Prediction of the Hepatic and Renal Clearance of Transporter Substrates in Rats Using in Vitro Uptake Experiments

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ABSTRACT:

The clearance route and the absolute values for hepatic and renal clearance of drugs are important criteria for the selection of drug candidates. Based on pharmacokinetic theory, by assuming that uptake is the rate-determining process for the biliary excretion of drugs, organ intrinsic clearance should be simply estimated by the intrinsic uptake. In this study, to investigate whether organ clearance can be predicted from the in vitro uptake activity, we performed uptake experiments using isolated hepatocytes and kidney slices, integration plot analyses, and in vivo pharmacokinetic studies using 12 barely metabolized drugs in rats. The in vivo hepatic and renal clearance could be approximated by uptake clearance estimated from integration plot analyses, except for the renal clearance of some drugs that was relatively small. The comparison of intrinsic uptake clearance from in vitro experiments and integration plot studies revealed that in vivo hepatic uptake was well explained by uptake into isolated hepatocytes, whereas in kidney, in vivo uptake clearance was 10 to 100 times that in kidney slices and a scaling factor is required for its prediction from in vitro experiments. The organ clearance and the fraction excreted into urine could be predicted from in vitro studies except for drugs whose renal clearance was relatively small. This study suggests that the uptake process is the determining factor for organ clearance of minimally metabolized drugs, and uptake assays using isolated hepatocytes and kidney slices are useful for evaluating the uptake clearance.

To select drug candidates rationally in the early stage of drug development, many characteristics of the drugs should be taken into account. The pharmacokinetic property of drugs determines their systemic exposure and local distribution, and thus it is an important factor for optimizing pharmacological and toxicological effects. It is determined by several intrinsic factors such as metabolism, membrane transport, and protein binding, and various in vitro experimental systems have been established (Roberts, 2001; Balani et al., 2005).

Drugs are cleared mainly from the blood circulation by the liver and kidney, and many kinds of metabolic enzymes and transporters are responsible for their elimination (Iio et al., 2005; Shitara et al., 2006). The relative contribution of the liver and kidney to the overall clearance of drugs is important for considering their pharmacological and toxicological effects. Much evidence has indicated that the function of metabolic enzymes and transporters is modified by several factors such as pathophysiological conditions, genetic polymorphisms, and drug-drug interactions (Shitara et al., 2005, 2006; Ieiri et al., 2006; König et al., 2006; Lynch and Price, 2007). Therefore, to avoid large changes in the pharmacokinetics of drugs in unusual circumstances, clearance from multiple elimination pathways in liver and kidney is thought to be a desirable feature for many drugs. For example, the plasma concentration of enalaprilat was significantly increased by reduced renal function, whereas that of temocaprilat was not significantly changed (Oguchi et al., 1993) because enalaprilat is predominately excreted from the kidney, whereas temocaprilat is excreted from both the liver and kidney, which can minimize the effect of renal dysfunction because of its alternative elimination route by the liver (Ishizuka et al., 1997, 1998). On the other hand, if the pharmacological target is the liver or kidney, efficient targeting of drugs is needed to maximize the pharmacological effect. Pravastatin, a hydrophilic HMG-CoA reductase inhibitor, is efficiently retained in the enterohpatic circulation, which enables the long-term inhibition of HMG-CoA reductase in liver and the avoidance of its systemic side effects such as myopathy (Kitamura et al., 2008b). Antibiotics excreted mainly into bile and urine are selected for the treatment of bile duct inflammation and infection of the urinary tract, respectively (Tsuji, 2006). Thus, the prediction of the elimination route of drugs is

ABBREVIATIONS: Oatp, organic anion transporting polypeptide; Oat, organic anion transporter; E₁7βG, estradiol-17β-glucuronide; PAH, p-aminophenacetate; PCG, benzylpenicillin; SD, Sprague-Dawley; LC, liquid chromatography; MS, mass spectrometry; AUC, area under the plasma concentration-time profile.
essential for the development of desirable drugs. However, in vitro experimental systems for this prediction have not been established yet.

Apparent intrinsic clearance (CL_{int, app}) consists of 1) uptake clearance from blood to an organ (P_1), 2) backflux clearance from the organ to the blood (P_2), and 3) metabolism, or biliary or urinary excretion (P_3) and can be described as eq. 1 (Shitara et al., 2006):

$$CL_{int, app} = \frac{P_1}{P_1 + P_2 + P_3}$$

When P_1 is much larger than P_2, apparent intrinsic clearance can approximate intrinsic uptake clearance (P_1). In this case, the uptake clearance is the sole determinant of the overall intrinsic clearance, and therefore the hepatic and renal clearance of drugs can be predicted by uptake clearance.

