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Quantitative Prediction of Intestinal Metabolism in Humans from a Simplified F_g Model and Empirical Scaling Factor

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Nonstandard Abbreviations: CL_{ab}: absorption clearance, CL_h: hepatic clearance, $CL_{int,intestine}$: intestinal intrinsic clearance, $CL_{int,liver}$: liver intrinsic clearance, CL_m: metabolic clearance, $CL_{m,index}$: intestinal intrinsic clearance corrected with that of midazolam, CL_r: renal clearance, CL_{tot}: total body clearance, CYP: cytochrome P450, DMSO: dimethyl sulfoxide, F_a: fraction of drug absorbed, F_g: intestinal availability, F_h: hepatic availability, HIMs: human intestinal microsomes, HLMs: human liver microsomes, LC-MS/MS: high-performance liquid chromatography with a tandem-mass spectrometry system, PAMPA: parallel artificial membrane permeability assays, P_{app}: apparent permeability coefficient, P-gp: P-glycoprotein, PS_{eff}: efflux clearance, PS_{inf}: apparent influx clearance, Q: luminal flow rate, Q_h: hepatic blood flow, R_b: blood-to-plasma concentration ratio, SF: scaling factor, α: empirical scaling factor

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Abstract

This study aimed to establish a practical and convenient method of predicting intestinal availability (F_g) in humans for highly permeable compounds at the drug discovery stage, with a focus on CYP3A4-mediated metabolism. We constructed a "simplified F_g model", described using only metabolic parameter, assuming that passive diffusion is dominant when permeability is high, and that the effect of transporters in epithelial cells is negligible. Five substrates for CYP3A4 (alprazolam, amlodipine, clonazepam, midazolam, and nifedipine) and 4 for both CYP3A4 and P-glycoprotein (P-gp) (nicardipine, quinidine, tacrolimus, and verapamil) were used as model compounds. Observed F_aF_g values for these compounds were calculated from *in vivo* pharmacokinetic parameters, while *in vitro* intestinal intrinsic clearance ($CL_{int,intestine}$) was determined using human intestinal microsomes. The $CL_{int,intestine}$ for the model compounds corrected with that of midazolam were defined as $CL_{m,index}$ and incorporated into simplified F_g model with empirical scaling factor. Regardless of whether or not the compound was a P-gp substrate, the F_aF_g could be reasonably fitted by simplified F_g model, and the value of the empirical scaling factor was well estimated. These results suggest that the effects of P-gp on F_a and F_g are substantially minor, at least in the case of highly permeable compounds. Further, liver intrinsic clearance ($CL_{int,liver}$) can be used as a surrogate index of intestinal metabolism based on the relationship between $CL_{int,liver}$ and $CL_{m,index}$. F_g can be easily predicted using simplified F_g model with the empirical scaling factor, enabling more confident selection of drug candidates with desirable pharmacokinetic profiles in

humans.

Introduction

Given the substantial time and cost associated with drug discovery and development, increasing importance has been placed on the prediction of pharmacokinetics (PK) in humans of drug candidates at the discovery stage to avoid later termination of development due to an undesirable PK profile.

Bioavailability (F) of an orally administered drug, which is the fraction of drug reaching systemic blood circulation, is expressed as the product of the fraction of the dose that enters the enterocyte (F_a), intestinal availability (F_g), and hepatic availability (F_h). Therefore, to effectively pass into systemic blood circulation, orally administered drugs must not only have high solubility and permeability in the gastrointestinal tract, but also be stable against metabolizing enzymes in the gastrointestinal tract and liver.

At the drug discovery stage, compounds selected as drug candidates using high throughput screening typically are those with high permeability, since intestinal permeability is one of the most important factors in determining the F of orally administered drugs. Although P-gp is highly expressed in intestinal epithelial cells and has the potential to reduce drug absorption, the effect on drug absorption is not quantitatively as important as suggested, particularly for highly permeable compounds (Chiou et al., 2001; Lin, 2004; Cao et al., 2005). Therefore, for highly permeable compounds, a relatively high F_a can be expected when dissolution in the gastrointestinal tract is not the rate-limiting step.

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CYP3A subfamily and P-gp are present in high levels in human intestinal epithelial cells as a metabolizing enzyme and an efflux transporter, respectively. Benet et al. (1999) proposed that the synergistic effects of CYP3A4-mediated metabolism and P-gp-mediated efflux in epithelial cells may result in an unexpectedly high first-pass metabolism in the intestine due to the overlapping substrate specificities of these proteins.

Ito et al. (1999) constructed a theoretical model for F_aF_g which took into consideration the integrated process of permeability and metabolism in epithelial cells:

$$F_aF_g = \frac{CL_{ab}}{CL_{ab}+CL_m} \times \left\{1-\exp\left(-\frac{PS_{inf}}{Q} \times \frac{CL_{ab}+CL_m}{PS_{eff}+CL_{ab}+CL_m}\right)\right\} \quad \text{(Equation 1)}$$

where PS_{inf} is the apparent influx clearance from the lumen to epithelial cells, PS_{eff} is the efflux clearance from cells to the lumen, CL_{ab} is the absorption clearance from cells to blood, CL_m is the metabolic clearance in cells, and Q is luminal flow rate. However, precise estimation of several parameters described in the theoretical model is difficult, and therefore application of the model has not been reported.

