. For in vivo or in situ intestinal perfusion studies in animals and for in vitro cell culture methods, 20 model drugs are recommended. To aid in defining the high permeability class boundary for a test compound, the use of a high permeability internal standard with permeability in close proximity to the low/high permeability class boundary is recommended. Metoprolol, for example, which has a 96% fraction absorbed (FA),[10] is cited in the BCS guidance as a potential internal reference standard. Drugs that have a permeability above that of metoprolol, i.e., the permeability ratio of test/metoprolol is greater than 1.0, would be considered highly permeable, and drugs with a permeability ratio lower than 1.0 would be considered low permeability. In practice, however, it is the borderline drugs, which have permeabilities close to that of the internal reference standard, that have garnered the most attention. These drugs are not as easy to assign permeability classification since the variability of the method used to determine permeability may blur the borderline between high and low permeability and the choice of internal reference standard can significantly impact the borderline between high and low permeability.

To address this issue, we adapted the "0.8−1.25" confidence interval rule that is typically used for assessing the BE of drug products to address these issues for permeability that are described in 21 CFR 320.33 and recommended by the FDA.[11] Using the in situ single pass perfusion model, we show that the permeability classification agrees with the % FA and allows for correct assignment of permeability class for a series of 20 test drugs. Metoprolol was selected as a high permeability internal standard since its reported extent of absorption is 96% and is close to the 90% FA permeability boundary for highly permeable drugs.[10] The methods and analysis described herein provide a robust means of assessing permeability for BCS classification.

Materials and Methods

Chemicals. The test drugs used in this study were the highest grade commercially available. Atenolol, carbamazepine, cimetidine, furosemide, hydrochlorothiazide, ketoprofen, metoprolol tartrate, naproxen sodium, PEG4000, piroxicam, propranolol HCl, and verapamil HCl were purchased from Spectrum Chemical; amoxicillin, antipyrine, caffeine, methyldopa, pindolol, and theophylline were purchased from Sigma; ranitidine HCl was purchased from Research Biochemicals International, mannitol was purchased from EM Science, and enalaprilat was purchased from USP. All other chemicals used in this study were purchased from

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Sigma. The HPLC solvents methanol, acetonitrile, triethylamine, orthophosphoric acid, and trifluoroacetic acid were purchased from Fisher Chemical and were all HPLC grade. ^{14}C-PEG 4000 was purchased from Amersham GE HealthCare.

In Situ Single Pass Perfusion. Rats were housed and handled according to TSRL, Inc., guidelines complying with the U.S. Public Health Service Policy for the Care and Use of Laboratory Animals. Male albino Sprague−Dawley rats, 9−10 weeks old and weighing 250−400 g, used in these studies were purchased from Charles Rivers Laboratory. Prior to each experiment, the rats were fasted for 18 h with free access to water. The procedure for the in situ single pass perfusion followed previously published reports.[12−14] Briefly, rats were anesthetized either with an im injection of ketamine/xylazine and butorphanol (87 mg/kg, 7 mg/kg, and 0.05 mg/kg body weight, respectively) or by 2% isoflurane. The abdomen was opened by a midline incision of 3−4 cm. A jejunal segment of approximately 10 cm was carefully exposed and cannulated on two ends with flexible PVC tubing. The tubing was incubated in a 37° water bath to maintain temperature, and a perfusion solution containing 10 mM MES buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.01% PEG 4000 with an osmolarity of approximately 290 mosm/L was pumped through the intestinal segment. The isolated segment was rinsed with blank perfusion buffer, pH 6.5 at a flow rate of 0.5 mL/min in order to clean out any residual debris.

