Quantitative Prediction of Intestinal Glucuronidation of Drugs in Rats Using In Vitro Metabolic Clearance Data

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Summary: UDP-glucuronosyltransferase (UGT) is highly expressed in the small intestine and catalyzes the glucuronidation of small molecules, which may affect the oral bioavailability of drugs. However, no method of predicting the in vivo observed fraction of absorbed drug (F_{aF_g}) affected by UGT has yet been established. Here, we investigated the relationship between F_{aF_g} and in vitro clearance of nine UGT substrates (ketoprofen, tolcapone, telmisartan, raloxifene, entacapone, resveratrol, buprenorphine, quercetin, and ezetimibe) via UGT in intestinal microsomes (CL_{int, UGT}) in rats. F_{aF_g} was calculated from pharmacokinetic parameters after intravenous and oral administration or using the portal-systemic concentration difference method, with values ranging from 0.027 (ezetimibe) to 1 (tolcapone). Glucuronides of model compounds were observed in the portal plasma after oral administration, with CL_{int, UGT} values ranging from 57.8 (tolcapone) to 19,200 µL/min/mg (resveratrol). An inverse correlation between F_{aF_g} and CL_{int, UGT} was observed for most compounds and was described using a simplified intestinal availability model reported previously. This model gave accurate predictions of F_{aF_g} values for three in-house compounds. Our results show that F_{aF_g} in rats is affected by UGT and can be predicted using CL_{int, UGT}. This work should hasten the development of a method to predict F_{aF_g} in humans.

Keywords: UDP-glucuronosyltransferase (UGT); glucuronidation; intestinal metabolism; quantitative prediction; in vitro–in vivo correlation

Introduction

Glucuronidation, which is catalyzed by UDP-glucuronosyltransferase (UGT), is an important reaction in the metabolism of drugs. UGT is widely expressed in various tissues including the liver, kidney, lung, and intestine. Although the liver is the primary organ contributing to drug metabolism, recent studies have indicated that intestinal glucuronidation may have a significant impact on the pharmacokinetics (PK) and oral bioavailability (F) of UGT substrates. For example, raloxifene is metabolized mainly via UGT in the intestine, resulting in F of only 2% in humans. Ezetimibe is also rapidly metabolized in the intestine of humans and rats, and its glucuronide is detected mainly in plasma after oral (p.o.) administration. As intestinal glucuronidation has been suggested to generate extremely low F values for drugs, prediction of the effects of intestinal glucuronidation would be valuable for selecting drug candidates with potentially favorable human PK profiles. However, adequate prediction methods have yet to be developed.

Investigation of the relationship between in vitro metabolic stability and in vivo PK parameters is useful for the prediction of F. For cytochrome P450 (CYP), several studies have proposed criteria for avoiding low F using in vitro metabolic stability. Kato et al. assessed the relationship between fraction absorbed (F_a), intestinal availability (F_g), hepatic availability (F_h), and in vitro intrinsic clearance (CL_{int}) using ten drugs which are CYP3A4 substrates, and showed that F_{aF_g} was markedly reduced when the CL_{int} in human liver microsomes was more than 78 µL/min/mg. Kadono et al. found an inverse correlation between F_{aF_g} and CL_{int} in human intestinal microsomes, and proposed a simplified F_g model to predict F_g using CL_{int}. However, due to limited...
information available for which drugs undergo intestinal glucuronidation in humans, similar approaches using glucuronidation have not been widely applied. In rats, evidence from in vivo and in vitro studies suggests that intestinal glucuronidation contributes to F. Ezetimibe was extensively absorbed and observed mainly as its glucuronide in portal plasma in rats. The same approach was applied to resveratrol, with similar findings. In addition, Mistry et al. showed a correlation between CLint (via UGT in intestinal microsomes) and in vivo intrinsic intestinal clearance using three opioid compounds. Studies using rat models are important in that detailed and reliable in vivo data, which complement the results of human studies, can be obtained for a wide range of compounds.

Here, we attempted to determine the in vitro—in vivo relationship of UGT substrates, and develop a method for predicting the effect of UGT on FFG from in vitro data using rats. We investigated the relationship between FFG data obtained from in vivo studies and CLint via UGT in intestinal microsomes in rats using nine UGT substrate drugs, and fitted this relationship to a simplified FFG model to obtain an equation for predicting the effect of UGT on FFG. In addition, portal plasma concentrations of glucuronides after p.o. administration of two typical intestinal UGT substrates, ezetimibe and raloxifene, were determined to obtain direct evidence for intestinal glucuronidation.

