Improved Prediction of *In Vivo* Peroral Absorption from *In Vitro* Intestinal Permeability Using an Internal Standard to Control for Intra- and Inter-Rat Variability

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Purpose. To evaluate the use of an *in vitro* intestinal permeability model to predict rat and human absorption as well as to evaluate the use of an internal standard to control for intra- and inter-rat variability.
Methods. *In vivo* peroral absorption and *in vitro* steady-state intestinal permeability coefficients were determined in the rat for a variety of structurally different compounds with different physicochemical properties including: progesterone, hydrocortisone, salicylic acid, caffeine, clonidine, p-aminoclonidine, UK-14304, oxymetazoline, mannitol, PEG 900, PEG 4000, and a number of novel hydrophilic chemical entities.
Results. The intestinal permeability coefficients determined *in vitro* could be used to predict the peroral absorption of a compound in both the rat and human. Normalizing the permeability of a test compound to an internal standard, e.g. mannitol, greatly improved the prediction of peroral absorption.
Conclusions. The use of an internal standard can aid in the prediction of the peroral absorption of a test compound, in particular, for one that has moderate absorption in the range of 20–80%. Moreover, these methods would appear to be a useful means to improve the prediction of other absorption models as well, such as the Caco-2 cell systems and in-situ perfusion methods.

KEY WORDS: peroral absorption; *in vitro*/*in vivo* correlation; *in vitro* intestinal permeability; *in vivo* mass-balance; Ussing chamber; rat.

INTRODUCTION

The vast majority of drugs, although intended for systemic action, are designed for peroral (PO) ingestion primarily for ease of administration and patient compliance. In order for a drug to reach the systemic circulation via the PO route, the compound must be able to survive and traverse many different environments in the stomach, intestine, and liver. Limiting factors which can decrease overall PO bioavailability of a drug include low solubility or chemical instability in the gastrointestinal tract, high gastrointestinal and/or hepatic metabolism, and poor intestinal membrane permeability. Low PO bioavailability of a drug is typically undesirable and can lead to significant intra- and inter-person variability in drug bioavailability and potentially, therefore, therapeutic performance. Large variability in turn will require that sufficient safety margins exist between therapeutic doses and those that produce unwanted side effects. In the event that bioavailability is low, variable, and undesirable, an understanding of the underlying reason can assist in the identification of ways to circumvent or correct the problem. This discussion will focus on the utilization of *in vitro* systems in Drug Discovery to determine *a priori* whether oral absorption will likely be a limiting factor in the overall PO bioavailability of a drug candidate.

There is a growing number of literature references that have shown good correlations between a compound's permeability coefficient calculated in various models and total percent systemic absorption in humans and rats (examples: 1–10). The purpose of the current study was to evaluate the *in vitro* rat intestinal segment preparation as a model of *in vivo* PO absorption. Furthermore, since variability can be an issue with any experimental system, this study has examined the use of an internal standard to control for intra- and inter-rat variation.

The excised intestinal system was choosen for various reasons some of which include ease of use and lower animal number requirements relative to the in-situ perfusion method. Moreover, the rat intestine allows for the examination of regional differences in permeability whereas the Caco-2 system is a single cell type. Nevertheless, the purpose of this study is not to advocate the use of any one system since each will have their advantages and disadvantages in a particular situation, but only to evaluate the *in vitro* rat intestinal segment preparation as another tool to examine the potential for molecules to be perorally absorbed both in the rat as well as in human subjects. The rat was selected both for convenience and because it has been shown to be a reasonable predictor of human PO absorption for compounds that tranverse the intestine by passive diffusion mechanisms (6,9–12).

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were analytical grade or better and used as received. All solutions were prepared using distilled water which had been passed through a Culligan Aqua-Summa II reagent grade water system. Novel chemical entities were synthesized at Procter & Gamble. ^{3}H-clonidine (68.6 Ci/mmol), ^{3}H-p-aminoclonidine (55.7 Ci/mmol), ^{3}H-UK-14304 (75.6 Ci/mmol), ^{3}H-PEG-900 (2.23 μ Ci/mg), ^{3}H-hydrocortisone (143 μCi/μg), ^{14}C-mannitol (55 Ci/mmol), ^{14}C-PEG-4000 (18 mCi/g), ^{14}C-progesterone (0.18 μCi/mg), and ^{14}C-salicylic acid (1.92 μCi/μg) were obtained from DuPont NEN (Boston, MA). ^{14}C-oxymetezoline (1 μCi/mg) and tritium-labeled novel chemical entities were prepared by Amersham (Arlington Heights, IL).