At the start of the study, perfusion solution containing a test drug and the internal permeability reference drug (metoprolol) was perfused through the intestinal segment at a flow rate of 0.2 mL/min. ^{14}C-PEG 4000 was added to the perfusion buffer as a nonabsorbable marker for measuring water flux. Test drug concentrations used in the perfusion studies were determined by dividing the highest prescribed dose by 250 mL, the accepted gastric volume, in order to represent the maximal drug concentration present in the intestinal segment and were within their solubility limits reported at pH 6.5.[15−17] After steady state was reached in the segment, as assessed by the inlet over outlet concentration ratio of PEG 4000 which approaches 1 at steady state, samples were taken in 10 min intervals for 1 h (10, 20, 30, 40, 50, and 60 min). All samples including perfusion samples at different time points, original drug solution, and inlet solution taken at the exit of the syringe were immediately assayed by HPLC.

Table 1. Summary of HPLC Mobile Phase and Temperature Conditions Used for Test Compounds

compounds	mobile phase[a] aqueous:organic	wavelength (nm)	retention time[b] (min)
amoxicillin	93/07	220	15
antipyrine	80/20	220	7.8
atenolol	80/20	220	4.4
caffeine	75/25	220	5
carbamazepine	40/60	230	7.0
cimetidine	87/13	220	4.6
enalaprilat	83/17	220	10.5
furosemide	70/30	272	9.3
hydrochlorothiazide	75/25	220	6
ketoprofen	55/45	254	8.8
mannitol[c]	na	na	na
methyldopa	90/10	220	4.3
metoprolol	80/20	220	9.0
naproxen	50/50	220	8.5
pindolol	80/20	220	6.1
piroxicam	75/25	220	28.1
propranolol	75/25	220	14.8
ranitidine	87/13	220	4.6
theophylline	93/7	280	8.0
verapamil	73/27	220	31

[a] The aqueous phase was 0.05% trifluoroacetic acid in water. The organic phase was 100% acetonitrile, with the following exceptions: for carbamazepine, the aqueous phase was water and the organic phase was methanol with 10% methylene chloride; for furosemide, the aqueous phase was 1.4% glacial acetic acid in water and the organic phase was tetrahydrofuran; and for theophylline, the aqueous phase was 10 mM sodium acetate with 0.5% glacial acetic acid in water and the organic phase was acetonitrile. [b] All separations were performed under ambient temperatures with the exception of amoxicillin (30 °C) and naproxen (35 °C). [c] Measured by radioactivity, not HPLC.

HPLC Analysis. All compounds were assayed following standard HPLC methodology developed at TSRL. Most compounds were separated on a Gemini 250 mm × 4.6 mm C-18 column (5 μm particle size, 110 Å pore size) with a 10 μL injection volume and a flow rate of 1 mL/min. For carbamazapine, a 3.9 × 300 mm, 5 μm particle size C18 column was used; for furosemide, a 4.6 × 250 mm, 5 μm particle size C18 column was used; for ketoprofen, a 3.9 × 300 mm C18 μBondapak column was used; and for theophylline a 4.0 × 300 mm 5 μm particle size C8 column was used. The mobile phase and detection wavelength used for the analysis are presented in Table 1.

Data Analysis. The effective permeability (P_{eff}) was determined assuming the "plug flow" model expressed in

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eq 1,[18]

$$P_{\text{eff}}(\text{cm/s}) = \frac{Q \ln(C'_{\text{out}}/C'_{\text{in}})}{2\pi RL} \quad (1)$$

where Q is the perfusion buffer flow rate, $C'_{\text{out}}/C'_{\text{in}}$ is the ratio of the outlet concentration of test drug and the inlet or starting concentration of test drug that has been adjusted for water transport (eq 2), R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the intestinal segment. The ratio of concentrations $C'_{\text{out}}/C'_{\text{in}}$ was adjusted to account for water transport that may have occurred during the perfusion. To correct for this, a nonabsorbed radioactive tracer, ^{14}C-PEG 4000, was included in the perfusion buffer. The measured $C_{\text{out}}/C_{\text{in}}$ ratio was corrected for water transport according to eq 2,

$$\frac{C'_{\text{out}}}{C'_{\text{in}}} = \frac{C_{\text{out}}}{C_{\text{in}}} \frac{(A_{\text{in}} - A_{\text{bkg}})}{(A_{\text{out}} - A_{\text{bkg}})} \quad (2)$$

where A_{in} is equal to the radioactive counts in the inlet sample, A_{out} is equal to the radioactive counts in the outlet sample, and A_{bkg} is the background counts. Except where noted, all experiments were done with an n equal to 4 rats with 6 samples collected per rat. Permeability values that calculate as a negative number were reported as zero, and outliers are identified by the Grubbs test for outlying observations.