Yang et al. (2007) constructed a "Q_{gut} model" for predicting F_g:

$$F_g = \frac{Q_{gut}}{Q_{gut}+fu_G \times CL_{int,intestine}} \quad \text{(Equation 2)}$$

$$Q_{gut} = \frac{Q_{villi} \times CL_{perm}}{Q_{villi}+CL_{perm}} \quad \text{(Equation 3)}$$

where CL_{perm} is the clearance defining permeability through the enterocyte, Q_{villi} is villous blood flow, fu_G is the fraction of unbound drug in the enterocyte, and Q_{gut} is a hybrid parameter of CL_{perm}

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and Q_{villi}. In their report, assuming fu_G to be unity and a Q_{villi} value of 18 L/h, the accuracy in predicting F_g was better than that of the "well-stirred" gut model. Therefore, if the values of CL_{perm} and $CL_{int,intestine}$ can be estimated precisely and easily, the Q_{gut} model may be useful in predicting F_g at the drug discovery stage.

In the present study, we aimed to establish a more practical and convenient method for predicting F_g by simplifying the theoretical model under conditions of high permeability. We focused on CYP3A4-mediated metabolism and examined intestinal intrinsic clearance ($CL_{int,intestine}$) of 5 model compounds for CYP3A4 substrates (alprazolam, amlodipine, clonazepam, midazolam, and nifedipine) and 4 model compounds for both CYP3A4 and P-gp substrates (nicardipine, quinidine, tacrolimus, and verapamil). The obtained $CL_{int,intestine}$ were corrected using the $CL_{int,intestine}$ of midazolam (reference compound). These corrected values, defined as $CL_{m,index}$, were incorporated into the simplified model. We examined whether or not F_g values for highly permeable compounds can be explained satisfactorily by the simplified model. In addition, the relationship between $CL_{m,index}$ and $CL_{int,liver}$ was examined for 35 compounds, and a reliable procedure for predicting F_g was proposed.

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Materials and Methods

Materials

All compounds except midazolam and tacrolimus were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Midazolam, tacrolimus, and in-house compounds were provided by Astellas Pharma Inc. (Tokyo, Japan). Pooled human intestinal microsomes (HIMs) were purchased from XenoTech LLC (Kansas City, KS, USA) and individual HIMs were purchased from KAC Co., Ltd. (Kyoto, Japan). Pooled human liver microsomes (HLMs) were purchased from XenoTech. All other chemicals and reagents were of analytical grade and purchased from commercial sources.

Permeability study using artificial membrane

Parallel artificial membrane permeability assays (PAMPA) conducted in this study utilized the PAMPA Evolution from *p*ION Inc. (Woburn, MA, USA). In a PAMPA, a "sandwich" is formed from a 96-well microtiter plate (*p*ION Inc., PN 110163) and a 96-well filter plate (IPVH; Millipore, Billerica, MA, USA), so that each composite well is divided into two chambers, with a donor at the bottom and an acceptor at the top, separated by a 125 μm thick microfilter disc (0.45 μm pores) coated with a 20% (w/v) dodecane solution of a lecithin mixture (*p*ION Inc., PN 110669). Drug samples were introduced via a 10 mM dimethyl sulfoxide (DMSO) stock solutions in a 96-well polypropylene microtiter plate. The robotic liquid handling system draws a 5 μL aliquot of the DMSO stock solution and mixes it into an aqueous buffer solution of 10% (v/v) DMSO to attain a

final typical sample concentration of 50 μM. The drug solutions were filtered using a 96-well filter plate (PVDF; Corning Life Sciences, Lowell, MA, USA), and added to the donor compartments. The donor solutions was adjusted to pH 6.5 (NaOH-treated universal buffer; *p*ION Inc., PN 110151), while the acceptor solution had a pH of 7.4 (*p*ION Inc., PN 110139). The plates were sandwiched together and incubated at 25 °C for 2 h in a humidity-saturated atmosphere. After incubation, the sandwiched plates were separated, and both the donor and acceptor compartments were assayed for the amount of material present by comparison with the UV spectrum (270–400 nm) obtained from reference standards. Mass balance was used to determine the amount of material remaining in the membrane barrier. The apparent permeability coefficient (P_{app}) was calculated using PAMPA Evolution software.

***In vitro* metabolism study in human intestinal microsomes**

$CL_{int,intestine}$ was calculated from the substrate disappearance rate in human intestinal microsomes (HIMs). Each compound was incubated with a reaction mixture (1,000 μL) consisting of human intestinal microsomal protein in 100 mM potassium phosphate buffer (pH 7.4) and 0.1 mM EDTA. After preincubation for 5 min at 37 °C, the enzyme reaction was initiated by adding 100 μL of 10 mM NADPH. The final concentration of each compound was 0.2 μM. The microsomal concentration used was 0.2 mg/mL except nicardipine with the concentration of 0.02 mg/mL. The final concentration of the organic solvent (acetonitrile) in the reaction mixture was 0.5% (v/v). For all compounds except tacrolimus and cyclosporine, 100 μL of the reaction mixture was moved into

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200 μL of acetonitrile containing internal standard (diazepam, 100 ng/mL) at appropriate time points for stopping the reaction. The reaction mixture was then centrifuged at 10,000g for 5 min, and 10 μL of the aliquot was injected into LC-MS/MS. With regard to tacrolimus, 100 μL of the reaction mixture was moved into 100 μL of acetonitrile containing internal standard (FR900520, 100 ng/mL) for stopping the reaction, and 500 μL of 0.01 M ammonium acetate buffer (pH 7.5) and 4.5 mL of tert-butyl methyl ether were added. The mixture was shaken for 20 sec and centrifuged at 1,870g for 10 min at 4 °C. Four milliliters of the organic layer was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved with 200 μL of 2 mM ammonium acetate in water with 75% methanol, and 10 μL of the aliquot was injected into LC-MS/MS. With regard to cyclosporine, 100 μL of the reaction mixture was moved into 100 μL of acetonitrile containing the internal standard (FR304906, 100 ng/mL) to stop the reaction, and 500 μL of distilled water and 4.5 mL of tert-butyl methyl ether were added. The mixture was shaken for 20 sec and centrifuged at 1,870×*g* for 10 min at 4 °C. Four milliliters of the organic layer was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was then dissolved with 200 μL of 10 mM ammonium acetate in water with 90% methanol, and 10 μL of the aliquot was injected into LC-MS/MS.