Animals

Male Sprague-Dawley rats (150–200g) were purchased from Charles River Laboratories. Rats were housed two to a cage and allowed to acclimate at least 3 days after arrival prior to being entered into a study. Rats were fed pelleted Rat Chow and water ad libitum and maintained on a 12 hour light/dark cycle. Research with animals adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

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In Vitro Permeability Studies

Rats were anesthetized with 60% CO_2/40% O_2 and the ileum was rapidly excised and placed in ice-cold, aerated (95% O_2/5% CO_2) Krebs bicarbonate buffer (114 mM NaCl, 5 mM KCl, 1.1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1.65 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, pH 7.4). A portion of tissue (without Peyer's patches), of approximately 2 cm, was cut and opened along the mesenteric border with surgical scissors. The tissue was mounted on a diffusion chamber system (Grass and Sweetana, 1988; Precision Instrument Design, Tahoe City, CA) and the seromuscular layer was removed using fine-tipped forceps and a dissection scope. The epithelial tissue was equilibrated with 5 mL aerated mucosal (Krebs + 10 mM mannitol) and serosal (Krebs + 10 mM D-glucose) buffers for about 30 min at 37°C prior to the start of the experiment. Various concentrations of the compound of interest were added to the mucosal or serosal side of the chamber in separate experiments: 0.1–12.5 mM clonidine, $1.9 \cdot 10^{-5}$-0.5 mM p-aminoclonidine, 0.1–0.5 mM UK-14304, 0.2 mM PEG-900, 0.01–0.1 mM PEG-4000, $9.38 \cdot 10^{-6}$ mM hydrocortisone, 0.5–1.0 mM mannitol, $4.10 \cdot 10^{-3}$ mM progesterone, $3.77 \cdot 10^{-3}$ mM salicylic acid, 0.5–2.5 mM oxymetazoline, and approximately 10^{-5}–2.5 mM of the novel chemical entities. In some cases, a final concentration of approximately 0.2 μCi/ml of ^{14}C-mannitol was added to the donor chamber as an internal standard. From 6 to 12 tissues from 2 to 4 rats were used for each compound. Samples were taken from both the donor chamber (0.1 mL) at the start and conclusion of the experiment, and receiver chamber (0.5 mL) at various times with sample volume replacement up to 3 hours. Solutions were circulated by gas lift (95% O_2/5% CO_2). The pH of the donor and receiver chambers was verified before and after the experiment. Samples were analyzed by HPLC and/or radiometric analysis.

The HPLC system consisted of a Waters 600 Solvent Controller, 996 PDA Detector, and 717 Autosampler (Waters Corp., Milford, MA). The column was a 250 × 4.6 mm YMCbasic reverse-phase column (YMC, Inc., Wilmington, NC, #042516303). The mobile phase composition was a methanol/0.03M phosphate buffer mixture, where the methanol content varied between 10 and 30% and pH varied from 3 to 7, depending on the molecule being analyzed. The flow rate was 1.0 mL/min, with UV detection between 210 and 254 nm, depending on the compound. Samples for radiometric quantitation were collected in scintillation vials, 10 mL of Ecoscint A (National Diagnostics, Atlanta, GA) was added, and samples were counted on a Beckman LS 6000SC scintillation counter using an external standardization method.

Metabolic products were looked for at the same time during the evaluation of the parent compounds with HPLC. Thin layer chromatography was used to evaluate the radiolabeled materials.

In-vitro ileum permeability constants, k_p (cm/min), were calculated at steady state under sink conditions according to Fick's laws of diffusion. This is mathematically described by the following equation:

$$k_p = [JV]/[AC_0]$$

where J is the solute flux rate in pmoles/ml/min, V is the volume of the receiver chamber in cm^3 (10 ml for our system), A is the cross-sectional area of the tissue surface exposed to solute transport in cm^2 (0.636 cm^2 for our system), and C_0 is the initial concentration of solute in the donor chamber in pmoles/ml.