Confidence Interval Analysis. Mean permeabilities (P_{eff}) and standard deviation of the test drugs and metoprolol were determined from the permeabilities obtained from 4 rats. For the confidence interval analysis, the permeability ratio of test drugs and metoprolol was calculated at each timed sample, 10−60 min. For each rat, then, the mean of 6 permeability ratios was calculated. The 90% confidence interval for each test compound was determined from the mean permeability ratio of 4 rats, as shown in eq 3, using the confidence function in Excel software with a significance level of 0.1,

$$\bar{x} \pm 1.645\left(\frac{\sigma}{\sqrt{n}}\right) \quad (3)$$

where $\bar{x}$ is the mean permeability ratio, σ is the standard deviation, and n is the sample size.

Results

We sought to test the suitability of the single pass in situ perfusion method for permeability determinations as described by the BCS guidance and to address the issue of "borderline" drugs, drugs that have a FA close to that of the high permeability reference standard and operationally defined as those drugs that have a permeability value close to that of the internal permeability standard. Metoprolol was chosen as the internal permeability standard because its FA is well documented and is 96%,[10] which is close to the 90% FA specified in the BCS guidance as the border for high permeability drugs. The permeabilities of 20 compounds were determined using the single pass perfusion technique in rats. These compounds were stable in perfusion buffer and in blank perfusate (perfusion buffer without drug that is passed through control intestinal segments) and were soluble at the experimental conditions used. The concentration for the test drugs was the maximum dose divided by 250 mL as is described in the BCS guidance for the definition of a highly soluble compound. The compounds were coperfused with metoprolol as an internal permeability standard.

The permeabilities and their ratios of test/metoprolol internal standard determined in the rat perfusion are presented as mean ± standard deviation in Table 2. Also included in this table are the literature values for human permeability, largely determined in the laboratories of Hans Lennernäs and G.L.A. using the Loc-I-Gut methodology, and the published

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Table 2. The Human and Rat Permeabilities of the Test Compounds

compounds (permeability class)	dose (mg)	human permeability[a] (10^{-4} cm/s)	FA[a] %	rat permeability (10^{-4} cm/s)	rat P_{eff} ratio test/IS
piroxicam (H)	20	10.4 ± 5.9	100	2.62 ± 0.37	28.1
ketoprofen (H)	75	8.4 ± 3.3	100	1.55 ± 0.34	7.9
carbamazepine (H)	200	4.3 ± 2.7	100	1.79 ± 0.11	7.1
naproxen (H)	500	8.3 ± 4.8	100	1.19 ± 0.12	10.1
caffeine (H)	200	nd[d]	100	1.19 ± 0.23	5.5
antipyrine (H)	188	5.6 ± 1.6	100	0.96 ± 0.13	4.5
theophylline (H)	300	nd	96	0.68 ± 0.19	3.8
verapamil[b] (H)	120	6.7 ± 2.9	100	0.65 ± 0.05	3.9
propranolol (H)	80	2.8 ± 1.3	100	0.49 ± 0.07	3.2
pindolol (H)	10	nd	89	0.293 ± 0.11	1.2
metoprolol (H)	100	1.5 ± 0.9	96	0.20 ± 0.04	1.0
furosemide (L)	80	0.05 ± 0.04	61	0.117 ± 0.08	0.69
amoxicillin[c] (L)	875	0.3	45−75	0.120 ± 0.11	0.50
cimetidine (L)	800	0.3 ± 0.05	60	0.105 ± 0.06	0.44
enalaprilat (L)	20	0.1 ± 0.3	8	0.057 ± 0.06	0.42
mannitol (L)		nd	16	0.121 ± 0.05	0.39
ranitidine[b] L)	10	0.2 ± 0.06	50	0.073 ± 0.06	0.30
atenolol (L)	100	0.2 ± 0.2	50	0.060 ± 0.06	0.06
methyldopa (L)	500	0.1	45	0.016 ± 0.004	0.10
hydrochlorothiazide (L)	50	0.04 ± 0.05	67	0.001 ± 0.001	0.002