In the process of the hepatic and renal uptake of organic anions, organic anion transporting polypeptide (Oatp) and organic anion transporter (Oat) family transporters, respectively, which are expressed on the basal membrane, are mainly involved (Shitara et al., 2006). For the characterization of uptake properties of drugs in the liver and kidney, isolated hepatocytes and kidney slices can be used (Shitara et al., 2006). Our group previously demonstrated that tissue uptake clearance of several compounds obtained from a multiple indicator dilution method is well explained by the in vitro uptake clearance into isolated hepatocytes (Miyauchi et al., 1993) in rats. Hasegawa et al. (2003) have shown that rat kidney slices can be useful for predicting the renal uptake clearance of compounds and the relative contribution of Oat1 and Oat3 to their overall uptake. These experimental systems can also be directly applied to humans using cryopreserved human hepatocytes and human kidney slices to predict the hepatic and renal uptake clearance (Hirano et al., 2004; Nozaki et al., 2004).

The purpose of this study was to examine whether hepatic and renal clearance could be predicted simply from in vitro uptake studies using isolated hepatocytes and kidney slices in rats using 12 minimally metabolized anionic drugs from four therapeutic categories (HMG-CoA reductase inhibitors, angiotensin II receptor antagonists, angiotensin-converting enzyme inhibitors, and β-lactam antibiotics), each of which has a different urinary and biliary excreted fraction.

Materials and Methods

Materials. [3H]Pravastatin (44.6 Ci/mmol), [3H]olmesartan (79 Ci/mmol), and [14C]temocaprilat (16.0 mCi/mmol) and unlabeled pravastatin, olmesartan, and temocaprilat were kindly donated by Daiichi-Sankyo Co. (Tokyo, Japan). [3H]Valsartan (81.0 Ci/mmol) and unlabeled valsartan were kindly donated by Novartis Pharma (Basel, Switzerland). [3H]Pitavastatin (16 Ci/mmol) was kindly donated by Kowa Co. (Tokyo, Japan), and [3H]rosuvastatin (79 Ci/mmol) was donated by AstraZeneca (London, UK). Unlabeled pitavastatin was synthesized by Nissan Chemical Industries (Chiba, Japan). Unlabeled rosuvastatin, candesartan, and benazeprilat were purchased from Toronto Research Chemicals (North York, ON, Canada). [14C]Estradiol-17β-glucuronide (E_217βG) (53 Ci/mmol), [14C]taurocholate (5.0 Ci/mmol), and [14C] hippuric acid (PAH) (4.1 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [14C]Benzylenepicillcin (PCG) (59 mCi/mmole) was purchased from GE Healthcare UK (Little Chalfont, Buckinghamshire, UK). Unlabeled E_217βG, taurocholate, PAH, cefixime, and cefmetazole were purchased from Sigma-Aldrich (St. Louis, MO). Unlabeled PCG and enalaprilat were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade and commercially available.

Animals. Female Sprague-Dawley (SD) rats (6–7 weeks old) were purchased from Nippon SLC (Shizuoka, Japan). All animals were maintained under standard conditions with a reverse dark-light cycle and were treated humanely. Food and water were available ad libitum. The studies reported in this manuscript were conducted in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

In Vivo Pharmacokinetic Study. Female SD rats weighing approximately 170 to 200 g were used for these experiments. Under light ether anesthesia, their femoral artery and vein were cannulated with polyethylene catheters (SP-31; Natsume Seisakusyo, Tokyo, Japan), the bile duct was cannulated with a polyethylene catheter (PE-10; Natsume Seisakusyo) for bile collection, and the bladder was cannulated with a polyethylene tube for urine collection. After the surgical procedures, the rats were placed in a restraining cage (Ball cage, Natsume Seisakusyo) and allowed to recover from the anesthesia. The rats received a bolus intravenous injection of 0.2 mg/kg pitavastatin, 0.5 mg/kg rosuvastatin, 0.5 mg/kg valsartan, 0.08 mg/kg olmesartan, 0.08 mg/kg candesartan, 0.5 mg/kg temocaprilat, 0.5 mg/kg enalaprilat, 2 mg/kg PCG, or 1 mg/kg cefixime or constant infusion of pravastatin (76 mg/min/kg after bolus intravenous injection of 0.67 mg/kg) or cefmetazole (0.1 mg/min/kg) into their femoral vein. Blood samples were collected from the femoral artery at designated times. Bile and urine were collected in preweighed test tubes at designated times. Plasma was prepared by centrifugation of the blood samples (15,000 g, 5 min, Microfuge; Beckman Coulter, Fullerton, CA). All samples were stored at −20°C until drug concentrations were measured by LC/MS.