Mass Balance Studies

Rats were fasted overnight prior to administration of the test material (water ad libitum) and for 6 hr post-dose, then fed ad libitum until termination of the study. A total of 5 rats each were used for IV and PO dosing. The concentrations at which the compounds were dosed, both for IV and PO administration, were as follows: (1) 100 μg/kg clonidine (20 μCi/kg ^{3}H-clonidine); (2) 100 μg/kg p-aminoclonidine (20 μCi/kg ^{3}H-p-aminoclonidine); (3) 92 μg/kg UK-14304 (20 μCi/kg ^{3}H-UK-14304); (4) 100 μg/kg hydrocortisone (20 μCi/kg ^{3}H-hydrocortisone); (5) 10.5 mg/kg (20 μCi/kg) ^{3}H-PEG-900; (6) 1.2 mg/kg (20 μCi/kg) ^{14}C-PEG-4000; (7) 2.26 mg/kg (0.4 μCi/kg) ^{14}C-progesterone; (8) 93 μg/kg salicylic acid (16.4 μCi/kg ^{14}C-salicylic acid); (9) 91.2 μg/kg (10 μCi/kg) ^{14}C-oxymetazoline; (10) and various concentrations of the novel chemical entities. The IV dose was administered in a volume of 1 mL/kg through a tail vein. The PO dose was administered in a volume of 10 mL/kg through an oral dosing tube. After dosing, rats were placed in metabolism cages where urine (frozen) and feces were collected over 48 hrs, which was determined to be enough time for complete excretion of all compounds. At the end of each study, rats were sacrificed by CO_2 inhalation, and the carcasses were frozen in liquid nitrogen and then ground in a Thomas-Whiley laboratory mill (Model 4, Philadelphia, PA). Aliquots of urine and cage wash samples were mixed with scintillation fluid and assayed by liquid scintillation counting (Packard 2200 or 2550 scintillation counter). The feces and carcass samples were homogenized with addition of water, aliquoted, and combusted in a Packard 307 oxidizer. The resulting ^{14}C-CO_2 or ^{3}H-H_2O was collected in a scintillation vial, mixed with a scintillation cocktail and assayed by liquid scintillation counting.

The fraction of compound perorally absorbed was calculated from the ratio of the mean % radioactivity excreted in the urine after PO administration and the mean % radioactivity excreted in the urine after IV administration and is based on the following equation: (% absorbed PO) = (% excreted in the urine PO)/(fraction excreted in the urine IV), which assumes that the metabolic profile of the drug and the proportion of drug eliminated through the urine and feces is similar after both IV and PO dosing.

Statistics

The confidence limits of oral absorption were calculated according to Fieller's thereom, which gives a confidence interval for the ratio of two random variables. The thereom is based on the normality of two independent means. Least-squares nonlinear regression of PO absorption (A) on the intestinal permeability coefficient (K_p) was performed via a logistic function (S curved):

$$A = \{Max\}/\{1 + \exp[B(\log K_p + C)]\}$$

where the three parameters, Max, B, and, C, in the regression function are non-linear least squares estimates, which are obtained through an iterative minimization procedure.

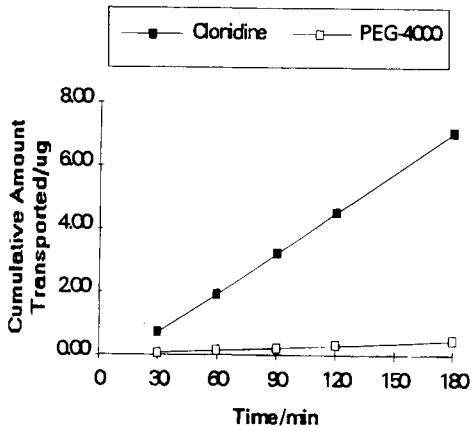

Fig. 1. Representive transport kinetic curves of clonidine (good absorption) and PEG-4000 (poor absorption) showing the linearity of transport for the duration of the experiment. These curves are also representative of the transport of the other compounds examined.

RESULTS AND DISCUSSION

In vitro tissue viability was monitored primarily through examination of the transport kinetics at steady state as well as by evaluation of the reproducibility of results. The amount of material being transported across the intestinal strip at steady state remained constant for the duration of the experiment suggesting that the rate-limiting step was also intact for this time. Representative transport curves are shown in Figure 1.

