Assessment of Intestinal Availability of Various Drugs
in Oral Absorption Process Using Portal Vein Cannulated Rats

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Abbreviations: F, bioavailability; Fa·Fg, intestinal availability; Fh, hepatic availability; kₐ, absorption rate constant; kₑ, elimination rate constant; CLₕ, hepatic clearance; IVIVE, in vitro-in vivo extrapolation; PBPK, physiologically based pharmacokinetic; α₁-AGP, alpha1-acid glycoprotein; Rₚ, blood / plasma concentration ratio; Qₚ₀, portal blood flow; Qₕ, hepatic blood flow; UGT, UDP-glucuronosyltransferase.
Abstract

In order to understand the rate-limiting process of oral drug absorption, not only total bioavailability (F) but also intestinal (Fa·Fg) and hepatic (Fh) availability after oral administration should be evaluated. Usually, Fa·Fg of drug is calculated from pharmacokinetic parameters after intravenous and oral administration. This approach is influenced markedly by the estimated value of Fh, which varies with the hepatic blood flow used in the calculations. In this study, portal vein cannulated rats were used to calculate the Fa·Fg of drugs from a single oral dosing experiment without data from intravenous injection. Portal vein cannulated rats were prepared by a new operative method that enables stable portal vein blood flow. This surgery had no effects on hepatic blood flow and metabolic activity. Our method for calculating Fa·Fg was validated by determining both portal and systemic plasma concentration profiles of various drugs possessing different pharmacokinetic properties following oral administration to the portal vein cannulated rats. Simulation of portal and systemic plasma concentrations by physiologically based pharmacokinetic modeling indicated that the balance of the absorption rate constant (ka) and elimination rate constant (ke) resulted in different patterns in portal and systemic plasma concentration-time profiles. This study is expected to provide a new experimental animal model that enables identification of the
factors that limit oral bioavailability and to provide pharmacokinetic information on the oral absorption process of drugs in the drug discovery.
Introduction

Current strategies in drug discovery/development to create innovative drugs that have high affinity and selectivity to target molecules has often produced new drug candidates with high molecular weight, high lipophilicity and low solubility in water. Most such compounds are easily recognized by metabolic enzymes and quickly inactivated after administration. Oral drugs may suffer from poor systemic exposure due to extensive first pass effects in the liver and intestine. Also, excretive transporters expressed in enterocytes, such as P-glycoprotein, work as a barrier to absorption by actively expelling compounds into the gastrointestinal (GI) tract (Thiebaut et al., 1987; Saito et al., 1995). In addition to metabolism and active efflux of compounds, low solubility or low membrane permeability also contribute to attenuation of their systemic exposure (Gertz et al., 2010; Nishimuta et al., 2011). When considering the feasibility of development of new oral drug candidates, it is quite important to estimate the effect of these factors on oral bioavailability (F) in humans and to understand the reasons for low systemic exposure.

Oral F of drugs can be expressed as

\[ F = F_a \cdot F_g \cdot F_h \]  (1)

where \( F_a \) is the fraction of the dose absorbed from the GI tract, and \( F_g \) and \( F_h \) are
fractions of the dose not metabolized in the intestine and liver, respectively, before reaching the systemic circulation. Fh is theoretically calculated by clearance theory from the ratio of hepatic clearance (CLh) and hepatic blood flow (Kato et al, 2003). CLh can be evaluated from kinetic analysis of in vivo blood concentration profiles of drugs after intravenous injection. Also, several in vitro methods using hepatocytes or their subcellular fractions have been established for in vitro-in vivo extrapolation (IVIVE) of CLh (Ito at al., 2005; Kilford et al., 2009).

Although use of IVIVE for hepatic metabolism is widely accepted, its use for intestinal metabolism is less definitive (Mizuma et al, 2002). Kinetic models to describe intestinal first pass metabolism have thus far not fully succeeded because the extent of intestinal metabolism is influenced not only by the intrinsic metabolic activity but also by physiological complexities that are unique to the GI tract.

Typically, Fa-Fg is calculated by dividing oral F by Fh, for which plasma concentration profiles after intravenous and oral administration are required (Kato et al, 2003). However, this approach is influenced markedly by the estimated value of Fh, which varies with hepatic blood flow used in the calculations. Also, extra-hepatic clearance must be evaluated, particularly for drugs with a high renal clearance.

The use of portal vein cannulated rats as a preclinical animal model allows the
unrestrained sampling of systemic and portal blood simultaneously without the necessity of anesthesia (Murakami et al., 2003; Kanazu et al., 2005; Kuze et al., 2009). Fa-Fg is evaluated by measuring the difference between portal and systemic blood concentrations after oral dosing (Kosaka et al., 2011; Furukawa et al., 2012). Thus, this method enables the estimation of Fa-Fg in individual animals without intravenous drug administration.