**Materials and Methods**

**Chemicals:** Pooled intestinal microsomes and S9 from male Sprague-Dawley (SD) rats (n = 110 and 97, respectively) were purchased from XenoTech (Lexena, KS, USA). Uridine 5′-diphosphate-glucuronic acid (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate (PAPS), ketoprofen, raloxifene, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). NADPH-4Na was purchased from Roche Diagnostics (Indianapolis, IN, USA). Tolcapone was purchased from United States Pharmacopeia (Rockville, MD, USA). Telmisartan was purchased from LKT Laboratories (St. Paul, MN, USA). Entacapone, raloxifene 4′-glucuronide (R4G), raloxifene 6-glucuronide (R6G), ezetimibe phenoxy glucuronide (EPG), and ezetimibe hydroxy glucuronide (EHG) were purchased from Toronto Research Chemicals (North York, Canada). Resveratrol was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Buprenorphine (Lepetan injection, 0.2 mg) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Ezetimibe was purchased from AK Scientific, Inc. (Union City, CA, USA). In-house compounds were obtained from Astellas Pharma Inc. (Tokyo, Japan). All other reagents and solutions were commercial products of analytical grade.

**Selection of model compounds:** We selected six compounds which undergo intestinal glucuronidation in vitro and/or in vivo (telmisartan, raloxifene, resveratrol, buprenorphine, quercetin, and ezetimibe), and three compounds whose glucuronides have been observed as major metabolites in rats (ketoprofen, tolcapone, and entacapone). For buprenorphine, quercetin, and tolcapone, major metabolites produced by enzymes other than UGT, were also detected in rats (CYP for buprenorphine and tolcapone, and sulfotransferases (SULT) for quercetin).

**Animals:** Male SD rats (Charles River, Yokohama, Japan), aged 8 weeks, weighing approximately 200 g were used. The animal experiments were carried out at Astellas Research Technology (Ibaraki, Japan) with the approval of the animal experiment ethics committee of Astellas Pharma Inc. in accordance with the “International Guiding Principles for Biomedical Research Involving Animals” developed by The Council for International Organization of Medical Science.

**Pharmacokinetic study in rats:** Animals (n = 3 for each dosing route) were fasted for approximately 17 h before dosing. Compounds were administered as a solution at 1 mg/kg. Before intravenous (i.v.) administrations, the femoral vein and artery were cannulated for administration and blood sampling, respectively, under isoflurane anesthesia. Blood samples were taken from the femoral artery at 0.1, 0.25, 0.5, 1, 2, 4, 6, and 24 h after i.v. administration using syringes containing heparin sodium. Samples were also taken from the tail vein at 0.25, 0.5, 1, 2, 4, 6, and 24 h after p.o. administration. Blood samples were kept on ice, and centrifuged at 16,000 × g for 2 min at 4°C to obtain plasma. Plasma was stored at −20°C prior to analysis.

The portal-systemic concentration difference method (P-S method) was carried out according to the method of Hoffman et al. Compounds were administered to rats (n = 3–4) as a solution at 1 mg/kg (3 mg/kg for resveratrol). At 8 time points within 360 min after p.o. administration, animals were sacrificed, and blood samples were collected simultaneously from the portal vein and abdominal aorta using syringes containing heparin sodium. Blood samples were processed as described above.

**Sample preparation for quantification:** The concentrations of tested compounds in rat plasma were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after sample preparation. The internal standards (IS) for each compound were tolmetin (for ketoprofen), diazepam (tolcapone, telmisartan, raloxifene, R4G, R6G, entacapone, buprenorphine, and in-house compounds 1 and 3), naringenin (for resveratrol and quercetin), diclofenac (for ezetimibe, EPG, and EHG), and an in-house compound (for in-house compound 2).

Sample preparation of resveratrol and quercetin was performed by modifying the method of Meng et al. and Lan et al. Briefly, to 0.1 mL of plasma, 0.01 mL of 0.5 mol/L acetic acid, 0.01 mL of 2 mg/mL ascorbic acid, and 0.01 mL of IS solution were added and mixed. For resveratrol, after adding 1 mL of ethyl acetate, the organic
layer remaining after centrifugation was collected and evaporated under a nitrogen stream. The residues were dissolved in 50% methanol and injected into the LC-MS/MS system. For quercetin, after adding 0.2 mL of acetonitrile, the supernatant after centrifugation was injected into the LC-MS/MS system.

For all other compounds, 0.03 mL of plasma, 0.03 mL of 50% acetonitrile, 0.03 mL of IS solution, and 0.15 mL of 0.1% formic acid in acetonitrile were combined. After centrifugation, the supernatant was injected into the LC-MS/MS system.