Calculated *in vivo* PO absorption values from mass-balance studies as well as *in vitro* steady-state ileum permeability coefficients for the compounds tested in the rat are presented in Table I. Permeability values were shown to be independent of donor solute concentration and/or similar in either the mucosal to serosal or serosal to mucosal direction, suggesting that the mechanism of absorption for these compounds was simple diffusion. In addition, none of the compounds were determined to be metabolized in the *in vitro* intestinal preparation. Human absorption data, octanol/water partition coefficients and other physicochemical properties are included in Table I for comparison. A graphical representation of the intestinal permeability and percent PO absorption in rat is presented in Figure 2. Least-squares non-linear regression of the data resulted in the following correlation ($r^2 = 0.840$):

$$A = \{96.2\}/\{1 + \exp[-6.25(\log K_p - 0.600)]\} \quad (1)$$

where A is total percent PO absorption of the compound in the rat and K_p is the corresponding permeability coefficient (10^{-4} cm/min) across the rat ileum. Furthermore, the correlation between rat intestinal permeability and percent PO absorption in human (for compounds with available data) is depicted in Figure 3. Least-squares non-linear regression of the data is represented by the following correlation ($r^2 = 0.996$):

$$A = \{98.3\}/\{1 + \exp[-8.81(\log K_p - 0.554)]\} \quad (2)$$

where A is total percent PO absorption of the compound in the human and K_p is the corresponding permeability coefficient (10^{-4} cm/min) across the rat ileum.

It is apparent that a reasonable prediction of PO absorption of a chemical entity can be made by measuring its absolute *in-vitro* intestinal permeability coefficient. However, it is also apparent from this work that the transition from poor absorption to good absorption occurs over approximately one order of magnitude (Figures 2 and 3). This transition phenomenon is characteristic of what other laboratories have seen in other model systems (2–4,7,8,10). This factor compounded with the reality of significant intra- and inter-rat variability can make it very difficult to rank order compounds with permeability coefficients within this transition region. However, it was discovered that by including an internal marker compound, in this case, mannitol, during the permeability studies, the predicted absorptions could significantly be improved by dividing the absolute permeability coefficient of the compound by the internal standard permeability coefficient.

This excercise was performed for the structuraly similar novel chemical entities, A through E (amino imidazolines), primarily because, at the time, the absorption model was going to be applied to this class of molecules. The ratio of the mean test compound permeability to the mean mannitol permeability is recorded in Table I. The subsequent least-squares non-linear regression of this data is plotted in Figure 4 and is represented by the following equation ($r^2 = 0.840$):

$$A = \{100\}/\{1 + \exp[-7.42(K_p \text{ ratio} - 0.812)]\} \quad (3)$$

where A is total percent PO absorption and K_p ratio is the mean permeability coefficient of the compound (10^{-4} cm/min) divided by the mean permeability coefficient of the internal standard, mannitol (10^{-4} cm/min).

The comparison between the actual PO absorption and those predicted from equation 1 and equation 3 is summarized in Table II. Novel chemical entities A through F are chemically similar amino imidazolines (NCE-F has a MW 190 and log P −3.2), while G (bisphosphonate: MW 281 and log P −0.2) and H (hydroxamate: MW 316 and log P −0.66) are two

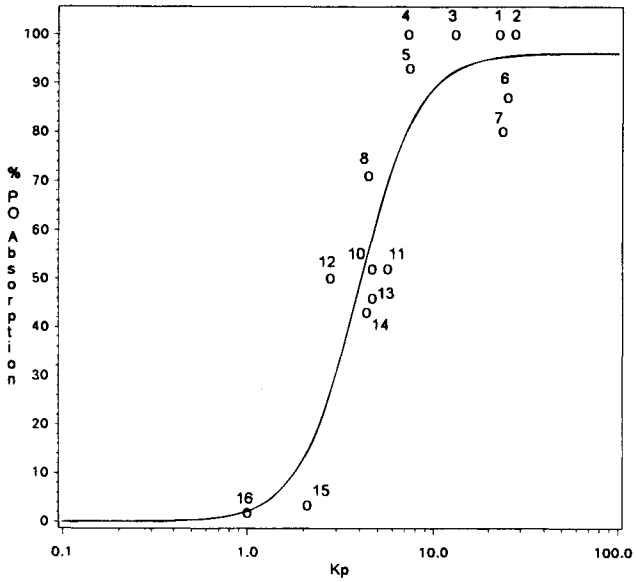

Fig. 2. Correlation between rat intestinal permeability (Kp/(10^{-4} cm/min)) and percent rat oral absorption for the compounds listed in Table I. Compounds are identified by their corresponding number from Table I. The sigmoid curve was obtained by least-squares non-linear regression.