The cannulation method in this study was improved upon from a previously reported method (Murakami et al., 2003; Kuze et al., 2009) in order to obtain stable blood flow in the portal vein, and Fa-Fg in the portal vein cannulated rats was assessed after oral administration of several types of drugs (indomethacin, midazolam, felodipine, fexofenadine, raloxifene, sulpyride and famotidine) with diverse pharmacokinetic properties. In addition, in order to analyze the relationship between portal and systemic plasma concentrations, portal and systemic concentration-time profiles were simulated by physiologically based pharmacokinetic (PBPK) modeling.

This study is expected to provide a new experimental animal model that enables the assessment of intestinal and hepatic availability separately under similar physiological conditions to untreated rats and to identify the factors limiting oral bioavailability in drug discovery.
Materials and Methods

Materials. Indomethacin, midazolam, antipyrine, lidocaine, and famotidine were purchased from Wako Pure Chemicals (Osaka, Japan), and felodipine, sulpiride, raloxifene, and fexofenadine were purchased from Sigma-Aldrich (St.Louis, MO, USA). The primary metabolites (1’- or 4’-hydroxylated metabolites) of midazolam and tolbutamide were purchased from Toronto Research Chemicals (North York, ON, Canada). 4’-Hydroxylated metabolite of mephenytoin was purchased from Ultrafine Chemicals (Manchester, England). All other chemicals used were reagent grade or better.

Animals. All animal procedures were conducted under protocols approved by the Kaken Institutional Animal Care and Use Committee. Cannulated and untreated male Sprague-Dawley rats (8 weeks old, 260-300 g body weight) were purchased from Charles River Laboratory Japan (Yokohama, Japan) and were kept in an experimental animal room with an ambient temperature of 22-24°C and a 12-h light-dark cycle for 6 days before use. The cannulated and untreated rats were shipped to our lab from Charles River Laboratory Japan 2 days after the surgical procedure and arrived the next day. The oral and intravenous administration studies were conducted on the 9th and 16th days.
after the procedure.

**Surgical procedure for portal vein cannulation.** Animals were implanted with catheters in the portal vein as follows. Rats were anaesthetized with ketamine and xylazine administered intraperitoneally. A mid-line incision 1-2 cm was made in the abdominal cavity and the portal vein was detached near the liver. To prevent bleeding, the portal vein was ligated temporarily as the catheter was inserted. The catheter (3.5Fr polyurethane tube, Access™ technologies Inc.) was inserted immediately and fixed by a purse-string suture on the portal vein. The time to reperfusion was about 1 min after intercepted blood flow. This method for insertion of catheter can avoid the occlusion of the vessel. In addition, a catheter with trumpet-shaped opening was used to prevent the catheter from slipping out of the vessel with minimizing the effect on blood flow. Another end of the catheter was passed subcutaneously to the dorsal base of the neck and the laparotomy was closed in two layers, with a 4/0 silk blade to the muscle, and a surgical clip to close the skin. Surgical procedures were approved by the Institutional Animal Care and Use Committee of Charles River Laboratory Japan. This surgical procedure allows the collection of blood samples without the necessity of restraints and anesthesia.
Blood / Plasma concentration ratio. The blood / plasma concentration ratio (Rb) was determined in vitro after incubation of the compounds with fresh pooled blood from 4 cannulated rats. Blood was preincubated at 37°C in a water bath, and spiked with the test compounds at 100 ng/mL. The blood samples were incubated at 37°C for 15 min. After centrifugation at 14,000 g for 10 min, the plasma samples were transferred into 4 volumes of methanol containing verapamil (IS) and then centrifuged. The concentrations of test compounds in the supernatant were determined by liquid chromatography / tandem mass spectrometry (LC-MS/MS).

Blood biochemical and hematological tests. 5 mL of blood was taken from fasted rats under isoflurane anesthesia at 1 and 9 days after surgery. After centrifugation at 14,000 g for 10 min, aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), total protein concentration (TP), total bilirubin concentration (TBIL), albumin concentration (ALB), total cholesterol concentration (TCHO), triglyceride concentration (TG) and glucose concentration (GLU) in plasma were determined using standard enzymatic methods on an automated analyzer (DRI-CHEM, Fuji Film). Concentration of alpha1-acid glycoprotein (α1-AGP) in plasma was determined with a
commercial rat AGP ELISA kit (Immunology Consultants Lab). Red blood cells (RBC), white blood cells (WBC), hematocrit (HCT) and platelets (PLT) were determined 9 days after surgery using an automated counter (KX-21NV, Sysmex).

**Preparation of hepatic microsomes.** To compare hepatic metabolic activities of the cannulated and untreated rats, hepatic microsomes were prepared using standard techniques (von Moltke et al., 1993) with some modifications. The liver samples were homogenized with a glass potter homogenizer and the homogenate was centrifuged at 9,000 g for 20 min at 4˚C. Supernatant was collected and centrifuged at 101,000 g for 1 h at 4˚C. Pellets were resuspended in 0.1 M sodium phosphate medium (pH 7.4) to obtain the microsomal fraction. Total protein concentration was measured using a BCA Protein Assay Kit (Nacalai tesque, Kyoto, Japan) using bovine serum albumin (BSA) as a standard. The microsomes were stored at -80˚C until use.