Integration Plot Analysis. Female SD rats weighing approximately 170 to 200 g were used for these experiments. Under light ether anesthesia, their femoral artery and vein were cannulated with polyethylene catheters (SP-31; Natsume Seisakusyo). The rats received a bolus intravenous injection of 0.1 mg/kg pravastatin, 0.2 mg/kg pitavastatin, 0.05 mg/kg rosuvastatin, 0.01 mg/kg valsartan, 0.02 mg/kg olmesartan, 0.02 mg/kg candesartan, 0.01 mg/kg temocaprilat, 0.5 mg/kg enalaprilat, 0.5 mg/kg benazeprilat, 1 mg/kg PCG, 1 mg/kg cefixime, or 2 mg/kg cefmetazole. Blood samples were collected from the femoral artery at 15, 30, 45, 60, 90, and 120 s. The rats were sacrificed at 30, 60, or 120 s after dosing, and their livers and kidneys were immediately removed. Plasma was prepared by centrifugation of the blood samples (15,000 g, 5 min, Microfuge). Phosphate-buffered saline was added to tissue samples and homogenized to make a 30% homogenate. All samples were stored at −20°C until drug concentrations were measured by LC/MS.

Determination of the Protein-Unbound Fraction and Blood/Plasma Concentration Ratio. Binding of drugs to plasma proteins was determined by an ultrafiltration method. Plasma was prepared by the centrifugation of blood samples (15,000 g, 5 min, Microfuge). The concentrations of the drugs in the filtrate and the plasma before filtration were determined by LC/MS. The adsorption of the drugs on the membrane was confirmed to be negligible.

To determine the blood/plasma concentration ratio (R_P) values, blood was obtained from female SD rats. Drugs were added to the blood samples individually, and they were incubated together at 37°C for 5 min. Plasma was prepared by centrifugation of the blood samples (15,000 g, 5 min, Microfuge). The concentrations of the drugs in the blood and the plasma samples were determined by LC/MS. The protein-unbound fraction in the blood (f_u) was calculated by dividing the protein-unbound fraction in plasma (f_u) by R_p.

Uptake Study Using Rat Freshly Isolated Hepatocytes. Isolation of hepatocytes and an uptake study were conducted as described previously (Yamazaki et al., 1993). Isolated hepatocytes (viability >85%) were suspended in Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.8 mM KCl, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl₂, pH 7.4) and stored on ice. Before the uptake study, hepatocytes were preincubated at 37°C for 3 min, and the uptake reaction was started by adding drugs to the hepatocyte suspension. After a designated time, the reaction was terminated by separating the cells from the medium using a centrifugal filtration technique. For this purpose, a 100-µl aliquot of incubation mixture was placed in a 0.4-ml centrifuge tube (Sarstedt, Nunhembrecht, Germany) containing 50 µl of 2 N sodium hydroxide for radiolabeled compounds or 100 µl of 5 M sodium acetate for unlabeled compounds under a 100-µl layer of an oil mixture (density, 1.05, mixture of silicone oil and mineral oil; Sigma-Aldrich). Samples were then centrifuged for 10 s in a Microfuge. During this process, the hepatocytes pass through the oil layer into the aqueous
solution (2 N NaOH or 5 M CH₃COONa). In the case of unlabeled compounds, tubes were frozen in liquid nitrogen immediately after centrifugation and stored at −20°C until drug measurement.

The concentrations of pravastatin, pitavastatin, rosuvastatin, valsartan, olmesartan, temocaprilat, PCG, E17βO, and taurocholate were determined by measuring their radioactivity. After overnight incubation at room temperature to dissolve the cells in alkali, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial. The compartment containing dissolved cells was neutralized with 50 μl of 2 N hydrochloric acid and mixed with scintillation cocktail (Clearass II; Nakalai Tesque, Kyoto, Japan), and the radioactivity was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter). The concentrations of candesartan, benazeprilat, enalaprilat, cefizoxime, and cefmetazole were determined by LC/MS. Cells in 5 M sodium acetate buffer were taken from the centrifuge tube and sonicated in a new tube to break them down. This sample was used for the measurement of drug concentrations by LC/MS.

Uptake Study Using Rat Kidney Slices. An uptake study using rat kidney slices was performed as described previously (Hasegawa et al., 2002). Kidney slices (300-μm thick) from female SD rats were kept in ice-cold buffer (120 mM NaCl, 16.2 mM KCl, 1 mM CaCl₂, and 1.2 mM MgSO₄ in 10 mM NaH₂PO₄-Na₂HPO₄, pH 7.5). Two slices, each weighing 15 to 25 mg, were determined by LC/MS. Cells in 5 M PBS (100 mM) were taken from the centrifuge tube and sonicated in a liquid scintillation counting (LS6000SE; Beckman Coulter). The concentrations of candesartan, benazeprilat, enalaprilat, cefizoxime, and cefmetazole were determined by LC/MS. PBS (100 μl) was added to the slices followed by sonication to break them down. This sample was used for the measurement of drug concentration by LC/MS.

Quantification of Drug Concentration by LC/MS. Samples were precipitated with 3 volumes of acetonitrile (for in vivo samples and kidney slices) or methanol (for hepatocytes) and centrifuged at 15,000g at 4°C for 10 min. The supernatants were subjected to LC/MS. An LCMS-2010 EV equipped with a 12-well plate with 1 ml of oxygenated buffer after preincubation of slices for 5 min at 37°C. After incubation for designated periods, each slice was rapidly removed from the incubation buffer, washed with ice-cold buffer, blotted on filter paper, and weighed.