Table I. Summary of Experimental and Literature Data Including Human and Rat Oral Absorption, Ileum Permeability Coefficient (K_p), Octanol/Water Partition Coefficient (log P), Molecular Weight (MW), and pK_a Data for Various Compounds as Well as a Number of Structurally Similar Novel Chemical Entities (Amino Imidazolines: NCE-A through E)

Compound	% PO Absorption[a] (human)	% PO Absorption[b] (rat)	*In Vitro* Ileum K_p[c] (rat)	mean K_p (solute)/ mean K_p (mannitol)[d]	log P (octanol/water, pH 7.4)	MW	pK_a
1. progesterone	100	100 (56–100)	23.1 (5.7)		3.89	315	
2. caffeine	100	100[e]	28.0 (3.6)		0.0	194	
3. salicylic acid	100	100 (100–101)	13.2 (1.6)		−2.14	138	3.0
4. NCE-A		100[f]	7.4 (1.7)	1.07	−1.64	215	9.7
5. hydrocortisone	89	93 (82–108)	7.5 (1.0)		1.53	362	
6. clonidine	95	87 (81–94)	25.5 (5.6)		0.85	230	8.2
7. UK-14304		80 (67–94)	23.9 (3.8)		0.31	292	7.4
8. NCE-B		71 (64–79)	4.5 (0.5)	0.98	−0.89	233	10.6
9. mannitol	65		4.2 (1.7)		−3.10	182	
10. p-amino-clonidine		52 (45–59)	4.7 (0.5)		−0.88	245	9.2
11. NCE-C		52 (46–59)	5.7 (1.1)	0.83	−0.29	261	9.3
12. NCE-D		50 (36–63)	2.8 (0.7)	0.85	−0.40	240	8.7
13. NCE-E		46 (36–56)	4.7 (1.0)	0.75	−1.70	229	10.5
14. oxymetazoline		43 (38–48)	4.4 (0.8)		−0.30	260	10.2
15. PEG-900	10	3.3 (2.5–4.1)	2.1 (0.4)			900	
16. PEG-4000	0	1.7 (1.5–1.9)	1.0 (0.2)		−5.10	4000	

[a] data from Artursson and Karlsson (2), Arnaud (13), Rubas et al. (3), Goodman and Gilman (14), Nasrallah and Iber (15). Note that the reported value for mannitol (15) is higher than other values reported in the literature. Some of this discrepancy may be due to the apparent hepatic metabolism of mannitol to CO_2, which was not accounted for in some studies. The higher value is consistent with the current data, in that compounds that had permeability ratios of less than 1 had absorptions less than 65%, while those with ratios approximately 1 or greater showed better absorption.
[b] number in parentheses represents 90% confidence interval.
[c] permeability coefficient/(10^{-4} cm/min); number in parentheses represents the standard deviation.
[d] the mean permeability coefficient of the test solute divided by the mean permeability coefficient of mannitol from the same tissue segments, not the mean mannitol value shown in the Table which was from a separate experiment.
[e] data from Latini et al. (16)
[f] data from a PO bioavailability study in rat.

structurally different molecules. It is apparent from the results that the ratio method provides a much better prediction of actual PO absorption for the compounds relative to those obtained using the absolute permeability only. It is also interesting to note that equation 3, while derived from a structurally similar class of molecules (A through E), is very useful to predict the PO absorption of other structurally different molecules (entities G and H in Table II). The improved prediction of PO absorption by using equation 3 over equation 1 assumes that the majority of deviation from the absolute permeability model was in the the measurement of the permeability coefficient and not from the PO absorption data.

The selection criteria for an internal standard are not completely clear at this time. However, the molecule of choice will have to be passively absorbed across the intestine. Mannitol was used in the present study primarily because it has been historically used in our laboratory as an internal control for tissue integrity.

Finally, although not shown graphically, it is clear that octanol/water partition coefficient does not provide a good prediction of PO absorption (linear regression: $r^2 = 0.375$; nonlinear regression: $r^2 = 0.357$), suggesting that octanol is not necessarily an appropriate surrogate for intestinal tissue, in particular for hydrophilic analogues. The reason for this is likely due to the availability of non-lipid pathways for absorption in the intestine.