**LC-MS/MS analysis:** The LC-MS/MS system for raloxifene, resveratrol and quercetin consisted of a LC-VP/ LC-20A series (Shimadzu, Kyoto, Japan) and an API-3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster, CA, USA). The system for all other compounds consisted of an Acquity UPLC (Waters, Milford, MA, USA) and a Quattro Ultima triple quadrupole mass spectrometer (Waters). The HPLC separation was performed at 40°C at a flow rate of 0.3–0.4 mL/min with a gradient of solvents A and B or C and D. Mobile phases A and B consisted of 0.1% formic acid and 10% (A) or 90% (B) acetonitrile in water. Mobile phases C and D consisted of 10mM/L ammonium acetate and 10% (C) or 90% (D) acetonitrile in water. For resveratrol, a gradient of 10% methanol and 70% methanol was used for the separation. Columns used for chromatographic separation were Inertsil ODS-4 (2.1 x 30 mm, 3 µm, GL Science, Tokyo, Japan) for tolcapone and entacapone, Supelco Discovery HS C18 (2.1 x 75 mm, 3 µm, Supelco, Bellefonte, PA, USA) for resveratrol, and Capcell PAK C18 MG (2.0 x 35 mm, 3 µm, Shiseido, Tokyo, Japan) for all other compounds. For the MS/MS analysis, a multiple reaction monitoring experiment was conducted in positive ion mode by monitoring selected ions as follows (precursor ion/product ion): ketoprofen (253.0/208.9), tolcapone (274.0/181.8), telmisartan (515.4/497.5), raloxifene (474.3/112.0), R4’G (650.1/474.2), R6G (650.1/474.2), entacapone (305.6/232.5), buprenorphine (468.2/396.2), in-house compound 1 (504.3/131.3), in-house compound 2 (462.3/251.2), and in-house compound 3 (532.2/404.3). For all other compounds, analyses were performed in negative ion mode by monitoring the selected ions as follows: resveratrol (227.1/185.1), quercetin (301.0/151.1), and ezetimibe (408.0/271.1), EPG (584.0/112.9), EHG (584.1/447.0).

**Quantification of compound concentration in plasma:** The peak areas of each compound were divided by the peak area of the IS to obtain the peak area ratio. Calibration curves for the compounds were constructed by a least-square linear regression of the peak area ratios of the standards versus the drug concentrations.

**Blood-to-plasma concentration ratio (Rb):** Blood was spiked with 50 µg/mL compound solution to obtain a final concentration of 1 µg/mL and incubated at 37°C for 20 min, except for quercetin (5 min). An aliquot (0.05 mL) of each blood sample was added to 0.1 mL of water for determination of the compound concentrations in blood. For quercetin, 2 mg/mL ascorbic acid was added instead of water. The remaining blood samples were centrifuged at 1,800 × g for 10 min at 4°C, to obtain plasma. The blood and plasma samples were treated as described above and analyzed using LC-MS/MS for measuring the peak-area ratio of each unchanged compound against IS. The Rb was calculated by dividing the concentration in the blood sample by that in the plasma sample. Assays were performed in triplicate.

**PK analysis:** The area under the plasma concentration-time curves (AUC) after i.v. and p.o. administration, and the total body clearance in plasma (CLt,plasma) after i.v. administration were calculated by model-independent analysis using a program developed in-house. F was calculated as a ratio of AUC after i.v. and p.o. administration. The FaFg from the i.v.-p.o. indirect method was calculated using Equations 1–4, assuming that the elimination of compounds from the body after i.v. administration consisted of liver metabolism and renal excretion:

\[ CL_{t,\text{blood}} = \frac{CL_t}{RB} \]  
\[ CL_h = CL_{t,\text{blood}} - CL_t \]  
\[ F_h = 1 - \frac{CL_h}{Q_h} \]  
\[ F_{Ag} = F/F_h \]

where \( CL_{t,\text{blood}} \) is the total body clearance in blood, \( CL_h \) is the hepatic clearance, \( CL_t \) is the renal clearance, and \( Q_h \) is the hepatic blood flow. \( CL_t \) is assumed to be 0. The value of \( Q_h \) in rats was 58.8 mL/min/kg. \( F_{Ag} \) was calculated from the mean values of the parameters \( F \) and \( F_h \).

The \( F_{Ag} \) using the P-S method was calculated using Equation 5:

\[ F_{Ag} = Q_{pv} \times R_h \times (AUC_{pv} - AUC_{sys}) / D \]

where \( Q_{pv} \) is the portal blood flow, \( AUC_{pv} \) is the AUC in the portal vein, \( AUC_{sys} \) is the AUC in the systemic circulation, and \( D \) is the dose. The value of \( Q_{pv} \) was 39.2 mL/min/kg. \( AUC_{sys} \) and \( AUC_{pv} \) were calculated from the mean concentration at each time point using the linear trapezoidal method.

**In vitro metabolic stability for UGT assay in intestinal microsomes:** The reaction mixture (1 mL) was prepared to a final concentration of 0.2 µmol/L each compound, 0.1 mg/mL rat intestinal microsomes, 50 mM/L Tris-HCl buffer (pH 7.4), 5 µg/mL alamethicin, and 8 mM/L MgCl₂. After preincubation for 30 min, the reaction was initiated by the addition of UDPGA to give a final concentration of 2 mM/L. After incubation at 37°C for 0, 10, 20, 30, 45, and 60 min (or 0, 1, 2, 5, 10, and 20 min for resveratrol and quercetin), a 50 µL aliquot of each reaction mixture was collected and added to 0.1 mL of acetonitrile containing 0.1% formic acid and IS to terminate the reaction. After centrifugation at 16,000 × g for 5 min at 4°C, the supernatant was injected into the LC-MS/MS system.
system described above to measure the peak-area ratio of each compound against IS. Assays were performed in duplicate.