**Measurement of metabolic activity.** Liver microsomes were diluted with 0.1 M potassium phosphate buffer (pH 7.4) to achieve a protein concentration of 10 mg/mL (1 mg/mL final concentration). Substrate solutions were prepared at a concentration of 1 mM (for tolbutamide and mephenytoin) and 1 mg/mL (for midazolam) in methanol (1%
final concentration). Substrate solutions were pre-incubated at 37°C for 5 min. The incubation was initiated by the addition of NADPH regenerating system (glucose 6-phosphate (25 mM), NADP (2.5 mM), glucose 6-phosphate dehydrogenase (2 units/mL), MgCl₂ (10 mM)). The samples were incubated for 20 min (for midazolam) and 60 min (for tolbutamide and mephenytoin), and reactions were terminated with 4 volumes of methanol containing verapamil (IS). After centrifugation at 14,000 g for 10 min, the hydroxyl metabolite concentrations of the test compounds in the supernatant were determined by LC-MS/MS.

**Pharmacokinetic Studies.** A total of 9 drugs (antipyrine, lidocaine, indomethacin, midazolam, felodipine, fexofenadine, raloxifene, sulpyride and famotidine) were used for the pharmacokinetics studies. Each of the drugs was suspended in aqueous 0.5% methyl cellulose and orally administered to the fasted rats at a dose of 5 mg/kg except for antipyrine (0.3 mg/kg), indomethacin (0.3 mg/kg) and midazolam (1 mg/kg) (n = 3). After oral administration of the drugs to the cannulated rats, blood samples were taken from the portal and caudal veins of the unanesthetized rats at 0.083, 0.25, 0.5, 1, 2, 4, 6, and 8 h under unrestricted conditions. In the case of antipyrine, indomethacin, and midazolam, an extra sampling point was added at 0.05 h. For intravenous administration,
each of the drugs was dissolved in a solution containing dimethyl sulfoxide, ethanol, cremophor EL and saline (1, 2.5, 2.5 and 94% final concentration, respectively) and intravenously administered to the fasted rats at a dose of 1 mg/kg except for antipyrine (0.3 mg/kg), indomethacin (0.1 mg/kg), and midazolam (0.3 mg/kg). Following administration, blood samples were taken from the caudal vein at 0.083, 0.25, 0.5, 1, 2, 4, 6, and 8 h. The plasma samples were separated by centrifugation at 14,000 g for 10 min at 4°C and stored at -30°C until use. The compound concentrations in the plasma were quantified using LC-MS/MS.

**LC-MS/MS analysis.** The LC-MS/MS system consisted of a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), Accela HPLC and TSQ Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA). LC conditions were as follows: column, CAPCELL PAK C18 ACR (1.5 mm I.D. × 35 mm, 3 µm; Shiseido, Tokyo, Japan), YMC-Triart C18 (2.0 mm I.D. × 30 mm, 3 µm; YMC, Kyoto, Japan); column temperature, 40°C; gradient elution at 0.3 mL/min with methanol and aqueous 0.1% formic acid; and injection volume, 15 µL. The main working parameters for mass spectrometers were as follows: ion mode, electrospray ionization, positive; spray voltage, 4000 V; sheath gas pressure, 30 Arb; auxiliary gas pressure, 35 Arb; capillary
temperature, 300˚C; multireaction monitoring method with transitions of \( m/z \) 360.4 → 141.0 for indomethacin, \( m/z \) 326.1 → 291.1 for midazolam, \( m/z \) 384.1 → 338.0 for felodipine, \( m/z \) 342.2 → 112.2 for sulpiride, \( m/z \) 474.2 → 112.1 for raloxifene, \( m/z \) 403.1 → 165.0 for famotidine, \( m/z \) 502.3 → 466.3 for fexofenadine, \( m/z \) 235.2 → 86.1 for lidocaine, \( m/z \) 189.1 → 77.1 for antipyrine, \( m/z \) 342.1 → 203.1 for 1’-hydroxylated midazolam, \( m/z \) 287.1 → 88.9 for 4’-hydroxylated tolbutamide, \( m/z \) 235.0 → 150.0 for 4’-hydroxylated mephenytoin, and \( m/z \) 455.2 → 165.1 for verapamil (IS). The lower limit of determination was 0.2 or 1 ng/mL and the linear detection range was up to 500 ng/mL.