[a] Human permeability and FA data were taken from refs 19−26. [b] Verapamil[27] and ranitidine[28] are potential efflux pump substrates. [c] Amoxicillin shows nonlinear absorption kinetics. The high dose (3000 mg) of drug shows a FA of 45% while the low dose (500 mg) shows a FA of 75%.[29] [d] Not determined.

values for the human % FA of these drugs. The human P_{eff} values are on average 3.8-fold higher than those values measured using the rat in situ perfusion studies, which agrees with published reports comparing human and rat permeabilities.[18] This is likely a result of different geometric and anatomical surface area between rats and humans.[30] A direct comparison of human and rat P_{eff} data shows that the two methods correctly assign a given drug's BCS permeability classification (Figure 1). The relatively large amount of scattering in the permeability correlation between humans and rats, particularly with the high permeability drugs, may be attributed to the different models, macroscopic versus microscopic, and experimental setup, Loc-I-Gut versus single pass perfusion, used when measuring human or rat permeability, respectively. Differential effects of efflux or absorptive mechanisms between human and rat may also account for the variability seen in Figure 1.

The permeability determinations for each test drug and the internal reference are plotted in Figure 2. The classification of such compounds as piroxicam as high permeability drugs is clearly evident from the figure, since their permeabilities are significantly higher than that of the internal standard, metoprolol. Similarly, the classification of the low permeability drug methyldopa, which has a permeability of 0.016 ± 0.004, is straightforward. However, the low permeability drugs, which have mean permeabilities less than that of the internal standard, exhibit considerable permeability distribution overlap with the internal standard (see standard deviation error bars in Figure 2). Thus, the current acceptance criteria of high permeability drugs based on the mean permeability may become problematic when the drug being tested has a borderline permeability where the permeability ratio with the internal standard is close to 1.

To address this issue, we employed a confidence interval analysis to compare a drug's permeability with that of the internal standard. Statistical analysis employing the "0.8−1.25" confidence interval rule has been an accepted means of analysis for in vitro bioequivalence studies and in vivo bioequivalence studies.[31,32] In this sense, if a permeability study using the in situ rat perfusion study is viewed as an "in situ bioequivalence study", then a similar rule may be adopted for the purpose of permeability classification. The