Since the activity of CYP enzymes in HIMs depends on the preparation method (mucosal scraping and enterocyte elution) (Galetin and Houston, 2006), the use of absolute value of $CL_{int,intestine}$ for F_g prediction is somewhat problematic. Therefore, in the present study, $CL_{int,intestine}$, expressed as mL/min/mg microsomal protein, was corrected for each compound with that of midazolam using Equation 4. This corrected value was defined as $CL_{m,index}$. The $CL_{m,index}$ values

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were used for metabolic parameter in intestine.

$$CL_{m,index} = \frac{CL_{int,intestine} \text{ of compound A}}{CL_{int,intestine} \text{ of midazolam}} \quad \text{(Equation 4)}$$

***In vitro* metabolism study in human liver microsomes**

$CL_{int,liver}$ was calculated from the substrate disappearance rate in human liver microsomes (HLMs). For nicardipine and felodipine, the microsomal concentrations used were 0.02 mg/mL and 0.05 mg/mL, respectively, while a concentration of 0.2 mg/mL was used for the remaining commercial compounds. For in-house compounds, the concentrations used ranged from 0.02 to 0.2 mg/mL. The other experimental conditions and sample preparation methods were the same as those employed in the HIMs *in vitro* metabolism study. $CL_{int,liver}$, expressed as mL/min/mg microsomal protein, was scaled to a whole body clearance (mL/min/kg) using physiological parameters of 32 mg microsomal protein/g liver (Barter et al., 2007) and 24.1 g liver/kg body weight (Davies and Morris, 1993).

Analytical method

The compounds were measured by LC-MS/MS using a Quattro Ultima (Waters, Milford, MA, USA) with an Alliance 2695 separation module (Waters). Multiple reaction monitoring mode was used to monitor ions as follows (precursor ion/product ion): alprazolam (309.0/281.0), amlodipine (409.0/238.0), clonazepam (315.9/270.0), midazolam (326.0/291.1), nifedipine (347.1/315.1),

nicardipine (480.0/315.0), quinidine (325.1/183.9), tacrolimus (821.5/768.3), verapamil (455.2/165.0), amitriptyline (278.0/91.0), cyclosporine (1219.2/1202.7), felodipine (383.9/337.9), propafenone (342.1/116.0), propranolol (260.1/182.9), timolol (317.1/261.0), diazepam (284.9/154.0), FR304906 (1280.9/1263.6), and FR900520 (809.6/756.2). With regard to alprazolam, amlodipine, clonazepam, nicardipine, and felodipine, samples were injected into an XTerra MC C18 column (3.5 μm, 4.6 mm×50 mm, Waters) warmed to 40 °C. Elution was conducted with 0.1% formic acid in water/acetonitrile (40:60) at a flow rate of 0.3 mL/min. With regard to midazolam, nifedipine, quinidine, verapamil, amitriptyline, propafenone, propranolol, and timolol, samples were injected into an Ascentis RP-Amide column (3 μm, 3 mm×30 mm, Supelco Inc., Bellefonte, PA, USA) warmed to 50 °C. Elution was conducted at flow rate of 0.5 mL/min by a linear gradient with the mobile phase, which consisted of A (20 mM ammonium acetate in water with 10% acetonitrile) and B (20 mM ammonium acetate in water with 90% acetonitrile). Except for timolol, the gradient condition was [min, B%] = [0, 0] - [0.5, 0] - [1, 70] - [3, 70] - [3.1, 0] - [4, 0]. The gradient condition for timolol was [min, B%] = [0, 0] - [0.5, 0] - [1, 70] - [3, 70] - [3.1, 0] - [4.5, 0]. With regard to tacrolimus, samples were injected into an XTerra MC C18 column (3.5 μm, 4.6 mm×50 mm, Waters) warmed to 55 °C. Elution was conducted at a flow rate of 0.4 mL/min with 2 mM ammonium acetate in water/2 mM ammonium acetate in methanol with 0.1% formic acid (20:80). With regard to cyclosporine, samples were injected into an XTerra MC C18 column (5 μm, 2.1 mm×50 mm, Waters) warmed to 40 °C. Elution was conducted at a flow rate of 0.2 mL/min with 10 mM ammonium acetate in water/methanol (10:90).

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Calculation of F_aF_g in humans

F_aF_g in humans was calculated using Equations 5-7 as follows,

$$CL_h = CL_{tot} - CL_r \quad \text{(Equation 5)}$$

$$F_h = 1 - \frac{CL_h}{Q_h} \quad \text{(Equation 6)}$$

$$F_aF_g = \frac{F}{F_h} \quad \text{(Equation 7)}$$

where CL_h is hepatic clearance, CL_{tot} is total body clearance, CL_r is renal clearance, and Q_h is hepatic blood flow in humans. Since the calculation of F_aF_g depends on Q_h, three different reference values (17.1, 20.7, and 25.5 mL/min/kg) were used for Q_h (Davies and Morris, 1993; Kato et al., 2003), and the pharmacokinetic parameters were derived from the literature. When pharmacokinetic parameters were obtained from more than one reference, the mean values were used. When CL_{tot} was obtained based on plasma concentration, it was converted to a blood-based value using the blood-to-plasma concentration ratio (R_b), which was obtained from the literature, or assumed to be unity if no value was available.

Simplification of the theoretical model of F_aF_g

The following assumptions were made to simplify the theoretical model:

Assumption 1: For highly permeable compounds, absorption is complete (Fa=1) when dissolution in the gastrointestinal tract is not the rate-limiting step.

Equation 1 can thus be simplified to Equation 8 by Assumption 1.