**Pharmacokinetic analysis.** Noncompartmental pharmacokinetics were calculated using Phoenix WinNonlin 6.1 (Pharsight, Mountain View, CA) for individual animals, and reported as the mean ± standard deviation of the group. Statistics were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA). Oral bioavailability (F) was calculated as the ratio of the dose-normalized AUC values for oral and intravenous dosed groups. Intestinal availability (Fa·Fg) was calculated using eq. (2):

\[
Fa\cdot Fg = \frac{Q_{por} \cdot R_b \cdot (AUC_{por} - AUC_{sys})}{Dose}
\]  

(2)

where \( Q_{por} \), \( R_b \), \( AUC_{por} \) and \( AUC_{sys} \) are the portal blood flow, the blood / plasma
concentration ratio, AUC in the portal vein and AUC in the systemic circulation, respectively. $Q_{por}$ was calculated as 32.9 mL/min/kg using eq. (3):

$$Q_{por} = F_a \cdot F_g \cdot \text{Dose} / (R_b \cdot (\text{AUC}_{por} - \text{AUC}_{sys})) \quad (3)$$

Antipyrine was used to estimate the portal blood flow because $F_a \cdot F_g$ of antipyrine is 1 according to published data (Takahashi et al., 2009). $F_h$ was calculated using eq. (4):

$$F_h = F / F_a \cdot F_g \quad (4)$$

**Simulation of plasma concentrations.** Simulations of portal and systemic plasma concentrations were performed using PBPK modeling based on Fig. 4. The following differential equations were solved by the Runge-Kutta-Gill method.

$$\frac{dC_{\text{Liver}}}{dt} = Q_a \cdot C_{\text{sys}} + Q_{por} \cdot C_{por} - Q_h \cdot C_{\text{Liver}} / K_p - CL_h \cdot C_{\text{Liver}} / K_p \quad (5)$$

$$\frac{dC_{por}}{dt} = Q_{por} \cdot C_{\text{sys}} + D \cdot k_a \cdot F_a \cdot F_g \cdot \exp(-k_a \cdot t) - Q_{por} \cdot C_{por} \quad (6)$$

$$\frac{dC_{\text{sys}}}{dt} = Q_h \cdot C_{\text{Liver}} / K_p - Q_h \cdot C_{\text{sys}} - CL_{NH} \cdot C_{\text{sys}} \quad (7)$$

$V_{\text{Liver}}, V_{por}$ and $V_{sys}$ were the volume in the liver, portal vein and systemic blood compartments, respectively, and $C_{\text{Liver}}, C_{por}$ and $C_{sys}$ were the concentrations in each respective compartment. $Q_a, Q_{por}$ and $Q_h$ ($= Q_a + Q_{por}$) were the blood flow at the hepatic artery, portal vein and hepatic vein. $D, k_a, CL_h, CL_{NH}$ and $K_p$ were oral dose, absorption rate constant, hepatic clearance, non-hepatic clearance and the liver / plasma
concentration ratio, respectively. The elimination rate constant ($k_e$) was calculated using eq. (8):

$$k_e = \frac{(CL_h + CL_{NH})}{(V_{liver} + V_{sys})}$$

\[ (8) \]

**Statistics.** The statistical significance of the difference between mean values was tested using a Student’s $t$ test. Differences with a $p$ value of less than 0.01 were considered to be statistically significant.
Results

Physiological condition of rats following the surgical procedure. To investigate the influence of the surgical cannulating procedure on the physiological condition of the rats, body weight, plasma biochemical parameters for hepatic function, $\alpha$1-AGP concentration in plasma, hematological condition and hepatic metabolic activities of the cannulated rats were measured and compared with those of untreated rats. As shown in Fig. 1, the rate of body weight gain in cannulated rats 2 days after the operation was significantly lower than in untreated rats (1.8 ± 2.5% in cannulated rats vs. 8.9 ± 1.5% in untreated rats, $p < 0.01$). However, body weight recovered quickly, and from 3 days (arrival at our lab) to 9 days after the surgery, the rate of gain was almost the same as that of untreated rats (21.1 ± 1.1% in cannulated rats vs. 19.0 ± 3.1% in untreated rats).

Biochemical tests were conducted in the cannulated rats 1 day and 9 days after the surgery (Table 1). Although plasma $\alpha$1-AGP concentration increased 1 day after the surgery, other clinical biochemical parameters showed no significant differences with untreated rats and were within normal range. The transient increase in plasma $\alpha$1-AGP concentrations in the cannulated rats 1 day after the operation was considered to be due to the inflammation caused by the surgery. Yasuhara et al. reported that rat plasma $\alpha$1-AGP concentration showed a marked elevation, reaching peak levels 2 to 4 days...
after laparotomy and then declining rapidly to control levels. Our data showing an initially elevated $\alpha_1$-AGP concentration that returned to baseline 9 days after the surgery are consistent with this report. Therefore, these data indicate that hepatic function was not severely impaired by the cannulating procedure employed in this study.

*In vivo* pharmacokinetics studies on the cannulated rats were performed 9 days after the surgical procedure. Changes in $\alpha_1$-AGP levels lead to a significant increase in protein binding and altered pharmacokinetics of basic drugs like propranolol (Yasuhara et al., 1985) and lidocaine (De Rick et al., 1987). In this study, no differences in pharmacokinetic parameters of lidocaine were observed between cannulated and untreated rats, again indicating that $\alpha_1$-AGP concentration in cannulated rats 9 days after surgery had declined to control levels.