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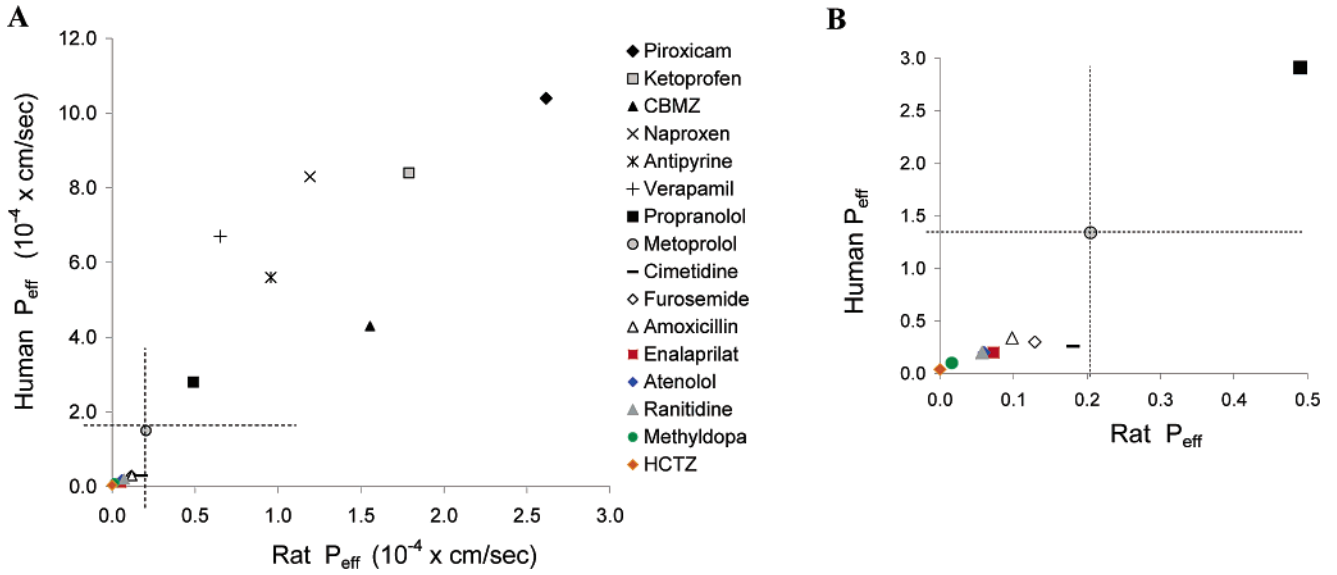

Figure 1. Comparison of permeabilities of test compounds in humans and rats. Panel A has all of the compounds (16 out of 20 compounds) which have a published human permeability value, and panel B is an expansion of the low permeability compounds and also includes the high permeability compounds metoprolol and propranolol. In both panels, the dashed lines represent the permeability value of metoprolol in human and rat. All data are taken from Table 2.

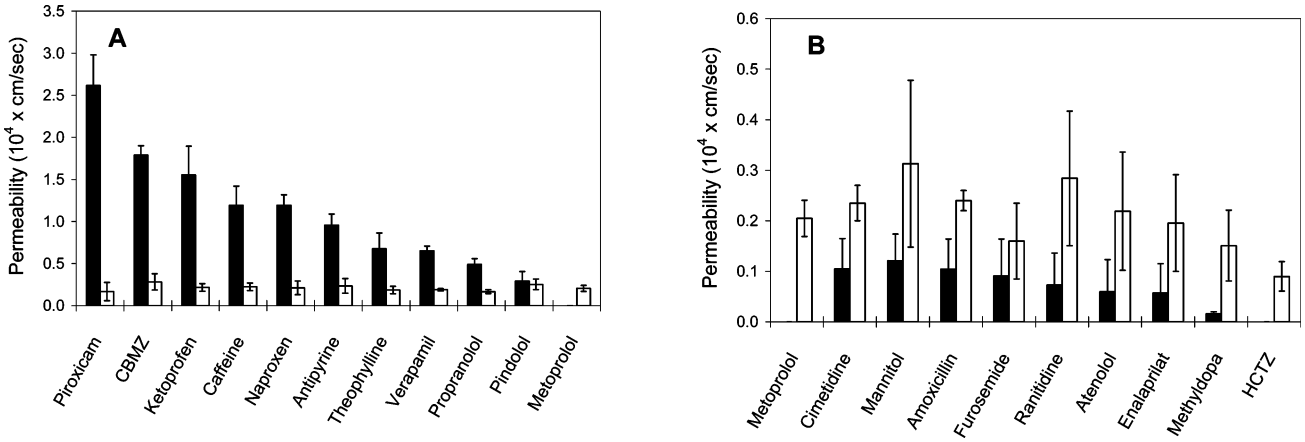

Figure 2. Comparison of the permeabilities of the test drugs with their coperfused internal rerference standard metoprolol (solid bars = test drug, open bars = metoprolol). Panel A contains the comparative values for the high permeability drugs, and Panel B contains the comparative permeability values for the low permeability drugs. CBMZ stands for carbamazepine, and HCTZ stands for hydrochlorothiazide.

acceptance criteria of a highly permeable drug would require that the lower limit of 90% confidence interval of the mean permeability ratio be greater than 0.8 while no upper limit needs to be specified. In our analysis, shown in graphical form in Figure 3, the 90% confidence interval was calculated from the mean permeability ratio of the test drug and the internal permeability standard (see eq 3 in the Materials and Methods Section).