The concentrations of pravastatin, pitavastatin, rosuvastatin, valsartan, olmesartan, temocaprilat, PCG, and PAH were determined by measuring their radioactivity. The slice was dissolved in 1 ml of Soluene 350 (PerkinElmer Life and Analytical Sciences). The radioactivity in scintillation cocktail was determined by liquid scintillation counting (LS6000SE; Beckman Coulter). The concentrations of candesartan, benazeprilat, enalaprilat, cefizoxime, and cefmetazole were determined by LC/MS. PBS (100 μl) was added to the slices followed by sonication to break them down. This sample was used for the measurement of drug concentration by LC/MS.

Pharmacokinetic Analysis. In vivo study. The area under the plasma concentration-time profile over 120 min (AUC₀–120) was calculated using a trapezoidal method. The plasma concentration-time profile was fitted to the two exponential equations using a nonlinear iterative least-squares method with MULTI software (Yamaoka et al., 1981) and AUC₀–120 was estimated by integration of the fitted equation from time 0 to infinity. The plasma clearance (CLtot, p), the biliary clearance based on the drug concentration in plasma (CLbile, p), and the renal clearance based on the drug concentration in plasma (CLrenal, p) were calculated using eqs. 2–4:

\[ \text{CL}_{\text{int}} = \frac{\text{Dose}}{\text{AUC}_{0-\infty}} \]  
\[ \text{CL}_{\text{bile, p}} = \frac{X_{\text{bile}}}{\text{AUC}_{0-120}} \]  
\[ \text{CL}_{\text{renal, p}} = \frac{X_{\text{renal}}}{\text{AUC}_{0-120}} \]  

where \( X_{\text{bile}} \) represents the cumulative excreted amount in bile over 120 min and \( X_{\text{renal}} \) represents the amount in urine over 120 min.

When the drugs were infused intravenously, CLtot, p, CLbile, p, and CLrenal, p were calculated using eqs. 5–7:

\[ \text{CL}_{\text{int, p}} = \frac{I}{C_{\text{ps}}} \]  
\[ \text{CL}_{\text{bile, p}} = \frac{V_{\text{bile}}}{C_{\text{ps}}} \]  
\[ \text{CL}_{\text{renal, p}} = \frac{V_{\text{renal}}}{C_{\text{ps}}} \]  

where \( I \) represents the infusion rate, \( C_{\text{ps}} \) represents the drug concentration in plasma at steady state, \( V_{\text{bile}} \) represents the biliary excretion rate at steady state, and \( V_{\text{renal}} \) represents the urinary excretion rate at steady state. \( C_{\text{ps}} \) was determined as the mean value of the plasma concentration at 30, 60, 90, and 120 min. \( V_{\text{bile}} \) and \( V_{\text{renal}} \) were determined as the mean value of the renal excretion rate from 30 to 60, 60 to 90, and 90 to 120 min. The clearances based on drug concentrations in whole blood (CLbile, B, CLrenal, B) were calculated by dividing CLbile, p, CLrenal, p, and CLrenal, p by \( R_{\text{B}} \) respectively.

Integration plot analysis. The AUC₀–120 was calculated using a trapezoidal method. The tissue uptake amount of drugs per gram of tissue (\( X_t \)) normalized by the plasma concentration (\( C_p \)) can be described as eq. 8:

\[ \frac{X_t(t)}{C_p(t)} = \text{CL}_{\text{uptake}} \times \frac{\text{AUC}_{0-\infty}}{C_p(t)} + \frac{X_t(0)}{C_p(0)} \]  

where \( \text{CL}_{\text{uptake}} \) represents tissue uptake clearance based on the drug concentration in plasma and \( X_t(0)/C_p(0) \) represents the initial distribution volume. Based on eq. 8, the AUC₀–120/C_p(t) value was plotted against the \( X_t(t)/C_p(t) \) value, and each plot was fitted to the straight line using a nonlinear iterative least-squares method. CLuptake, p was assumed as a slope of the fitted line. The uptake clearance based on drug concentration in whole blood (CLuptake, B) was calculated by dividing CLuptake, p by \( R_{\text{B}} \). Because renal clearance includes glomerular filtration and tubular secretion in the kidney, the tubular secretion clearance, which corresponds to in vitro uptake clearance in kidney slices, was estimated by subtracting \( f_D \times \text{glomerular filtration rate (GFR)} \) (12 ml/min/kg) from CLuptake, p.