Hematological conditions in cannulated rats were similar to those of untreated rats 9 days after surgery (Table 2). Down-regulation of cytochrome P450 in the liver due to inflammatory stimuli has been documented, and indwelling catheters in rats decrease hepatic metabolism of antipyrine (Chindavijak et al., 1988). Table 2 shows hepatic metabolic activities in microsomes generated from cannulated rats were measured using midazolam, tolbutamide and mephenytoin as P450 specific probes. For all drugs, no significant changes were observed in *in vitro* hepatic metabolic activity. In addition,
there were no alterations in the pharmacokinetics of antipyrine (Table 3), the systemic clearance of which is mainly determined by protein binding and hepatic intrinsic clearance (Rane et al., 1977). These results suggest that hepatic metabolic activity in the cannulated rats was not significantly different from the controls after a 9 day recovery period had taken place.

**Evaluation of hepatic blood flow and hepatic metabolic activities.** Lidocaine (1 mg/mL/kg) and antipyrine (0.3 mg/mL/kg) were intravenously administered to cannulated and untreated rats and plasma levels of unmetabolized drugs were measured (Fig. 2A and 2B) to calculate pharmacokinetic parameters (Table 3). The rate-limiting step of systemic clearance for lidocaine is hepatic blood flow (Wilkinson et al., 1975), while that for antipyrine is hepatic metabolism (Chindavijak et al., 1988). Systemic clearances of lidocaine in cannulated and untreated rats was $67.5 \pm 7.9$ and $69.3 \pm 1.1$ mL/min/kg, respectively. On the other hand, systemic clearance of antipyrine was very low in both groups. All pharmacokinetic parameters of drugs with both high and low hepatic extraction showed no significant differences between cannulated and untreated rats.
Estimation of portal blood flow using antipyrine. In order to estimate the portal blood flow rate, antipyrine was used as a reference drug. Since antipyrine has a high permeability to the intestinal membrane and is scarcely metabolized in the intestinal tract, \( Fa \cdot Fg \) was reported to be 1 (Takahashi et al., 2009). Antipyrine (0.3 mg/5mL/kg) was orally administered to cannulated and untreated rats (Fig. 2C and Table 3). No significant difference in oral \( F \) was observed between cannulated (0.76 ± 0.05) and untreated rats (0.86 ± 0.19). From 0.05 to 1 h, antipyrine plasma concentration in the portal vein was higher than that in the systemic circulation of cannulated rats, and AUC values during first hour were 369.8 ± 21.5 and 211.5 ± 7.9 ng·h/mL, respectively (Fig. 3). The plasma / blood concentration ratio (\( Rb \)) of antipyrine was 1.0. The portal blood flow was calculated by eq. (3) with these results, and was estimated to be 32.9 mL/min/kg.

Estimation of \( Fa \cdot Fg \) of commercial drugs. Various commercial drugs were orally and intravenously administered to cannulated rats and intestinal and hepatic availability were assessed separately. As shown in Fig. 3 and Table 4, plasma concentrations of all drugs in the portal vein just after oral administration were higher than in systemic circulation. Hucker et al. and Tolle-Sander et al. reported that indomethacin was
completely absorbed and did not undergo first-pass metabolism, whereas midazolam was highly permeable but extensively metabolized by CYP3A. Fa-Fg, Fh and F were 0.90, 1.09 and 0.98, respectively, for indomethacin, and 0.71, 0.05 and 0.03, respectively, for midazolam. Other compounds were reported to show low intestinal absorption due to poor solubility, permeability and metabolism. Fa-Fg was 0.26 for felodipine, 0.26 for sulpiride, 0.22 for raloxifene, 0.19 for famotidine and 0.11 for fexofenadine.

Analysis of portal and systemic plasma concentration-time profile. Patterns of portal and systemic plasma concentration profiles of tested drugs were analyzed using a computer simulation method based on the PBPK model indicated in Fig. 4. As shown in Fig. 5, simulation of plasma concentration patterns clearly showed that patterns of portal and systemic concentrations were classified by the balance between $k_a$ and $k_e$. In the case of $k_a > k_e$ (Fig. 5A), both concentrations overlapped following oral administration, whereas in the case of $k_a < k_e$ (Fig. 5B), portal concentration became higher than systemic, and both concentrations transitioned in parallel over the duration of the experiment. Fig. 3 shows that antipyrine, indomethacin, midazolam, felodipine and famotidin showed a $k_a > k_e$ pattern, whereas raloxifene, sulpiride and fexofenadine
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showed a $k_a < k_e$ pattern.
Discussion