In this figure, the middle line represents the mean permeability ratio while the top and bottom lines are the upper and lower limit of 90% confidence limits of the mean, respectively. Under the current BCS permeability criterion, a drug fails to be classified as a high permeability drug if the permeability is less than that of metoprolol regardless of the accuracy or precision of the data. The ≥0.8-90% confidence interval criterion suggested in this study takes the variability into consideration when determining permeability class. It would be possible under the "in situ bioequivalence" criteria for a drug with permeability less than that of metoprolol to still be classified in the high permeability class if its lower confidence interval were above 0.8. However, depending on the variability of the methodology, large numbers of data points (animals) may be required for drugs whose permeability ratio with metoprolol is below 1.0. The number of rats (n) required to ensure that the permeability ratio is above 0.8 at the 90% confidence level is related to the square of the relative standard deviation and can be estimated from eq 4,

$$n = \left[z_c \sigma \frac{P_{mean}}{(P_{mean} - 0.8)} \right]^2 \quad (4)$$

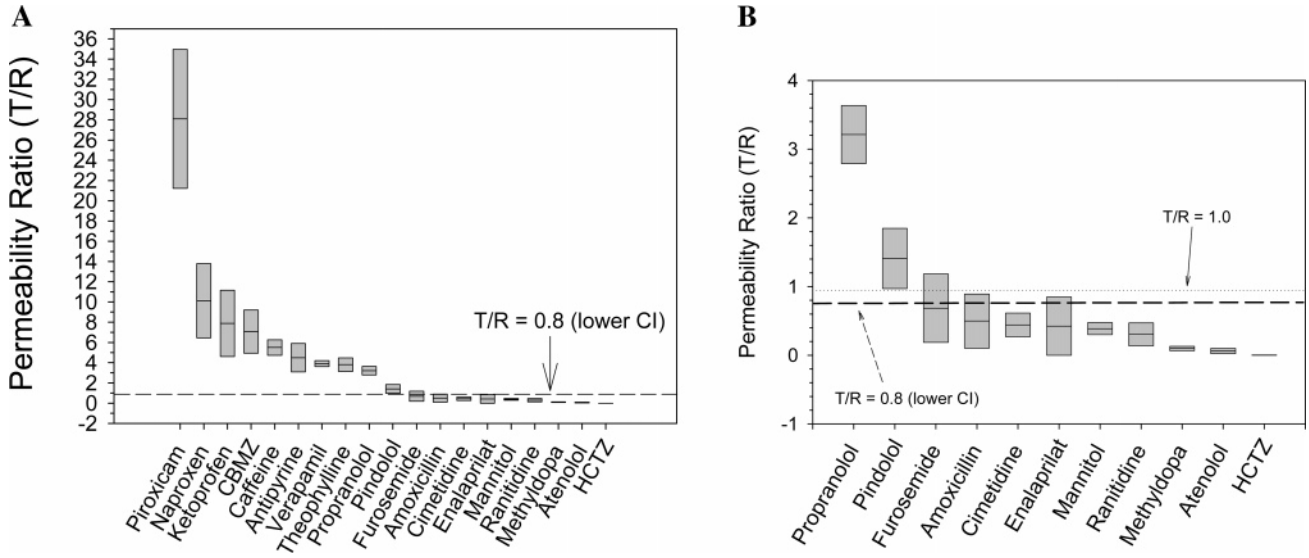

Figure 3. 90% confidence interval analysis of the permeability ratio of the test drugs and metoprolol (coperfused internal reference standard) determined using the single pass in situ permeability method. Panel A contains both high and low permeability drugs. Panel B shows all of the low permeability drug ratios along with two high permeability drugs, pindolol and propranolol. In both panels, the dashed line represents the lower 80% confidence limit. In panel B, the dotted line represents a test to internal reference standard permeability ratio of 1.