In the dispersion model, organ clearance (CLorg) is expressed as a function of the intrinsic clearance (CLint), organ blood flow rate (Q), and dispersion distance (\( D_Q \)):

\[ \text{CL}_{\text{org}} = Q \times (1 - F) \]  
\[ F = \frac{4a}{(1 + a)^2 \cdot \exp[(a - 1)/2D_Q] - (1 - a)^2 \cdot \exp[(-a + 1)/2D_Q]} \]  
\[ a = (1 + 4R_{\text{S}} \times D_Q)^{1/2} \]  
\[ R_{\text{S}} = f_D \times \text{CL}_{\text{uptake}}/Q \]  

The blood flow rate in the liver was set at 60 ml/min/kg and in the kidney at 40 ml/min/kg (Davies and Morris, 1993), and \( D_Q \) was set at 0.17 (Roberts and Rowland, 1986; Iwasubo et al., 1996). Because the in vivo intrinsic uptake clearance from blood to tissue (\( P_{\text{vivo}} \)) can be regarded as the organ intrinsic clearance (CLorg) when the rate-limiting step of the overall clearance is the uptake process, \( P_{\text{vivo}} = \text{CL}_{\text{pake, B}} \) was calculated by assigning the CLpake, B value to the CLorg in eqs. 9 to 12. When \( P_{\text{vivo}} \) values were calculated, drugs whose clearance was close to the blood flow (extraction ratio >0.7) were excluded because we cannot obtain the accurate \( P_{\text{vivo}} \) value. Detailed procedures for calculation of the parameters for the in vivo uptake clearance of valsartan as an example can be found in the Supplemental Appendix and Supplemental Fig. S1.

In vitro studies. The in vitro intrinsic uptake clearance (\( P_{\text{vivo}} \)) was calculated by dividing initial uptake velocity by the drug concentration in the
The relationship between the true intrinsic uptake clearance (P1KID,corrected) and the observed uptake clearance in kidney slices (P1KID) was explained theoretically by the simple mathematical model as shown in this figure. The details are described under Materials and Methods.

**Fig. 1.** A simple mathematical model for explaining the relationship between the true intrinsic uptake clearance (P1KID,corrected) and observed uptake clearance in kidney slices (P1KID). The third layer of kidney, the calculated CLorg corresponds to the secretion clearance, and the predicted renal clearance (CLr, predicted) was calculated using eq. 13:

\[
\frac{dC_s}{dt} = P_{21} \times C_s + \frac{f_{\text{R}}}{{\text{GFR}}} \times A + \frac{\text{CL}_{\text{org}}}{\text{CL}_{\text{r, predicted}}} + \frac{\text{CL}_{\text{b, predicted}}}{\text{CL}_{\text{r, predicted}}} \cdot A
\]

The detailed calculation procedures of the pharmacokinetic parameters estimated from in vitro assay of valsartan as an example can be found in the Supplemental Appendix and Supplemental Figs. S2 and S3. The predicted value of the fraction excreted into urine (\(f_{\text{urine, predicted}}\)) was calculated using eq. 14:

\[
f_{\text{urine, predicted}} = \frac{\text{CL}_{\text{r, predicted}}}{\text{CL}_{\text{r, predicted}} + \text{CL}_{\text{b, predicted}}} \cdot A
\]

In the process of in vitro-in vivo scale-up, we used the following parameters: 1.25 \times 10^8 hepatocytes/g of liver, 38.3 g of liver/kg b.wt., and 7.27 g of kidney/kg b.wt.

**Simple mathematical model for explaining the relationship between the true intrinsic uptake clearance (P1KID,corrected) and observed uptake clearance in kidney slices (P1KID).** One of the possible reasons for the discrepancy between predicted and observed intrinsic uptake clearance in the kidney was that the drug concentration inside the cells of kidney slices was lower than that in the buffer because a kidney slice consists of multiple cell layers and drugs cannot easily penetrate the multilayered cells (see also Results). Therefore, to explain the relationship between the true intrinsic uptake clearance (P1KID,corrected) and the observed uptake clearance in kidney slices (P1KID), we constructed the simple mathematical model as shown in Fig. 1.

We assumed that a kidney slice consists of multiple cell monolayers (number of monolayers = \(N\)), and that \(V_i\), \(C_{i0}\), and \(X_{i0}\) represent the volume of extracellular fluid compartment for each layer, the drug concentration in an extracellular fluid compartment at the \(n\)th layer, and the amount of drug taken up into an extracellular fluid compartment at the \(n\)th layer. In this model, we assumed that the drug is unidirectionally transported from an extracellular fluid compartment at the \(n\)th layer to that at the \((n+1)\)th layer with a clearance of \(P_{21}\) and that the drug is also taken up into an intracellular compartment at each layer from an extracellular fluid compartment at the same layer with a clearance of \(P_{1i}'\). We also assumed that the drug concentration in an intracellular fluid compartment at the first layer is regarded as that in the incubation medium (set as \(A\)).