For pharmaceutical companies, oral absorbability of new drug candidates is evaluated during the discovery to preclinical stage. Various in vivo and in vitro methods are used to assess oral F and the extent of first-pass metabolism in the liver and intestine (Ito et al., 2005; Gertz et al., 2010; Nishimuta et al., 2011). Usually, Fa-Fg and Fh are estimated from PK analysis of in vivo intravenous and oral administration studies using eq. (9) and (10):

\[ F_h = 1 - \frac{CL_h}{Q_h} \]  \hspace{1cm} (9)

\[ Fa \cdot F_g = \frac{F}{F_h} \] \hspace{1cm} (10)

where CLh is hepatic clearance after intravenous administration and Qh is hepatic blood flow. However, as shown in Fig. 6A, Fa-Fg and Fh calculated by eq. (9-10) are significantly affected by the hepatic blood flow used for the calculations. When hepatic blood flow changes only 1.3-fold (60 to 80 mL/min/kg), Fa-Fg shifts from 0.43 to 1.15 (2.7-fold) in eq. (9-10). This means that inter-individual differences in hepatic blood flow used for intravenous and oral administration studies may result in the inaccurate estimation of oral absorbability of test compounds. In addition, for compounds with multiple pathways of elimination, particularly those with a high real clearance, estimation of CLh is sometimes difficult and time-wasting.
On the contrary, as shown in Fig. 6B, Fa·Fg value estimated from eq. (2) in our study using cannulated rats was relatively stable, changing only 1.3-fold when the portal blood flow changed 1.3-fold (30 to 40 mL/min/kg). An additional advantage of our method is that Fa·Fg can be calculated from a single oral dosing; intravenous administration studies and estimation of non-hepatic clearance are not required.

In this study, several drugs with different pharmacokinetic properties were orally administered to cannulated rats, and their Fa·Fg and Fh were calculated separately. In the case of midazolam, several reports have shown its intestinal and hepatic availability in rats but the estimated values are highly variable. Although midazolam is a specific Cyp3a substrate in rats, it is still unclear whether midazolam is extensively metabolized in rat intestine. Strelevitz et al. reported that midazolam suffered high first pass extraction in the intestine after oral dosing and estimated Fa·Fg at 0.03 (CLh = 22.8 mL/min/kg, Qh = 70 mL/min/kg). On the other hand, Kotegawa et al. found that midazolam was primarily extracted in the liver and estimated Fa·Fg at 0.4 to 0.9 (CLh = 79 mL/min/kg, Qh = 90-110 mL/min/kg). Both reports showed different systemic clearance and hepatic blood flow, resulting in different Fa·Fg values. In this study, approximately 500-fold difference between portal and systemic concentrations of midazolam 3 min after oral administration was observed, suggesting that midazolam
was absorbed rapidly from the GI tract. Our method in using eq. (2) yielded a fairly high Fa·Fg value for midazolam (0.71), suggesting that the main factor limiting oral bioavailability of midazolam is hepatic first-pass metabolism.

Murakami et al. have reported the estimation of Fa·Fg and Fh from unrestricted and conscious cannulated rats. However, in that study, pharmacokinetic parameters from cannulated and untreated rats were not compared. Moreover, their cannulating method consisted of insertion of the catheter from the iliac vein to the portal vein, and therefore hepatic blood flow may have been altered. In contrast, in this study, a catheter was inserted to the portal vein and the edge of the catheter was processed such as a flange to avoid inhibiting blood flow as much as possible. Data from Tables 1 and 2 confirm that the physiological condition of the rats 9 days after the surgical procedure, including metabolic activity in the liver and plasma protein binding capacity, had recovered to normal. In addition, our method of portal vein cannulation does not affect hepatic blood flow as calculated from the clearance of lidocaine (Table 3).

Indomethacin has been reported to be absorbed completely in the GI tract (Hucker et al., 1966), and Fa·Fg of indomethacin was 0.90 in the present study. The low Fa·Fg of sulpiride and fexofenadine was probably due to brush border P-glycoprotein efflux (Watanabe et al., 2002; Kamath et al., 2005). Additionally, the low Fh of fexofenadine is
likely due to excretion to bile in the liver (Kamath et al., 2005). The Fa·Fg of famotidine was 0.19, as it appears to be absorbed predominantly via the paracellular pathway (Lee et al. 1993). Previous reports showed that Fa·Fg of raloxifene in Gunn rats deficient in UDP-glucuronosyl transferase (UGT) 1A and wild-type Wister rats was 0.63 and 0.34, respectively (Kosaka et al, 2011), suggesting that the low Fa·Fg of raloxifene was due to glucronidation and low absorption in the intestine. Taken together, the results in this study of intestinal and hepatic availability in cannulated rats were in agreement with these previous reports.