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$$F_a F_g = F_g = \frac{CL_{ab}}{CL_{ab} + CL_m} = \frac{1}{1 + \frac{CL_m}{CL_{ab}}} \quad \text{(Equation 8)}$$

According to Equation 8, F_g value is determined by the balance of CL_{ab} and CL_m.

Assumption 2: The difference in CL_{ab} values between highly permeable compounds is minor, and F_g value is mainly determined by CL_m.

Equation 8 can thus be simplified to Equation 9 by Assumption 2,

$$F_g = \frac{1}{1 + A \times CL_m} \quad \text{(Equation 9)}$$

where A is the reciprocal value of CL_{ab} and assumed to be treated as a constant value.

Assumption 3: CL_m is proportional to $CL_{m,index}$.

As shown in Equation 4, $CL_{m,index}$ is a metabolic parameter which has been corrected using reference compound (midazolam).

$$CL_m = SF \times CL_{m,index} \quad \text{(Equation 10)}$$

where SF is the scaling factor between CL_m and $CL_{m,index}$.

Combining Equation 9 and 10 gives Equation 11,

$$F_g = \frac{1}{1 + \alpha \times CL_{m,index}} \quad \text{(Equation 11)}$$

$$\alpha = A \times SF \quad \text{(Equation 12)}$$

where α is defined as the empirical scaling factor.

In the present study, Equation 11 is referred to as the "simplified F_g model." We examined

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whether or not F_g values for highly permeable compounds can be satisfactorily explained using this simplified F_g model.

Estimation of the empirical scaling factor (α) of simplified F_g model

Alprazolam, amlodipine, clonazepam, midazolam, nifedipine, nicardipine, quinidine, tacrolimus, and verapamil were used to establish the simplified F_g model. The value of α was estimated from a fitting study between human F_aF_g and the $CL_{m,index}$ values of these model compounds using a non-linear least squares method, MULTI (Yamaoka et al., 1981).

Results

Initially, permeability of the model compounds (alprazolam, amlodipine, clonazepam, midazolam, nifedipine, nicardipine, quinidine, tacrolimus, and verapamil) was examined using an artificial membrane, and the values of $P_{app,PAMPA}$ were compared with those of 25 reference compounds having known F_a in humans (Zhu et al., 2002) (Table 1). Figure 1 shows the correlation of $P_{app,PAMPA}$ versus F_a of the reference compounds. Based on the correlation, high F_a can be expected when $P_{app,PAMPA}$ value is $>1.0\times10^{-6}$ cm/sec. Since the all model compounds for CYP3A4 substrates showed high permeability ($\geqq7.0\times10^{-6}$ cm/sec), the F_aF_g values were assumed equal to F_g values ($F_a=1$).

Table 2 shows the $CL_{int,intestine}$ of the model compounds obtained from *in vitro* metabolism study in human intestinal microsomes (HIMs). On comparison of $CL_{int,intestine}$ for 4 HIMs from different lots, an approximately 3-fold variation in the $CL_{int,intestine}$ was observed. $CL_{int,intestine}$ ranged from 0.167 to 0.534 mL/min/mg for midazolam, 1.287 to 3.600 mL/min/mg for nicardipine, and 0.107 to 0.291 mL/min/mg for verapamil. However, similar values were observed after correction using Equation 1 to obtain $CL_{m,index}$, with values ranging from 0.07 to 0.09 for amlodipine, 0.53 to 0.64 for nifedipine, 6.74 to 7.71 for nicardipine, 2.82 to 3.36 for tacrolimus, and 0.54 to 0.64 for verapamil. Therefore, in the present study, mean values of $CL_{m,index}$ were used for the fitting study with simplified F_g model.

Table 3 summarizes the pharmacokinetic parameters of the model compounds in humans as well as values for F_aF_g calculated from Equations 5-7 under 3 different Q_h conditions. F_aF_g values

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for the model compounds represented a wide range. For example, under high Q_h conditions (25.5 mL/min/kg), high F_aF_g values were observed for alprazolam, amlodipine, clonazepam, nifedipine, and quinidine (0.730-0.932), middle-range values for midazolam and verapamil (0.482-0.509), and low values for nicardipine and tacrolimus (0.106-0.185). However, under low Q_h conditions (17.1 mL/min/kg), F_aF_g values for amlodipine, nifedipine, and verapamil were all calculated at >1. When F_aF_g was calculated at >1, it was treated as 1 for the estimation of α.

Figure 2 shows the relationship between the F_aF_g and $CL_{m,index}$ of the model compounds under 3 different Q_h conditions. Regardless of whether or not the compound was a P-gp substrate, F_aF_g of the model compounds decreased as $CL_{m,index}$ increased, and reasonably good fits were obtained using simplified F_g model, at least in the case of middle and high Q_h conditions (20.7 and 25.5 mL/min/kg, respectively). The α values obtained for the Q_h conditions of 17.1, 20.1, and 25.5 mL/min/kg were 0.43, 0.64, and 1.13, respectively (Table 4).

The observed F_aF_g and predicted F_g for felodipine and cyclosporine, which are CYP3A4 substrates with high permeability and outside of the model compounds used to construct simplified F_g model, are presented in Tables 5 and 6. F_g prediction was conducted using a simplified F_g model with the α values listed in Table 4. For felodipine, extensive metabolism in HIMs was observed ($CL_{m,index}$ was 2.80), and the predicted F_g ranged from 0.240 to 0.454. The predicted values were close to the observed F_aF_g values (0.222 to 0.291). In contrast, for cyclosporine, mild metabolism in HIMs was observed ($CL_{m,index}$ was 0.30), and the predicted F_g ranged from 0.747 to 0.886. The predicted values were slightly higher than the observed F_aF_g values (0.567 to 0.684).