In this condition, the mass-balance equation for an extracellular fluid compartment at the \(n\)th layer \([n = 2 \sim (N - 1)]\) can be described as the following equation:

\[
V \frac{dC_n}{dt} = P_{1i}' C_{(n-1)} - P_{21} C_n
\]

The equation for an extracellular fluid compartment at the \(N\)th layer is as follows:

\[
V \frac{dC_N}{dt} = P_{21} C_{(N-1)} - P_{1i}' C_N
\]

At the steady state, eqs. 15 and 16 can be converted to eqs. 17 and 18:

\[
C_s = \frac{P_{21}}{P_{1i}' + P_{21}} C_{(n-1)} = \left(\frac{P_{21}}{P_{1i}' + P_{21}}\right)^{n-1} A \quad (n = 2 \sim (N - 1)),
\]

\[
C_N = \frac{P_{21}}{P_{1i}' + P_{21}} C_{(N-1)} = \frac{P_{21}}{P_{1i}'} \left(\frac{P_{21}}{P_{1i}' + P_{21}}\right)^{N-2} A
\]

The velocity of drug uptake into an intracellular compartment at the \(n\)th layer is as follows (eq. 19):

\[
\frac{dX_n}{dt} = P_{1i}' C_n
\]

The velocity of drug uptake into the whole kidney slice \((v)\) can be described as the sum of the uptake velocity into an intracellular compartment at each layer.

\[
v = \sum_{i=1}^{N} dX_i = \sum_{i=1}^{N} P_{1i}' C_i
\]

P1KID is defined as the intrinsic uptake clearance for each cell monolayer in a kidney slice, whereas P1KID,corrected is defined as the “true” intrinsic uptake clearance for the whole kidney slice.

\[
P_{1\text{KID,corrected}} = N \cdot P_{1i}'
\]

Thus, when eq. 22 is assigned to eq. 21, the following equation can be obtained:

\[
P_{1\text{KID,corrected}} = N \cdot (P_{1\text{KID}} - P_{21})
\]
We also measured the plasma mainly eliminated from the body in an unchanged form in female SD rats. Pitavastatin (64%), suggesting that drugs used in this study were dose was excreted into bile and urine in an unchanged form, except for infusion study (ceftizoxime and pravastatin), more than 80% of the sensitivity in LC/MS analysis. Therefore, pravastatin and cefmetazole were quantitatively because of its low absorption and elimination (Fig. 2). In the kidney, the P1vivo value for each compound was approximately 10 to 100 times larger than the in vitro P1KID value (Fig. 3B). One of the possible reasons for the discrepancy is that the drug concentration inside the cells of kidney slices was lower than that in the buffer because a kidney slice consists of multiple cell layers and drugs cannot easily penetrate the multilayered cells, whereas in the physiological condition, drugs can access every cell through blood perfusion. Therefore, the uptake clearance was calculated by the uptake amount per unit weight of kidney slice normalized by the drug concentration in the medium might underestimate the true intrinsic uptake clearance. To mimic this situation, we constructed the simple model to explain the relationship between in vitro P1KID and in vivo P1vivo values and...
estimate the true intrinsic uptake clearance (P_{IKID,corrected}) based on the results of an in vitro uptake assay using kidney slices (see Materials and Methods). Because of analyses using this model, the relationship between P_{IKID} and P_{IKID,corrected} could be described as the following equation by fitting the in vitro P_{IKID} and in vivo P_{IVivo} values for each compound to eq. 23:

\[
P_{IKID,corrected} = 32.5 \times P_{IKID} - 7.05
\]

Thus, the P_{IKID,corrected} values were well explained by the P_{IVivo} values and used for the further analyses as an intrinsic uptake clearance in kidney.

**Prediction of the Organ Clearance from in Vitro Uptake Clearances.** Based on the intrinsic uptake clearance obtained from in vitro studies (P_{IHEP} and P_{IKID}), the hepatic and renal clearances of all test compounds were calculated by the simple scale-up method and dispersion model. In the case of kidney, P_{IKID,corrected} values instead of P_{IKID} values were used for intrinsic uptake clearance. The predicted organ clearances were compared with the in vivo observed values (Fig. 4). As a result, the hepatic and renal clearance could be predicted from the results of the in vitro uptake assay using isolated hepatocytes and kidney slices, except for the renal clearances of pitavastatin, valsartan, olmesartan, and candesartan, which were relatively small.

**Prediction of the Elimination Routes of Drugs from the in Vitro Uptake Assay.** Using the predicted hepatic and renal clearances (CL_{h, predicted} and CL_{r, predicted}), the fraction excreted into urine (f_{urine}) was calculated by eq. 14 and compared with the observed values. As shown in Fig. 5, f_{urine} values for nine compounds could be reasonably predicted from the results of the in vitro assay, but in the case of pitavastatin, valsartan, olmesartan, and candesartan, the predicted f_{urine} values overestimate the real f_{urine} values because of the underestimation of the renal clearances predicted from the in vitro assay.