Fig. 3 shows the different patterns in portal and systemic plasma concentrations that were observed. Portal and systemic plasma concentrations of antipyrine, indomethacin, midazolam and felodipine became equal at the terminal phase, whereas those of sulpiride, raloxifene and fexofenadine were parallel until 8 h after oral dosing. Simulation of portal and systemic plasma concentrations by the PBPK model suggested that the balance of k_a and k_e resulted in the different patterns in portal and systemic plasma concentrations seen in Fig. 5. In the case of k_a > k_e, absorption from the intestinal tract terminated quickly, resulting in negligible contribution of the absorption process to the concentration in the portal vein. In contrast, when the balance was k_a < k_e, the terminal phase of both plasma concentrations reflected the absorption process but
not the elimination process because of the flip-flop relation between absorption and elimination. In this case, terminal concentration in the portal plasma was kept higher than that in the systemic plasma and shifted in parallel. Accordingly, the analysis of both portal and systemic plasma drug concentrations in cannulated rats can yield pharmacokinetic information on the oral absorption process of drugs that is also important when considering drug oral absorbability.

In conclusion, use of a novel method to obtain portal vein cannulated rats described herein enables the assessment of intestinal availability under similar physiological conditions to untreated rats. This method also allows the determination of the pharmacokinetic properties of drugs relating to the oral absorption and systemic elimination rate constant. In the drug discovery stage, use of this experimental system is highly advantageous in identifying factors that limit oral bioavailability when considering development of oral drug candidates.
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Authorship Contributions

Participated in research design: Matsuda, Konno, Satsukawa and Yamashita

Conducted experiments: Matsuda, Konno

Contributed new reagents or analytic tools: Kobayashi, Takimoto, Morisaki

Performed data analysis: Matsuda

Wrote or contributed to the writing of the manuscript: Matsuda and Yamashita
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Figure Legends

Fig. 1. Effects of the surgical procedure on body weight in cannulated and untreated rats.
●, cannulated rats; ○, untreated rats. The rats were shipped to our lab 2 days after the operation and arrived the next day. Each symbol represents the mean ± S.D. of 4 rats.

Fig. 2. Systemic plasma concentration-time profile in cannulated and untreated rats. (A), lidocaine intravenous administration (1 mg/mL/kg); (B), antipyrine intravenous administration (0.3 mg/mL/kg); (C), antipyrine oral administration (0.3 mg/5 mL/kg). ●, cannulated rats; ○, untreated rats. Each symbol represents the mean ± S.D. of 3 rats.

Fig. 3. Systemic and portal plasma concentration-time profiles of several drugs following oral administration in cannulated rats. (A), antipyrine (0.3 mg/kg); (B), indomethacin (0.3 mg/kg); (C), midazolam (1 mg/kg); (D), felodipine (5 mg/kg); (E), famotidine (5 mg/kg); (F), raloxifene (5 mg/kg); (G), sulpiride (5 mg/kg); (H), fexofenadine (5 mg/kg). ●, systemic plasma concentration; ○, portal plasma concentration. Each symbol represents the mean ± S.D. of 3 to 4 rats.

Fig. 4. Physiologically based pharmacokinetic modeling consisting of GI tract, portal
vein, hepatic and systemic compartments. \( V_{\text{por}} \), \( V_{\text{liver}} \) and \( V_{\text{sys}} \) are volume in portal vein, hepatic and systemic compartments, respectively and \( C_{\text{liver}} \), \( C_{\text{por}} \) and \( C_{\text{sys}} \) are concentrations in each respective compartment. \( Q_{\text{a}} \), \( Q_{\text{por}} \) and \( Q_{\text{h}} \) were the blood flow at the hepatic artery, portal vein and hepatic vein. \( D \), \( k_{\text{a}} \), \( CL_{\text{h}} \), \( CL_{\text{NH}} \) and \( K_{\text{p}} \) are oral dose, absorption rate constant, hepatic clearance, non-hepatic clearance and the liver / plasma concentration ratio, respectively.

Fig. 5. Simulated portal and systemic plasma concentration-time profiles after oral administration in a physiological model. (A), \( k_{\text{a}} = 0.20 \text{ min}^{-1} \) and \( k_{\text{e}} = 0.02 \text{ min}^{-1} \); (B), \( k_{\text{a}} = 0.02 \text{ min}^{-1} \) and \( k_{\text{e}} = 0.20 \text{ min}^{-1} \).

Fig. 6. Relation of hepatic and portal blood flow to predicted \( Fa \cdot Fg \) and \( Fh \) after oral administration of felodipine in rats. (A), \( Fa \cdot Fg \) and \( Fh \) were calculated using equation (9) and (10), \( F = 0.16 \) and \( CL_{\text{h}} = 52.2 \text{ mL/min/kg} \); (B), \( Fa \cdot Fg \) and \( Fh \) were calculated using equation (2) and (4), \( F = 0.16 \).
TABLE 1

Blood biochemical test and α1-acid glycoprotein (AGP) levels in the plasma of cannulated and untreated rats 1 or 9 days after surgery

Values represent the mean ± S.D. of 4 rats.