In vitro metabolic stability for CYP assay in intestinal microsomes: Reaction mixtures (1 mL) were prepared to give a final concentration of 0.2 µmol/L each compound, 0.2 mg/mL intestinal microsomes, 100 mmol/L Na₂K-phosphate buffer (pH 7.4), and 1 mmol/L EDTA. After preincubation for 5–10 min, the reaction was initiated by the addition of NADPH at a final concentration of 1 mmol/L. After incubation at 37°C for 0, 10, 20, 30, 45, and 60 min, a 0.05 mL aliquot of each reaction mixture was collected and added to 0.1 mL of acetonitrile containing 0.1% formic acid and IS to terminate the reaction. After centrifugation at 16,000 × g for 5 min at 4°C, the supernatant was injected into the LC-MS/MS system described above to measure the peak-area ratio of each compound compared with IS.

In vitro metabolic stability for SULT assay in intestinal microsomes: The reaction mixture (1 mL) was prepared to a final concentration of 0.2 µmol/L telmisartan, 0.1 mg/mL intestinal S9, and 10 mmol/L potassium phosphate buffer (pH 7.4). After preincubation for 5 min, the reaction was initiated by the addition of PAPS at a final concentration of 0.02 mmol/L. After incubation at 37°C for 0, 1, 2, 5, and 10 min, a 0.05 mL aliquot of each reaction mixture was collected and added to 0.1 mL of acetonitrile containing 0.1% formic acid and IS to terminate the reaction. After centrifugation at 16,000 × g for 5 min at 4°C, the supernatant was injected into the LC-MS/MS system described above to measure the peak-area ratio of each compound compared with IS.

Calculation of CL\textsubscript{int}: CL\textsubscript{int} from UGT assay (CL\textsubscript{int,UGT}), CYP assay (CL\textsubscript{int,CYP}), and SULT assay (CL\textsubscript{int,SULT}) were calculated using the following equation (Equation 6) based on the time course of the residual ratio of the unchanged drugs as determined using least-squares linear regression:

\[ \text{CL}_{\text{int}} (\mu L/\text{min/mg protein}) = k_e / \text{microsomal protein concentration} \]  

where \( k_e \) is the disappearance rate constant.

Parallel artificial membrane permeability assay (PAMPA): The PAMPA method was carried out using a PAMPA Evolution instrument from pION (Woburn, MA, USA) according to the method of Kadono, et al. The apparent permeability coefficient (\( P_{\text{app}} \)) was calculated using PAMPA Evolution software (pION).

Application of a simplified \( F_g \) model to the relationship between CL\textsubscript{int,UGT} and F\textsubscript{Fg}: Kadono et al. constructed a simplified \( F_g \) model as follows. \( F_g \) was represented by assuming that absorption was nearly complete for highly permeable compounds (\( F_g = 1 \)):

\[ F_g = \frac{\text{CL}_{\text{ab}}}{(\text{CL}_{\text{ab}} + \text{CL}_{\text{m}})} \]  

where CL\textsubscript{ab} is the absorption clearance and CL\textsubscript{m} is the metabolic clearance. Equation 7 was simplified to Equation 8 by assuming that CL\textsubscript{ab} values among highly permeable compounds were similar and that \( F_g \) is mainly determined by CL\textsubscript{m}:

\[ F_g = 1/(1 + A \times \text{CL}_{\text{m}}) \]  

where \( A \) is the reciprocal of CL\textsubscript{ab} (A = 1/CL\textsubscript{ab}) and is treated as a constant value. Assuming that CL\textsubscript{m} is proportional to CL\textsubscript{int}, Equation 8 can be represented as follows:

\[ F_g = 1/(1 + \alpha \times \text{CL}_{\text{int}}) \]  

where \( \alpha \), which is proportional to \( A \), is a constant value defined as the empirical scaling factor.

Model compounds (except buprenorphine and quercetin) were used to establish the simplified \( F_g \) model for intestinal glucuronidation. The value of \( \alpha \) was estimated from a fitting study between \( F_{\text{Fg}} \) and CL\textsubscript{int,UGT} in rats for model compounds using a previously described non-linear least-squares method called MULTI.

Results

PK parameters of model compounds and \( F_{\text{Fg}} \) using the i.v.-p.o. indirect method: PK parameters of model compounds and \( F_{\text{Fg}} \) calculated using the i.v.-p.o. indirect method are shown in Table 1. The \( F_g \) values for ketoprofen and tolcapone in rats were both 0.97, and the \( F_{\text{Fg}} \) values were calculated to be approximately 1. For telmisartan, which showed low CL\textsubscript{int, blood} (10.0 mL/min/kg), the \( F_g \) was 0.51 and the \( F_{\text{Fg}} \) value was calculated as 0.61. For raloxifene, entacapone, resveratrol, buprenorphine, quercetin, and ezetimibe, the CL\textsubscript{int, blood} values exceeded the Qs; therefore, due to the inapplicability of Equation 3, the \( F_{\text{Fg}} \) values were calculated using the P-S method as described below.