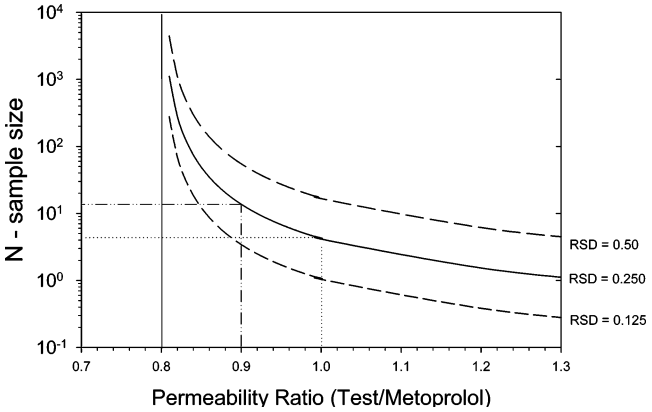

Figure 4. The effect of variability (RSD) on the sample size required to meet the >0.8 confidence interval rule for "in situ bioequivalence". The dotted line extending to 1.0 on the *X* axis indicates the sample size (4.227) at an RSD of 0.25. At a permeability ratio of 0.9 and with an RSD of 0.25, the sample size is 13.7 (dashed line).

where P_{mean} is the mean of the permeability ratio and σ is the relative standard deviation (RSD) obtained from in situ perfusion study using 4 rats, and z_c (1.645) is the confidence coefficient at the 90% level. This relationship is illustrated in Figure 4, in which the sample size is plotted against the permeability ratio in which the RSD values were 0.125, 0.25, and 0.5.

For the drugs with high permeability (ratio >1.0) and 25% RSD, a minimal sample size is required. However, increasing numbers of rats will be required for drugs whose permeability ratios fall below 1.0. For example, for test drugs with mean permeability ratios of 0.9, approximately 14 rats will be required if the RSD of the permeability determination is 25%.

Discussion

A considerable amount of permeability data has been generated in humans.[4,18,23−25,33−35] The human permeability database has played an important role for estimating human absorption and for classifying drugs into their appropriate BCS category, which can be used in setting bioequivalence standards for drug product approval. However, human permeability determinations come with a high cost and extensive experimental effort. Thus, alternative models for prediction of drug absorption and permeability have garnered increasing attention. The BCS Guidance lists in situ perfusion as one possible permeability classification tool along with others such as Caco-2 monolayer and excised tissue. Interspecies correlations have been attempted with data generated in humans, the in situ single pass rat perfusion model, and a variety of other models.[18,36] One advantage of the in situ rat perfusion approach is that the in situ single pass perfusion model appears to correlate best with the human data[12,18] for

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Table 3. Summary of Current Acceptance Criteria for in Vitro and in Vivo Bioequivalence Studies and Proposed BCS Permeability Studies

study	parameters	acceptance criteria
in vitro BE	Langmuir isotherms, k_1, k_2 (bile acid sequestrant)	0.8 < 90% CI of k_2 < 1.2
		0.8 < k_1 ratio < 1.2
in vivo BE	C_{max}, AUC	0.8 < 90% CI < 1.25
in situ BE (BCS permeability)	permeability ratio	ratio > 1.0 (current FDA BCS Guidance)
		0.8 < lower limit of 90% CI (suggested for "borderline" permeability drugs)

the drugs that are transported via passive diffusion mechanism. More complex transport mechanisms that rely on protein carriers and cellular energy systems, such as carrier mediated transport, are more problematic since the types of transporters and their expression levels may vary between species.