**Discussion**

In this study, to demonstrate whether the hepatic and renal clearances of hardly metabolized drugs can be predicted from the results of an in vitro uptake assay using isolated hepatocytes and kidney slices, assuming that the intrinsic uptake clearance approximates the organ intrinsic clearance, we investigated the relationship between the predicted organ clearance calculated from in vivo integration plot anal-

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**Table 2**

<table>
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<tr>
<th>Compound</th>
<th>f_{h}</th>
<th>R_{h}</th>
<th>f_{r}</th>
<th>R_{r}</th>
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<tr>
<td>Pravastatin</td>
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<td>0.769 ± 0.038</td>
<td>0.662 ± 0.069</td>
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<td>Pitavastatin</td>
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<td>0.539 ± 0.012</td>
<td>0.012 ± 0.003</td>
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<td>Rosuvastatin</td>
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<td>0.631 ± 0.029</td>
<td>0.083 ± 0.010</td>
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<td>Valsartan</td>
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<td>0.702 ± 0.059</td>
<td>0.014 ± 0.002</td>
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<tr>
<td>Olmesartan</td>
<td>0.00861 ± 0.00067</td>
<td>0.843 ± 0.02</td>
<td>0.010 ± 0.001</td>
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<tr>
<td>Candesartan</td>
<td>0.000515 ± 0.00150</td>
<td>0.578 ± 0.02</td>
<td>0.011 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>Temocaprilat</td>
<td>0.0639 ± 0.0099</td>
<td>0.780 ± 0.055</td>
<td>0.082 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Enalapril</td>
<td>0.420 ± 0.003</td>
<td>0.751 ± 0.010</td>
<td>0.559 ± 0.0086</td>
<td></td>
</tr>
<tr>
<td>Benazepril</td>
<td>0.245 ± 0.076</td>
<td>0.670 ± 0.042</td>
<td>0.366 ± 0.116</td>
<td></td>
</tr>
<tr>
<td>PCG</td>
<td>0.454 ± 0.085</td>
<td>0.664 ± 0.014</td>
<td>0.684 ± 0.129</td>
<td></td>
</tr>
<tr>
<td>Cefazolin</td>
<td>0.654 ± 0.070</td>
<td>0.767 ± 0.032</td>
<td>0.853 ± 0.098</td>
<td></td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>0.598 ± 0.063</td>
<td>0.691 ± 0.021</td>
<td>0.865 ± 0.095</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL_{bile,B} (mL/min/kg)</th>
<th>CL_{renal,B} (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pravastatin</td>
<td>57.3 ± 9.4</td>
<td>27.1 ± 3.5</td>
</tr>
<tr>
<td>Pitavastain</td>
<td>63.7 ± 1.3</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>66.1 ± 9.5</td>
<td>20.9 ± 1.2</td>
</tr>
<tr>
<td>Valsartan</td>
<td>33.3 ± 3.6</td>
<td>5.30 ± 0.53</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>13.7 ± 0.3</td>
<td>2.46 ± 0.52</td>
</tr>
<tr>
<td>Candesartan</td>
<td>6.18 ± 1.52</td>
<td>0.932 ± 0.206</td>
</tr>
<tr>
<td>Temocaprilat</td>
<td>45.6 ± 0.9</td>
<td>20.5 ± 0.7</td>
</tr>
<tr>
<td>Enalapril</td>
<td>4.54 ± 0.75</td>
<td>18.7 ± 1.8</td>
</tr>
<tr>
<td>Benazepril</td>
<td>16.0 ± 2.0</td>
<td>29.2 ± 0.8</td>
</tr>
<tr>
<td>PCG</td>
<td>49.7 ± 3.3</td>
<td>44.8 ± 11.5</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>2.89 ± 0.03</td>
<td>12.5 ± 1.1</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>37.6 ± 1.2</td>
<td>47.2 ± 0.3</td>
</tr>
</tbody>
</table>

**Fig. 2.** Comparison between in vivo organ clearance (CL_{bile,B} and CL_{renal,B}) and organ uptake clearance (CL_{uptake,B, liver} and CL_{uptake,B, kidney}) in liver and kidney. CL_{uptake,B} values for the liver and kidney were obtained from integration plot analyses. CL_{bile,B} and CL_{renal,B} (for the liver) are plotted in A, and CL_{renal,B} and CL_{uptake,B, kidney} are plotted in B. An enlarged view of B (range of x-axis and y-axis: 1–100 mL/min/kg; plots inside the circle are shown in C. Points represent the following: 1, pravastatin; 2, pitavastatin; 3, rosuvastatin; 4, valsartan; 5, olmesartan; 6, candesartan; 7, temocaprilat; 8, enalapril; 9, benazepril; 10, PCG; 11, ceftizoxime, and 12, cefmetazole. The solid line represents the line of unity, and the dashed lines represent the lines of 1:2 and 2:1 correlations.
ytes and in vitro uptake assays and the observed organ clearance for 12 drugs in rats. These drugs are anionic at neutral pH and are excreted mainly into bile or urine in an unchanged form with minimal metabolism. In addition, these drugs are known to be substrates of drug transporters such as Oatps and Oats.