Calculations of \( F_{\text{Fg}} \) using the P-S method: The AUC\textsubscript{pv}, AUC\textsubscript{sys}, and \( F_{\text{Fg}} \) values for raloxifene, entacapone, resveratrol, buprenorphine, quercetin, and ezetimibe are also shown in Table 1. The values of \( F_{\text{Fg}} \) of raloxifene and entacapone were 0.30 and 0.20, respectively, moderately low compared with the compounds assessed by the i.v-p.o. indirect method. The \( F_{\text{Fg}} \) values of resveratrol, buprenorphine, quercetin, and ezetimibe were markedly lower, all being under 0.10.

Rat \( F_g \) and Permeability in artificial membranes: To consider the contribution of \( F_g \) to \( F_{\text{Fg}} \), rat \( F_g \) values from published studies and \( P_{\text{app}} \) values from PAMPA were obtained. These results are shown in Table 2. The reported \( F_g \) values for the model compounds were all moderate to high (greater than 0.5). \( P_{\text{app}} \) values from PAMPA for ketoprofen, tolcapone, telmisartan, raloxifene, entacapone, and resveratrol were measured to be >1.0 × 10^(-6) cm/s, meaning that the \( F_g \) values were estimated to be high. For buprenorphine, quercetin, and ezetimibe, we were unable
than unchanged compound. Peaks corresponding to the glucuronides demonstrated that the compounds underwent intestinal glucuronidation following delivery to the intestinal lumen. Glucuronides of model compounds in portal plasma after p.o. administration for the P-S method were monitored to estimate the F_{app} values because the sensitivity of quantification and/or the solubility in the test solution were low.

Detection of glucuronides in portal plasma: Glucuronides of model compounds in portal plasma after p.o. administration for the P-S method were monitored to demonstrate that the compounds underwent intestinal glucuronidation following delivery to the intestinal lumen. Peaks corresponding to the glucuronides (176 Da higher than unchanged compound) were observed for all tested compounds (data not shown). For ezetimibe and raloxifene, both of which are drugs that typically undergo intestinal glucuronidation, concentrations of glucuronides were determined using synthetic standards. Figure 1 shows portal plasma concentration-time profiles of unchanged compounds and their glucuronides in a 60 min period after p.o. administration, which includes the time taken to reach maximum concentration (T_{max}). For ezetimibe, the presence of EPG and EHG (the major and minor glucuronide metabolites, respectively) were quantified. The concentration of EPG in the portal plasma reached >10 times that of unchanged ezetimibe, while EHG was not detected. For raloxifene, the metabolites R4G and R6G were quantified. The total concentration of the glucuronides was 3–8 times higher than that of unchanged raloxifene. R6G, which is reported to be the major metabolite in rats, was detected at >10 times higher concentrations than R4G.

In vitro metabolic stability in intestinal microsomes: C_{int} values for the model compounds in rat intestinal microsomes were estimated (Table 3). It can be seen that there is an inverse relationship between C_{int, UGT} and F_{app} such that compounds with low F_{app} had high values for C_{int, UGT} (e.g. resveratrol, quercetin, and ezetimibe), whereas compounds with high F_{app} had correspondingly low C_{int, UGT} (e.g. ketoprofen and tolcapone). The C_{int, UGT} for all model compounds exhibited approximately the same values at concentrations within the 0.2–2 µmol/L range (data not shown).

<table>
<thead>
<tr>
<th>Compound</th>
<th>F_{app}</th>
<th>Reference for F_{app}</th>
<th>P_{app} in PAMPA (×10^{-6} cm/s)</th>
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</thead>
<tbody>
<tr>
<td>Ketoprofen</td>
<td>0.99</td>
<td>—</td>
<td>7.0</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>0.83</td>
<td>Drug Approval Package for Tasmar</td>
<td>18</td>
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<td>Telmisartan</td>
<td>0.79–0.86</td>
<td>Shimasaki et al.</td>
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<td>Raloxifene</td>
<td>0.71</td>
<td>Lindstrom et al.</td>
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<tr>
<td>Entacapone</td>
<td>0.54</td>
<td>Drug Approval Package for Comtan</td>
<td>4.1</td>
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<tr>
<td>Resveratrol</td>
<td>0.77–0.80</td>
<td>Wenzel et al.</td>
<td>1.7</td>
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<tr>
<td>Buprenorphine</td>
<td>0.88–1.0</td>
<td>Mistry et al.</td>
<td>Not tested</td>
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<tr>
<td>Quercetin</td>
<td>0.59</td>
<td>Chen et al.</td>
<td>&lt;1.5</td>
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<td>Ezetimibe</td>
<td>0.40–0.66</td>
<td>Drug Approval Package for Zetia</td>
<td>&lt;21</td>
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</tbody>
</table>

*Calculated using PK parameters after i.v. and p.o. administration in this study.
†Excretion of radioactivity in bile and urine after p.o. administration of radiolabeled compound.
‡Absorption of radiolabeled telmisartan with the ligated loop method.
§Calculated from PK parameters of radioactivity after i.v. and intraduodenal administration of radiolabeled buprenorphine.
¶Calculated from PK parameters of quercetin and its metabolites after i.v. and p.o. administration.