Figure 3 shows the relationship between $CL_{m,index}$ and $CL_{int,liver}$ for 35 compounds containing the model compounds, additional 5 drug compounds (amitriptyline, felodipine, propafenone, propranolol, and timolol), and 21 in-house compounds. The results showed reasonably good correlation between these parameters, for example, when $CL_{int,liver}$ was <100 mL/min/kg, $CL_{m,index}$ of almost all compounds were <0.2. On the other hand, when $CL_{int,liver}$ was >100 mL/min/kg, most $CL_{m,index}$ increased. Furthermore, when $CL_{int,liver}$ was >1,000 mL/min/kg, $CL_{m,index}$ of all compounds exceeded 1.0. However, some compounds had $CL_{m,index}$ <0.2 despite having a $CL_{int,liver}$ >100 mL/min/kg.

Discussion

The importance of the intestine on drug metabolism has been well recognized (Wacher et al., 2001), however, it is difficult to estimate precisely all the important factors for intestinal metabolism at the drug discovery stage. Rats, dogs, and monkeys have been widely used in the prediction of PK in humans at the drug discovery stage, and evaluation using rats has been particularly useful for satisfactorily predicting F_a in humans (Zhao et al., 2003). However, expression levels and patterns for metabolizing enzymes in intestine differ distinctly between the two species (Cao et al., 2006). Further, with regard to dogs, intestinal enzymes are generally less active than in humans (Prueksaritanont et al., 1996), and although monkeys are genetically similar to humans, some drugs have shown remarkably lower F_aF_g in monkeys than in humans, possibly due to activity of metabolizing enzymes and efflux transporters being higher in monkey intestine than in human intestine (Takahashi et al., 2009; Akabane et al., 2010). These experimental animals are therefore unsuitable for predicting F_g in humans.

In the present study, we aimed to develop a practical and convenient method for predicting F_g of highly permeable compounds using simplified F_g model and empirical scaling factor, α. Our results showed that, regardless of whether or not a compound was a P-gp substrate, F_aF_g of the model compounds decreased as the $CL_{m,index}$ increased, and the F_aF_g could be reasonably fitted using simplified F_g model (Figure 2, Table 4). If P-gp-mediated efflux has a significant impact on F_a, and if synergistic effects of CYP3A4-mediated metabolism and P-gp-mediated efflux result in an unexpectedly low F_g, F_aF_g of P-gp and non P-gp substrates cannot be well fitted by the same curve

obtained from simplified F_g model. This finding suggests that, at least for highly permeable compounds, the effect of P-gp on F_a and the synergistic effects of CYP3A4 and P-gp on F_g are substantially minor.

Further, to verify the reliability of the simplified F_g model and α values, F_g prediction using simplified F_g model was conducted for cyclosporine and felodipine, which are CYP3A4 substrates having high permeability (Table 5, Table 6). With regard to cyclosporine, the predicted F_g value was slightly higher than the observed F_aF_g value. This discrepancy may be due to the dissolution characteristics of cyclosporine being sufficiently poor to cause low F_a (Varma and Panchagnula, 2005). With regard to felodipine, the predicted F_g value was close to the observed F_aF_g value.

According to the Q_{gut} model (Yang et al., 2007), when permeability is high ($CL_{perm} >> Q_{villi}$), Equation 2 can be transformed into Equation 13.

$$F_g = \frac{Q_{villi}}{Q_{villi} + fu_G \times CL_{int,intestine}} \quad \text{(Equation 13)}$$

Provided fu_G value does not differ significantly among compounds, F_g value can be determined using only the metabolic parameter, as the simplified F_g model. This may explain why the simplified F_g model was acceptable for predicting F_g of highly permeable compounds.

However, for compounds with low permeability, the simplified F_g model with α estimated here may actually overestimate the observed F_g. Residence time in epithelial cells for these low-permeability compounds may be longer than that of compounds with high permeability in the absorption process, and thus these compounds may have greater exposure to metabolizing enzymes

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within cells than more permeable compounds. Further, according to the Q_{gut} model (Yang et al., 2007), when permeability is low (Q_{villi}>>CL_{perm}), Equation 2 can be transformed into Equation 14. F_g values for low-permeability compounds are determined by the balance of the permeability and metabolic parameter.

$$F_g = \frac{CL_{perm}}{CL_{perm} + fu_G \times CL_{int,intestine}} \quad \text{(Equation 14)}$$

In the present study, we examined the relationship between $CL_{m,index}$ and $CL_{int,liver}$ for 35 compounds (Figure 3). While results showed reasonably good correlation between these parameters, several compounds had $CL_{m,index}$ <0.2 despite having a $CL_{int,liver}$ >100 mL/min/kg. For example, while the model compounds for CYP3A4 substrates showed good correlation, propafenone, which is mainly metabolized by CYP2D6 (Dilger et al., 2000), had $CL_{m,index}$ <0.2 despite having a $CL_{int,liver}$ of approximately 700 mL/min/kg. Further, in-house compound A, which is mainly metabolized by CYP1A2 and CYP2D6, had a $CL_{m,index}$ of 0.01 despite having a $CL_{int,liver}$ of approximately 600 mL/min/kg. Although Galetin and Houston (2006) reported that the rank order of CL_{int} values for CYP3A4, CYP2C9, CYP2C19, and CYP2D6 substrates was consistent between HLMs and HIMs, Paine et al. (2006) reported minimal contribution of CYP2D6 for intestinal metabolism. For example, CL_{int} value for metoprolol, a substrate for CYP2D6, was much lower in HIMs compared with HLMs (0.7 vs. 19.7 μL/min/mg). Further, Kato et al. (2003) reported that F_aF_g values of CYP3A4 substrates, but not non-CYP3A4 substrates, were reduced when $CL_{int,liver}$ exceeded 100 mL/min/kg. These previous findings therefore suggest that metabolism in the intestine is probably

minor for non-CYP3A4 substrates, even if metabolism in the liver is extensive. To obtain a more reliable prediction of F_g, the value of $CL_{m,index}$ should be used rather than $CL_{int,liver}$.