<table>
<thead>
<tr>
<th></th>
<th>1 day after the operation</th>
<th>9 days after the operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated rats</td>
<td>Cannulated rats</td>
</tr>
<tr>
<td>AST U/L</td>
<td>76 ± 14</td>
<td>89 ± 13</td>
</tr>
<tr>
<td>ALT U/L</td>
<td>30 ± 5</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>TP g/dL</td>
<td>5.5 ± 0.1</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>TBIL mg/dL</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>ALB g/dL</td>
<td>4.1 ± 0.3</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>TCHO mg/dL</td>
<td>86 ± 7</td>
<td>92 ± 13</td>
</tr>
<tr>
<td>TG mg/dL</td>
<td>54 ± 14</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>GLU mg/dL</td>
<td>101 ± 8</td>
<td>129 ± 15</td>
</tr>
<tr>
<td>α1-AGP μg/mL</td>
<td>45.7 ± 4.8</td>
<td>136.5* ± 13.0</td>
</tr>
</tbody>
</table>

*, p < 0.01, significantly different from the untreated rats using the student’s t-test.
TABLE 2

Hematological test, hepatic metabolic activity and liver weight in cannulated and untreated rats 9 days after surgery

Values represent the mean ± S.D. of 4 rats.

<table>
<thead>
<tr>
<th></th>
<th>Untreated rats</th>
<th>Cannulated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC</strong> $\times 10^4/\mu L$</td>
<td>689 ± 15</td>
<td>682 ± 37</td>
</tr>
<tr>
<td><strong>WBC</strong> $\times 10^2/\mu L$</td>
<td>70.8 ± 16.3</td>
<td>78.3 ± 25.2</td>
</tr>
<tr>
<td><strong>HCT</strong> %</td>
<td>43.5 ± 0.8</td>
<td>43.6 ± 2.2</td>
</tr>
<tr>
<td><strong>PLT</strong> $\times 10^4/\mu L$</td>
<td>110 ± 6</td>
<td>122 ± 11</td>
</tr>
<tr>
<td>1-hydroxylation of midazolam $pmol/mg/min$</td>
<td>106 ± 30</td>
<td>130 ± 20</td>
</tr>
<tr>
<td>4-hydroxylation of tolbutamide $pmol/mg/min$</td>
<td>10.4 ± 2.9</td>
<td>7.6 ± 2.5</td>
</tr>
<tr>
<td>4-hydroxylation of mephenytoin $pmol/mg/min$</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Liver weight $g$ liver/kg</td>
<td>28.1 ± 0.9</td>
<td>28.4 ± 1.6</td>
</tr>
</tbody>
</table>
TABLE 3

Pharmacokinetic parameters after administration of lidocaine and antipyrine in cannulated and untreated rats

Pharmacokinetic parameters were calculated from plasma concentration after intravenous administration of lidocaine (1 mg/mL/kg) and antipyrine (0.3 mg/mL/kg). Portal blood flow was calculated from plasma concentration after oral administration of antipyrine (0.3 mg/5mL/kg). Values represent the mean ± S.D. of 3 rats.

<table>
<thead>
<tr>
<th></th>
<th>Lidocaine</th>
<th>Antipyrine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated rats</td>
<td>Cannulated rats</td>
</tr>
<tr>
<td>Intravenous administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>ng·h/mL</td>
<td>240 ± 4</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>h</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>CL&lt;sub&gt;tot&lt;/sub&gt;</td>
<td>mL/min/kg</td>
<td>69.3 ± 1.1</td>
</tr>
<tr>
<td>V&lt;sub&gt;dss&lt;/sub&gt;</td>
<td>L/kg</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>Oral administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q&lt;sub&gt;por&lt;/sub&gt;</td>
<td>mL/min/kg</td>
<td>NT</td>
</tr>
<tr>
<td>F</td>
<td>mL/min/kg</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested; NC, Not calculated.
TABLE 4

Estimated organ bioavailability of each drug after intravenous and oral administration in cannulated rats

Values represent the mean of 3 to 4 rats.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>Rb</th>
<th>F</th>
<th>Fa-Fg</th>
<th>Fh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>po</td>
<td>iv</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.3</td>
<td>0.1</td>
<td>0.7</td>
<td>0.98</td>
<td>0.90</td>
</tr>
<tr>
<td>Midazolam</td>
<td>1</td>
<td>0.3</td>
<td>1.2</td>
<td>0.03</td>
<td>0.71</td>
</tr>
<tr>
<td>Felodipine</td>
<td>5</td>
<td>1</td>
<td>0.7</td>
<td>0.16</td>
<td>0.26</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>5</td>
<td>1</td>
<td>1.5</td>
<td>0.06</td>
<td>0.26</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>5</td>
<td>1</td>
<td>1.0</td>
<td>0.15</td>
<td>0.22</td>
</tr>
<tr>
<td>Famotidine</td>
<td>5</td>
<td>1</td>
<td>0.9</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>5</td>
<td>1</td>
<td>1.0</td>
<td>0.02</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6