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with its CL\textsubscript{int, UGT} (95 µL/min/mg). CYP activity may thus contribute to the low FaFg value recorded for this drug. Similarly, quercetin, which is metabolized by SULT as well as by UGT, gave a CL\textsubscript{int, SULT} value of 302 µL/min/mg when we tested in vitro metabolic stability in rat intestinal S9 in the presence of PAPS, suggesting that SULT might be also involved in the intestinal metabolism of quercetin.

**Prediction of F\textsubscript{g} using the relationship between FaFg and CL\textsubscript{int, UGT}**

To assess the trend between FaFg and CL\textsubscript{int, UGT}, we performed the analysis shown in Figure 2. We found an inverse correlation between FaFg and CL\textsubscript{int, UGT} for most drugs, although the metabolism of buprenorphine (compound 8 in Fig. 2) by CYP complicated the in vitro–in vivo relationship and impaired the correlation for this compound. To construct a practical equation for prediction of F\textsubscript{g}, we assessed whether the simplified F\textsubscript{g} model proposed by Kadono et al. could be applied to the in vitro–in vivo relationship for UGT substrates. The FaFg values for the model compounds were assumed to be equal to F\textsubscript{g} for this analysis. Buprenorphine and quercetin, which are metabolized by additional enzymes other than UGT in intestinal microsomes, were excluded from the model compounds. The CL\textsubscript{int, UGT} for ketoprofen, which showed no depletion in the in vitro metabolic study, was assigned an arbitrarily low value of 10 µL/min/mg. Curve-fitting to the data from the seven model compounds produced the simplified F\textsubscript{g} model shown in Figure 2. The in vitro–in vivo relationship fitted well with the curve, and the empirical scaling factor, α, was estimated to be 0.0050.

To verify the reliability of the obtained equation with α, F\textsubscript{g} values for three in-house UGT substrate compounds were predicted. P\textsubscript{app} values for compounds 1–3 in PAMPA were $>1 \times 10^{-6}$ cm/s. Therefore, the F\textsubscript{g} values were assumed to be high and the FaFg values were assumed to be equal to the F\textsubscript{g} values. F\textsubscript{g} values for compounds 1–3 (predicted using our equation and the CL\textsubscript{int, UGT}) were close to the observed FaFg (Table 4).

**Discussion**

Intestinal metabolism has the potential to affect the F of drugs as much as absorption and hepatic metabolism, which necessitates careful evaluation during drug discovery of how intestinal glucuronidation might affect the F value of putative drug candidates. In the case of intestinal CYP3A substrates,
Kadono et al. report the relationship between \( F_a \) and \( \text{CL}_{\text{int}, \text{UGT}} \), and a convenient method for predicting \( F_g \) using in vitro data.\(^7\) However, due to the limited number of drugs which are reported to undergo intestinal glucuronidation in not only humans, but also in animals, little information exists on in vitro–in vivo relationships for UGT, except for the findings of a single study in rats using three opioid-type compounds.\(^10\) Here, we selected nine UGT substrates with various chemical structures that are reported to undergo intestinal glucuronidation, or whose glucuronide was a major metabolite in rats to investigate the relationship between \( F_{aF_g} \) and \( \text{CL}_{\text{int}, \text{UGT}} \) in rats. Studies in rats are important in that detailed and reliable in vivo data can be obtained for a wide range of compounds and are necessary for constructing a method to predict human \( F_{aF_g} \).

The i.v.-p.o. indirect method, using PK parameters after i.v. and p.o. administration, was used as the first method for calculation of \( F_{aF_g} \). This method is useful in calculating \( F_{aF_g} \) in animals in the drug discovery stage and in actual clinical practice due to its convenience and noninvasiveness, and was used to calculate the \( F_{aF_g} \) for ketoprofen, tolcapone, and telmisartan, which showed low \( \text{CL}_{t} \) (Table 1). This method, however, is limited when \( \text{CL}_{t} \) exceeds \( Q_h \). As an alternative direct approach to assessing \( F_{aF_g} \), we conducted a P-S evaluation for raloxifene, entacapone, resveratrol, buprenorphine, quercetin, and ezetimibe, for all of which \( \text{CL}_{t} \) exceeded \( Q_h \) (Table 1). This method is more convenient than other direct techniques such as in situ perfusion.\(^23\)

We would stress that it is crucial that the required convenience and the PK profile of compounds are taken into account when selecting methods for studying \( F_{aF_g} \) during drug discovery investigations.