CONCLUSIONS

The *in vitro* permeability coefficient in rat intestine has been shown to be a reasonable predictor of not only *in vivo*

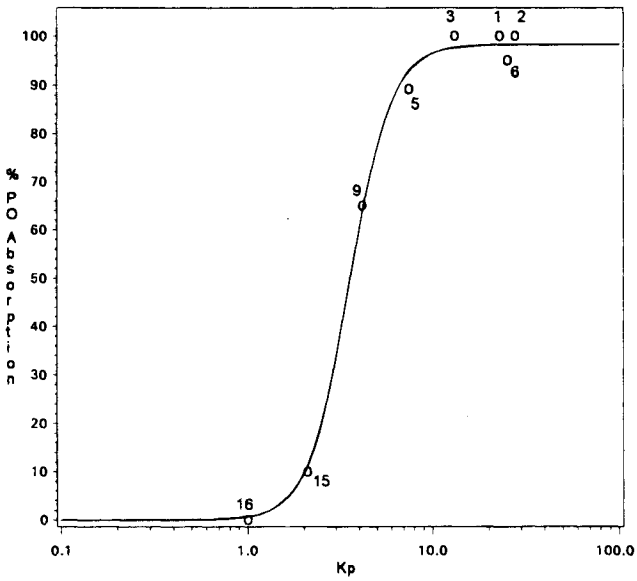

Fig. 3. Correlation between rat intestinal permeability (Kp/10^{-4} cm/min) and percent human oral absorption for the compounds listed in Table I. Compounds are identified by their corresponding number from Table I. The sigmoid curve was obtained by least-squares non-linear regression.

PO absorption in rats but also in humans. Moreover, it appears that a significantly better prediction of PO absorption can be made by normalizing against an internal standard, i.e. mannitol in this case, to control for intra- and inter-rat variability. This will be particularly important to rank order compounds that have moderate PO absorption in the 20 to 80% range. The ratio method will likely improve the predictiveness of other

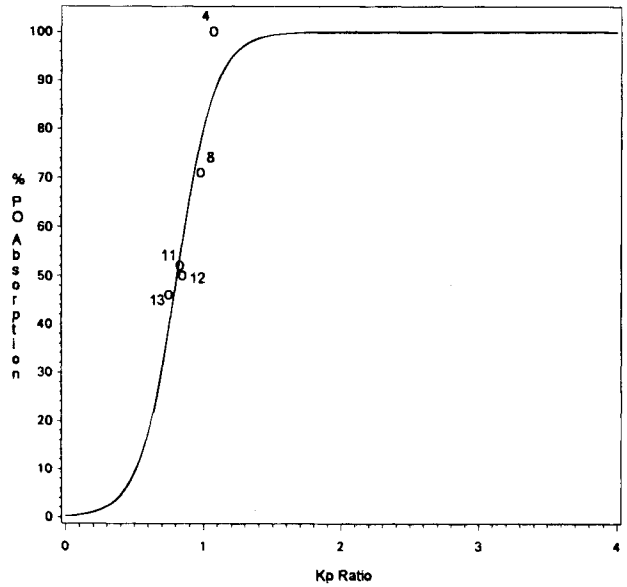

Fig. 4. Correlation between the ratio of the mean rat ileum permeability of a series of structurally related analogues (amino imidazolines: NCE-A through E) to the mean mannitol permeability (Kp/10^{-4} cm/min) in the same tissue and percent PO absorption in the rat. Compounds are identified by their corresponding number from Table I. The sigmoid curve was obtained by least-squares non-linear regression.

Table II. Comparison Between the Actual PO Absorption and Those Predicted from the Absolute Ileum Permeability Coefficient or from the Ratio of the Drug and Internal Standard Permeability Coefficients for a Number of Structurally Related Novel Compounds, A through F (Amino Imidazolines), and Two Other Chemically Different Novel Chemical Entities, G (Bisphosphonate) and H (Hydroxymate)

Novel chemical entity	Actual PO absorption	Predicted PO absorption from absolute ileum permeability	Predicted PO absorption from ratio of drug and internal standard permeabilities
NCE-A	100	78	87
NCE-B	71	40	78
NCE-C	52	69	53
NCE-D	50	35	57
NCE-E	46	61	39
NCE-F	>82	50	96
NCE-G	0.1–2	9	2
NCE-H	57	43	61

absorption models as well as allow for the facilitation of the comparison of permeability coefficients obtained from different labs, especially in light of the amount of variability seen with, for example, Caco-2 models (7,17).

The successful prediction of *in vivo* PO absorption using this model will depend on the following: (1) the compound being examined traverses the intestinal mucosa via simple diffusion mechanisms; and (2) the amount of compound that is delivered can be solubilized and is stable in the gastrointestinal fluid. Predicted absorption values can be used in combination with *a priori* knowledge of metabolism potential, chemical and enzymatic stability, and aqueous solubility of the compound to estimate the rate-limitations to overall PO bioavailability. In effect, the most efficient strategies to improve PO bioavailability can then be implemented.

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