Based on the results, we proposed a reliable procedure for predicting F_g in humans at the drug discovery stage. When $CL_{int,liver}$ of candidate compound is >100 mL/min/kg, $CL_{m,index}$ is typically >0.2, which results in a predicted F_g of <0.814 when using a high α (1.13), and an *in vitro* metabolism study in HIMs should therefore be performed to predict F_g from simplified F_g model. To avoid underestimating F_g values of drug candidates, the use of a high α value (1.13) may be desirable at the drug discovery stage.

Although the present study focused on CYP3A4-mediated intestinal metabolism, conjugate enzymes such as UDP-glucuronyltransferase and sulfotransferase have also been found to be expressed in human intestine (Glatt et al., 2001; Cao et al., 2006). In fact, the intestinal conjugative metabolism exerts a measurable impact on F in humans (Mizuma et al., 2005). If values of F_g in humans of substrates for conjugate enzymes can be fully compiled, estimation of empirical scaling factor related to conjugation may be possible from simplified F_g model, as in CYP3A4 substrates.

In conclusion, we developed a practical and convenient method for predicting F_g of highly permeable compounds from simplified F_g model and the results of *in vitro* metabolism study in HIMs, focusing on CYP3A4-mediated metabolism. Using this method, F_g can be easily predicted at the drug discovery stage, enabling more confident selection of drug candidates with desirable PK profiles in humans.

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Footnote

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Legends for figures

Figure 1. Correlation between F_a in humans and permeability in artificial membrane of 25 reference compounds

P_{app} <0.1×10 $^{-6}$ cm/sec was plotted as 0.1×10 $^{-6}$ cm/sec. F_a values were quoted from Zhu et al. (2002).

Figure 2. Relationship between human F_aF_g and $CL_{m,index}$ of 9 model compounds under 3 Q_h conditions

Solid lines were obtained by a fitting study with simplified F_g model using a non-linear least square method, MULTI. When observed F_aF_g was calculated at >1, it was treated as 1. When $CL_{m,index}$ was <0.01, it was treated as 0.01. Open circles represent non-P-gp substrates, and closed circles represent P-gp substrates. 1, alprazolam; 2, amlodipine; 3, clonazepam; 4, midazolam; 5, nifedipine; 6, nicardipine; 7, quinidine; 8, tacrolimus; 9, verapamil.

Figure 3. Relationship between $CL_{m,index}$ and $CL_{int,liver}$

$CL_{m,index}$ <0.01 was plotted as 0.01. $CL_{int,liver}$ <10 mL/min/kg was plotted as 10 mL/min/kg. The size of the balls reflects the F_g predicted from simplified F_g, with large balls representing high F_g and small balls low F_g. Closed circles represent the 9 model compounds, and open circles represent 5 commercial and 21 in-house compounds. 1, alprazolam; 2, amlodipine; 3, clonazepam; 4,

midazolam; 5, nifedipine; 6, nicardipine; 7, quinidine; 8, tacrolimus; 9, verapamil; 10, amitriptyline; 11, felodipine; 12, propafenone; 13, propranolol; 14, timolol.

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Table 1. Permeability in artificial membranes and F_a in humans

Reference Compounds	P_{app} ($\times 10^{-6}$ cm/sec)	F_a [a]	Model Compounds	P_{app} ($\times 10^{-6}$ cm/sec)
Acetaminophen	2.8	0.80	Alprazolam	7.0
Acyclovir	0.0	0.21	Amlodipine	19.5
Alprenolol	37.2	0.94	Clonazepam	18.6
Amphotericin B	0.0	0.05	Midazolam	30.8
Antipyrine	2.6	1.00	Nifedipine	13.4
Atenolol	0.1	0.52	Nicardipine	31.5
Caffeine	2.0	1.00	Quinidine	17.9
Carbamazepine	27.7	1.00	Tacrolimus	34.3
Dicrofenac	32.9	1.00	Verapamil	35.8
Furosemide	0.2	0.60		
Hydrochlorothiazide	0.1	0.70		
Indomethacin	22.4	1.00		
Ketoprofen	12.7	1.00		
Ketorolac	4.0	1.00		
Metoprolol	11.3	0.95		

Nadolol	0.0	0.32
Naproxen	11.1	0.98
Piroxicam	16.1	1.00
Prednisone	14.3	0.99
Propranolol	37.4	0.93
Ranitidine	0.1	0.55
Sulpiride	0.0	0.36
Terbutaline	0.7	0.68
Theophylline	1.8	0.97
Warfarin	12.9	0.97

[a] Quoted from Zhu et al. (2002).

Table 2. Intestinal intrinsic clearance and $CL_{m,index}$ of 9 model compounds in 4 different pooled human intestinal microsomes

	HIMs-1 [a]		HIMs-2 [a]		HIMs-3 [b]		HIMs-4 [b]		Mean
	$CL_{int,intestine}$ (mL/min/mg)	$CL_{m,index}$	$CL_{int,intestine}$ (mL/min/mg)	$CL_{m,index}$	$CL_{int,intestine}$ (mL/min/mg)	$CL_{m,index}$	$CL_{int,intestine}$ (mL/min/mg)	$CL_{m,index}$	$CL_{m,index}$
Alprazolam	NT	-	ND	0.00	NT	-	ND	0.00	0.00
Amlodipine	NT	-	0.029	0.07	NT	-	0.022	0.09	0.08
Clonazepam	NT	-	ND	0.00	NT	-	ND	0.00	0.00
Midazolam	0.534	1.00	0.414	1.00	0.167	1.00	0.251	1.00	1.00
Nifedipine	0.340	0.64	NT	-	0.088	0.53	NT	-	0.58
Nicardipine	3.600	6.74	NT	-	1.287	7.71	NT	-	7.22
Quinidine	ND	0.00	NT	-	ND	0.00	NT	-	0.00
Tacrolimus	1.792	3.36	NT	-	NT	-	0.706	2.82	3.09
Verapamil	0.291	0.54	NT	-	0.107	0.64	NT	-	0.59

$CL_{m,index}$ were calculated from Equation 1. When no depletion was observed, the $CL_{m,index}$ was treated as zero.