To estimate the contribution of \( F_a \) to \( F_{aF_g} \), literature values of \( F_a \) in rats and \( P_{\text{app}} \) values from PAMPA were obtained (Table 2). For ketoprofen, tolcapone, telmisartan, raloxifene, resveratrol, and buprenorphine, the reported \( F_a \) values are high (>0.7), indicating that \( F_{aF_g} \) can be simplified to \( F_a \). For entacapone, it is possible that the \( F_a \) value is higher than 0.54, because the total recovery of radioactivity (sum of the radioactivity excreted in bile, urine, and feces) was approximately 80% in the referenced data, indicating that a part of the remaining 20% was likely absorbed. For quercetin and ezetimibe, the \( F_a \) values are moderate (~0.5), while the obtained \( F_{aF_g} \) values were markedly low (0.033 and 0.027, respectively), suggesting that \( F_g \) more significantly lowers the value of \( F_{aF_g} \) than \( F_a \). Values of \( P_{\text{app}} \) obtained in PAMPA for ketoprofen, tolcapone, telmisartan, raloxifene, entacapone, and resveratrol were high (>1.0 \( \times \) 10\(^{-6} \) cm/s), which was consistent with the reported \( F_a \) and suggests that these compounds possibly have high permeability in vivo.

Monitoring glucuronides in the portal plasma after p.o. administration is important to obtain direct evidence that drugs undergo glucuronidation in the intestine. For ezetimibe, a significant amount of EPG was observed in portal plasma shortly after p.o. administration (Fig. 1A). It has been reported that ezetimibe is extensively absorbed and is detected mainly as EPG (with <5% of the total detected drug being in the unmodified form) in the portal blood in rats.\(^5\) Our data are consistent with this report, indicating strong evidence of intestinal glucuronidation. The conjugate in portal plasma did not come from the enterohepatic circulation because EPG is hardly absorbed (<5%).\(^5\) Additionally, the total concentration of the raloxifene glucuronides R6G and R4\(^+\)G in portal plasma within 60 min of p.o. administration was approximately 3–8 times higher than that of the unchanged raloxifene (Fig. 1B). The major glucuronide of raloxifene in rat portal plasma was found to be R6G, which was has been shown to be the major metabolite in rats,\(^14\) and these results bear similarity to those of Jeong et al. from studies in rat intestinal microsomes.\(^30\) These results for raloxifene are, to our knowledge, the first direct evidence that raloxifene undergoes intestinal glucuronidation in vivo and confirm several in vitro studies showing that this compound is metabolized by intestinal UGT. For entacapone, resveratrol, buprenorphine, and quercetin, peaks corresponding with the glucuronides were detected in rat portal plasma after p.o. administration (data not shown). These results indicate that our model compounds underwent glucuronidation in the rat intestine.

Intestinal metabolism can cause low \( F_g \), resulting in low \( F \) values. Therefore, prediction of \( F_g \) in the drug discovery stage is crucial in selecting drug candidates with potentially favorable PK. However, quantitative prediction of intestinal glucuronidation has been minimally discussed as a potential method. As shown in Figure 2, an inverse correlation between \( F_{aF_g} \) and \( \text{CL}_{\text{int}, \text{UGT}} \) for model compounds was observed (with the exception of buprenorphine). This result suggests that a strong direct relationship was evident between the in vitro- and in vivo intestinal glucuronidation for most of the tested compounds, and that \( F_{aF_g} \) can be predicted using in vitro data, similar to CYP3A substrates.

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Table 4. Pharmacokinetics parameters and predicted \( F_g \) of in-house compounds

<table>
<thead>
<tr>
<th>In-house compound</th>
<th>Functional group</th>
<th>( P_{\text{app}} ) in PAMPA (( \times 10^{-6} ) cm/s)</th>
<th>( \text{CL}_{\text{int}, \text{UGT}} ) (( \mu L/\text{min}/\text{mg} ))</th>
<th>( R_h )</th>
<th>( \text{CL}_{t} ) ( \text{plasma} ) (( \text{mL}/\text{min}/\text{kg} ))</th>
<th>( \text{CL}_{t} ) ( \text{blood} ) (( \text{mL}/\text{min}/\text{kg} ))</th>
<th>( F )</th>
<th>( F_{aF_g} )</th>
<th>Predicted ( F_{aF_g} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>COOH</td>
<td>22</td>
<td>160</td>
<td>0.50</td>
<td>13.8</td>
<td>27.6</td>
<td>0.32</td>
<td>0.60</td>
<td>0.56</td>
</tr>
<tr>
<td>Compound 2</td>
<td>COOH</td>
<td>&gt;30</td>
<td>248</td>
<td>0.63</td>
<td>15.5</td>
<td>24.5</td>
<td>0.24</td>
<td>0.41</td>
<td>0.45</td>
</tr>
<tr>
<td>Compound 3</td>
<td>OH</td>
<td>&gt;30</td>
<td>596</td>
<td>0.70</td>
<td>27.2</td>
<td>38.9</td>
<td>0.079</td>
<td>0.23</td>
<td>0.25</td>
</tr>
</tbody>
</table>

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We applied a simplified Fg model proposed by Kadono et al. to the correlation of seven model compounds (ketoprofen, tolcapone, telmisartan, rapoxifene, entacapone, reserterol, and ezetimibe) for the prediction of Fg. This model is convenient and practical for drug discovery, because (if assuming high Fg) the only components are Fg, Clint, and the empirical scaling factor (α). The curve obtained using Equation 9 (on the assumption of FgFg = Fg) was closely fitted to the observed values for model compounds, and α was calculated as 0.0050.