The objective of the study was to determine the suitability of the in situ perfusion model for permeability classification of 20 selected test compounds following the BCS Guidance and using an appropriate statistical method. The current acceptance criterion from the BCS Guidance for the high permeability class of drug is that the ratio of the permeability of the test drug to that of a highly permeable internal standard be greater than 1.0. While this rule may be workable for highly permeable drugs, we note that for those test drugs that are of borderline permeability, that is, their permeability approaches that of the internal standard, a simple determination of ratio may be difficult to interpret due to the inherent variability of the methodology.

To address this issue, we developed a confidence interval approach for the permeability classification that includes elements of existing FDA acceptance criteria for bioequivalence testing. The 0.8−1.25 rule is commonly used in those studies where the 90% confidence interval of the mean of the parameters should be within the range 0.8− 1.25. As an example, for the in vitro bioequivalence study for bile acid sequestrants, the current acceptance criteria are that (i) the 90% confidence interval of binding capacity constant (k_2) is within 0.8 and 1.2 and (ii) the test/reference ratio of mean binding affinity constant (k_1) is within ±20%.[32] For in vivo bioequivalence studies, the 90% confidence interval for the ratio of the mean response (C_{max} and AUC) of generic products to that of an innovator product should be within the limit of 0.8 and 1.25.[31] Table 3 shows the comparison of the acceptance criteria set for different bioequivalence studies.

We reasoned that since the extent of drug absorption is well correlated with its permeability in humans and animals, the acceptance criteria for a highly permeable drug should require that the lower limit of the 90% confidence interval of the mean permeability ratio between the test and the internal reference standard be greater than 0.8. For the data set in the current work, the selected test drugs fell into their respective permeability classes (high, ratio >1; low, ratio <1) according to their permeability class determined in humans.

It is also important to note that the "in situ bioequivalence" may allow for classification of drugs with a permeability value less than that of the internal standard as high permeability, as long as the lower limit of the 90% confidence of the mean is above 0.8. Theoretically, if the variability of the method could be controlled throughout the permeability testing, a drug with a test/IS permeability ratio of 0.81 could be classified as a high permeability drug. However, practically speaking, this would require a minimum of 1109 rats at a RSD of 25% and thus would be neither time nor cost efficient.

Finally, the 90% FA level for a class one drug is considered conservative by many since the experimentally determined FA is seen to be less than 90% for many drugs that are generally considered completely absorbed. Recommendations from industrial, academic, and regulatory personnel include modifying the cutoff level for highly permeable drugs for the purpose of obtaining biowaivers from the current FA level of 90% to 85% or 80%.[37−41] A practical approach is suggested by our present work for accommodating the reduction of the FA cutoff level. That is, a reduction in the FA level can be approximated by a reduction in the drug to internal standard (metoprolol) permeability ratio. This, in turn, would adjust the lower limit of the 90% CI (0.8) for the permeability ratio. Since metoprolol is experimentally estimated to have a fraction absorbed of 96%,[10] FA cutoff values of 90%, 85%, or 80% would lower the mean permeability ratio (test to metoprolol) to 0.94 (90/96), 0.88 (85/96), or 0.83 (80/96), respectively, and lower the 90% confidence interval cutoff value to 0.75, 0.71, or 0.67, respectively. Discussion on the acceptability of lowered FA cutoff values is ongoing.

In summary, the rat single pass perfusion method accurately categorized the selected test drugs into the proper

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permeability class as determined in human experiments. This positive correlation will allow for the use of the rat perfusion system to categorize drugs into their appropriate BCS permeability class. In addition, the development of the "in situ bioequivalence" criteria allows for a regulatory relevant and robust means to assess drug permeability. Thus, the in situ rat perfusion model proved to be reliable permeability determination which will more accurately facilitate drug discovery and development as well as regulatory standards for marketed products.

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