ND, no depletion; NT, not tested.

[a] Individual microsomes.

[b] Pooled microsomes.

Table 3. Pharmacokinetic parameters, F, F_h, and F_aF_g of 9 model compounds in humans

	Substrate	$CL_{tot,p}$ (mL/min/kg)	R_b	$CL_{tot,b}$ (mL/min/kg)	CL_r (mL/min/kg)	CL_h (mL/min/kg)	F	Q_h (mL/min/kg) 17.1 F_h	17.1 F_aF_g	20.7 F_h	20.7 F_aF_g	25.5 F_h	25.5 F_aF_g
Alprazolam	CYP3A4	0.74	0.78 [a]	0.95	0 [d]	0.95	0.880	0.945	0.932	0.954	0.922	0.963	0.914
Amlodipine	CYP3A4	7.00	1.00 [d]	7.00	0 [d]	7.00	0.640	0.591	1.084	0.662	0.967	0.725	0.882
Clonazepam	CYP3A4	0.87	1.00 [d]	0.87	0 [d]	0.87	0.900	0.949	0.948	0.958	0.939	0.966	0.932
Midazolam	CYP3A4	5.77	0.53 [a]	10.89	0 [d]	10.89	0.276	0.363	0.760	0.474	0.582	0.573	0.482
Nifedipine	CYP3A4	8.22	0.74 [b]	11.11	0 [d]	11.11	0.412	0.350	1.176	0.463	0.889	0.564	0.730
Nicardipine	CYP3A4, P-gp	7.00	0.71 [c]	9.86	0 [d]	9.86	0.065	0.423	0.154	0.524	0.124	0.613	0.106
Quinidine	CYP3A4, P-gp	3.86	0.92 [a]	4.20	1.37	2.83	0.764	0.835	0.915	0.863	0.885	0.889	0.859
Tacrolimus	CYP3A4, P-gp	-	-	0.70	0 [d]	0.70	0.180	0.959	0.188	0.966	0.186	0.973	0.185
Verapamil	CYP3A4, P-gp	12.15	0.77 [a]	15.78	0 [d]	15.78	0.194	0.077	2.512	0.238	0.816	0.381	0.509

$CL_{tot,p}$, total body clearance in plasma base; $CL_{tot,b}$, total body clearance in blood base. Pharmacokinetic parameters were quoted from reference as follows: alprazolam (Smith et al., 1984); amlodipine (Faulkner et al., 1986); clonazepam (Crevoisier et al., 2003); midazolam (Kupferschmidt et al., 1995; Thummel et al., 1996; Tsunoda et al., 1999); nifedipine (Holtbecker et al., 1996); nicardipine (Higuchi and Shiobara, 1980); quinidine (Greenblatt et al., 1977; Rakhit et al., 1984); tacrolimus (Floren et al., 1997; Moller et al., 1999); verapamil (McAllister and Kirsten, 1982; Eichelbaum et al., 1984).

[a] Quoted from Obach (1999).

[b] Quoted from Holtbecker et al. (1996).

[c] Quoted from *Goodman and Gilman's textbook, The Pharmacological Basis of Therapeutics, 9th ed.*

[d] Assumed value.

Table 4. Empirical scaling factor of the simplified F_g model obtained from 9 model compounds under 3 Q_h conditions

Q_h (mL/min/kg)	17.1	20.7	25.5
α	0.43	0.64	1.13

α values were estimated from a fitting study between human F_aF_g and $CL_{m,index}$ of 9 model compounds with simplified F_g model using a non-linear least square method, MULTI.

Table 5. Pharmacokinetic parameters, F, F_h, and F_aF_g of cyclosporine and felodipne in humans

	$CL_{tot,p}$ (mL/min/kg)	R_b	$CL_{tot,b}$ (mL/min/kg)	CL_r (mL/min/kg)	CL_h (mL/min/kg)	F	Q_h (mL/min/kg) 17.1 F_h	17.1 F_aF_g	20.7 F_h	20.7 F_aF_g	25.5 F_h	25.5 F_aF_g
Cyclosporine	-	-	6.60	0 [a]	6.60	0.420	0.614	0.684	0.681	0.617	0.741	0.567
Felodipine	12.00	1.45	8.23	0 [a]	8.23	0.150	0.516	0.291	0.600	0.250	0.675	0.222

Pharmacokinetic parameters were quoted from reference as follows: cyclosporine (Gupta et al., 1990); felodipine (*Goodman and Gilman's textbook, The Pharmacological Basis of Therapeutics, 9th ed*).

[a] Assumed value.

Table 6. *In vitro* parameters and predicted F_g of cyclosporine and felodipne

			Predicted F_g [b]		
	P_{app} [a]	$CL_{m,index}$		α	
	(×10 $^{-6}$ cm/sec)		0.43	0.64	1.13
Cyclosporine	15.9	0.30	0.886	0.839	0.747
Felodipine	9.9	2.80	0.454	0.358	0.240

[a] Estimated from PAMPA.

[b] Predicted from simplified F_g model.

Figure 1.