To verify the reliability of the obtained Fg model with α, we used it to predict Fg for three in-house compounds, which are known substrates of UGT and have high permeability. These compounds are derivatives with different functional glucuronidation target groups (COOH and OH), and were therefore appropriate to verify the usefulness of this model. Predicted Fg values for compounds 1–3 were very similar to observed Fg values, indicating that the effects of UGT on Fg can be predicted with this model (Table 4). In this study, we used seven compounds to construct the Fg model, but increasing this number may further improve its reliability.

Kadono et al. reported previously that a simplified Fg model could be applied to describe the relationship between FgFg and Clint, CYP in humans using CYP3A substrates with high absorption.7 Our present results indicate that the simplified Fg model is also useful for predicting intestinal glucuronidation in rats, suggesting that it can also be applied to human intestinal glucuronidation. The value of α, which is an empirical scaling factor consisting of the reciprocal of Clint and a factor connecting Clint, UGT and Clm, may differ among intestinal enzymes and between species, because physiological parameters related to absorption and metabolism, such as intestinal pH and volume, organ-specific blood flows, and expressed enzyme levels, also differ between species. Further studies using a sufficient number of human UGT substrates to apply to the Fg model are needed to determine the α for intestinal glucuronidation in humans to allow comparisons with data obtained in rats.

In the intestine of humans and various animal species, mRNA expression of approximately ten UGT isoforms has been reported.2,12–14 In humans, UGT1A7, 1A8, and 1A10, which are expressed selectively in the intestine,21 have been reported to be critical for the intestinal glucuronidation of rapoxifene51 and various flavonoids.35 In rats, UGT1A2, 1A3, and 1A7 were reported to be specific to the intestine,32 and may play a significant role in rat intestinal glucuronidation. However, until further studies confirm the expression and glucuronidation activity of UGT isoforms in the rat intestine, we cannot take into consideration which UGT isoforms contribute to the intestinal glucuronidation of the tested compounds.

Although the present study focused on UGT substrates, other enzymes such as CYP, SULT, and esterases are known to exist in the intestine, suggesting that some compounds may come under the influence of more than one enzyme in this environment. Indeed, buprenorphine, which has not been well characterized with respect to intestinal CYP metabolism, was metabolized in intestinal microsomes not only by UGT but also CYP (Table 3). The Clint, CYP value was half that of the Clint, UGT value, suggesting that CYP was also involved in intestinal metabolism of buprenorphine. The CYP-mediated oxidative metabolism of buprenorphine is known to be less prevalent in female than in male rats.16

Using female rats, Mistry et al. found good correlation between Clint, UGT and in vivo intestinal clearance for three opioid-type compounds including buprenorphine.10 However, in the present study using male rats, where CYP-mediated metabolism is a complicating factor, buprenorphine was an outlier for the otherwise good correlation between Clint, UGT and FgFg, as shown in Figure 2. We conclude that buprenorphine was metabolized in the intestine by both CYP and UGT, resulting in a markedly low FgFg value. Prediction of Fg for drugs metabolized by more than one intestinal enzyme will be complicated and requires further study.

Quercetin was metabolized in intestinal S9 by SULT (Table 3), suggesting that intestinal SULT may also affect the FgFg. Therefore, we excluded quercetin from the compounds used to construct the Fg model, despite the Clint, UGT and FgFg values fitting well to the correlation shown in Figure 2. The importance of intestinal glucuronidation and/or sulfation to the PK profile of quercetin in rats was reported by Chen et al.,17 but the conjugates were analyzed without separation, and consequently the major conjugate was unclear. Several studies have suggested that intestinal SULT may affect the Fg of drugs and xenobiotics. For example, flavonoids with a polyphenol structure have been reported to be metabolized in human intestinal S9 or cytosol with PAPS.36,37 Mizuma et al. reported that the Fg values of three SULT substrates were low (0.1–0.7), resulting in low F in humans.38 Further studies investigating the relationship between Clint, SULT and Fg will be required to reveal the impact of intestinal SULT on Fg.

In conclusion, we constructed a simplified intestinal Fg model and found a good correlation between FgFg and Clint, UGT for seven compounds that undergo intestinal glucuronidation in rats. In addition, this model enabled us to predict the FgFg of three in-house compounds, indicating that in vitro–in vivo prediction is achievable for intestinal glucuronidation. We anticipate that this study will expedite the development of a method for predicting FgFg due to glucuronidation in humans.

Acknowledgments: The authors would like to thank Yoshimitsu Nakajima and Masahiro Hirano for their help with the animal experiments, and Kouichi Tanaka for his help with the PAMPA experiments. The authors would also like to thank Keitaro Kadono and Akiko Koakutsu for useful discussion.
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