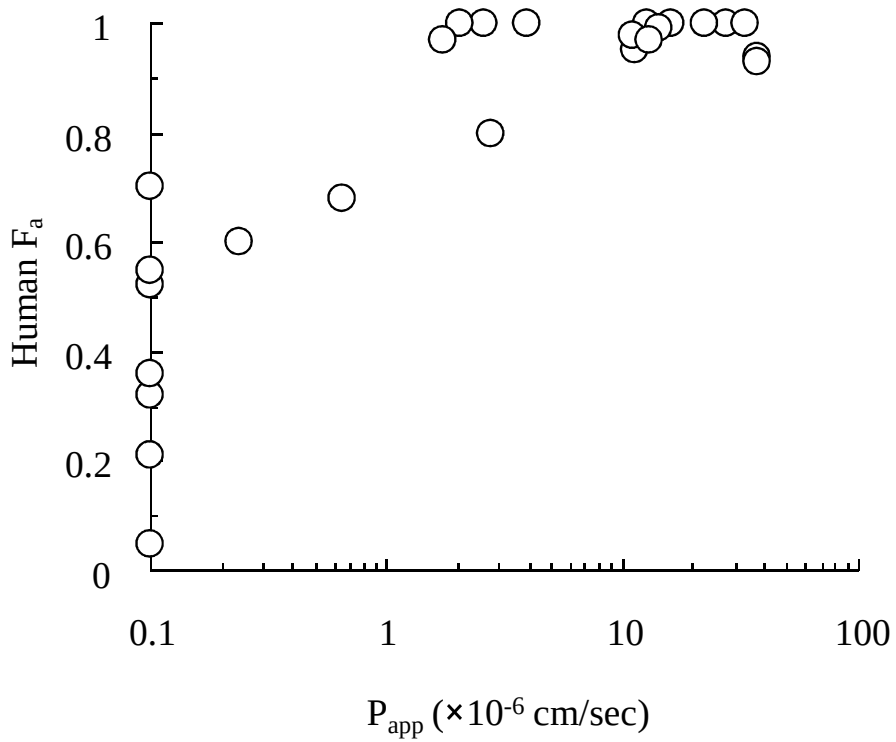

Figure 2.

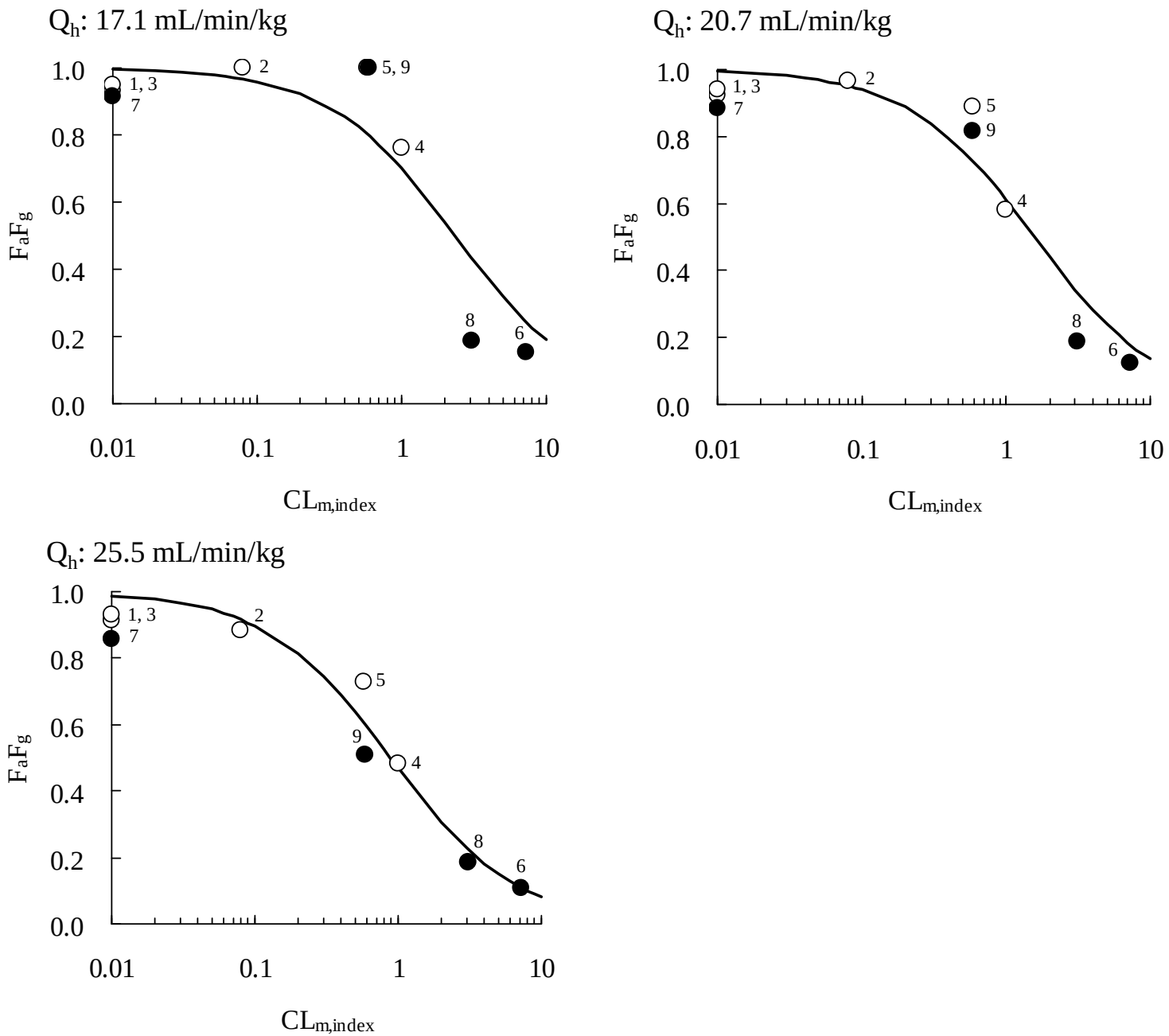

Figure 3.