To examine the importance of the tissue uptake process in the overall clearance of test drugs, the organ clearance (CL_{\text{org}}, B or CL_{\text{renal}}, B) was compared with the respective uptake clearance measured by integration plot analyses (CL_{\text{uptake}}, B, liver or CL_{\text{uptake}}, B, kidney) in rats. CL_{\text{bile}, B} was comparable to CL_{\text{uptake}, B, liver}, suggesting that the hepatic uptake of compounds was thought to be a determinant factor for overall hepatic clearance (Fig. 2A), whereas CL_{\text{renal}, B} was comparable to CL_{\text{uptake}, B, kidney} for eight drugs, but not for pitavastatin, valsartan, olmesartan, or candesartan (Fig. 2B). The renal clearance of these exceptional drugs was relatively small and underestimates their CL_{\text{uptake}, B, kidney} values, suggesting that the uptake process was not a rate-limiting step in their overall renal clearance. Thus, it is possible that backflux from kidney to blood is larger than elimination from kidney to urine or that significant reabsorption from urine to blood is involved in their renal excretion.

### TABLE 4
Uptake clearance of drugs in rat isolated hepatocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>P_{\text{HEP}} (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pravastatin</td>
<td>0.36 ± 0.22</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>Losartan</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Candesartan</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Temocaprilat</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Enalapril</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Benazepril</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>PCG</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Cefixime</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Ticagrelate</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

### TABLE 5
Uptake clearance of drugs in rat kidney slices

<table>
<thead>
<tr>
<th>Compound</th>
<th>P_{\text{KID}} (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pravastatin</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>Valsartan</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Candesartan</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Temocaprilat</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Enalapril</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Benazepril</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>PCG</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Cefixime</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Ticagrelate</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

Next, to examine whether the vivo uptake clearance could be predicted from in vitro uptake assays, the intrinsic uptake clearance obtained from integration plot analyses (P_{\text{Ivivo}}) was compared with the in vivo uptake clearance (P_{\text{IHEP}} or P_{\text{IKID}}) (Fig. 3). P_{\text{IHEP}} was
almost comparable to $P_{\text{vivo, liver}}$ (Fig. 3A), indicating that hepatic intrinsic uptake clearance can be predicted from in vitro uptake studies. Previous reports have also demonstrated that the in vivo uptake clearance collected using a multiple indicator dilution method and integration plot analysis could be predicted from the in vitro uptake clearance using isolated hepatocytes by simple scale-up calculations (Miyauchi et al., 1993; Kato et al., 1999). On the other hand, because it is difficult to isolate the renal tubular cells, kidney slices are proposed as a good in vitro tool for the characterization of renal uptake. Hasegawa et al. (2003) characterized active uptake of drugs and established a methodology for examining the contribution of Oat1 and Oat3 to the overall renal uptake of drugs. However, it remains to be clarified whether the in vivo renal clearance can be predicted using in vitro uptake assays. As a result, $P_{\text{vivo, kidney}}$ was 10 to 100 times larger than $P_{\text{KID, corrected}}$ (Fig. 3B). We hypothesized that this apparent discrepancy was caused by the decreased exposure of drugs to the inner cell layer of kidney slices. Then, to determine the theoretical relationship between the apparent uptake clearance per unit weight of kidney slices and true intrinsic clearance, we constructed the simple mathematical model and derived eq. 24 (see Materials and Methods). The slope of this equation (32.5) represents half of the number of cell layers in a kidney slice, assuming that the drug-containing buffer can access both sides of the kidney slice equally. The thickness of the kidney slice was approximately 300 μm, and the size of cells is generally 3 to 10 μm, suggesting that a kidney slice consists of 30 to 100 cell layers, which is comparable to the estimated number of cell layers. Thus, we corrected the uptake clearance in kidney slices to the true intrinsic uptake clearance ($P_{\text{KID, corrected}}$) using eq. 24.

Finally, in vivo organ clearance was estimated from the results of an in vitro uptake study (Fig. 4). When calculating the organ clearance from intrinsic clearance, we used a dispersion model with $D_N$ of 0.17 because a previous report suggested that it is the best prediction method compared with other models regardless of the magnitude of the clearance (Roberts and Rowland, 1986). The predicted hepatic clearance ($CL_{\text{h, predicted}}$) was almost comparable to the observed one ($CL_{\text{h, true}}$) (Fig. 4 A), suggesting that the hepatic clearance of compounds could be predicted from the results of the uptake assay using isolated hepatocytes. The renal clearance was calculated in the same way as that of liver using the corrected intrinsic uptake clearance ($P_{\text{KID, corrected}}$). The observed renal clearance ($CL_{\text{renal, true}}$) was correlated with the predicted clearance ($CL_{\text{renal, predicted}}$) for each drug within a 2-fold range of difference, except for pitavastatin, valsartan, olmesartan, and candesartan, whose renal clearances were not well predicted from their uptake clearances (Fig. 4B).

Regarding the backflux in the liver, multidrug resistance-associated proteins 3 and 4 on the sinusoidal membrane of hepatocytes are involved in the sinusoidal efflux of several glucuronide and sulfate conjugates, and parent compounds such as fexofenadine and methotrexate (Kitamura et al., 2008a; Matsushima et al., 2008; Tian et al., 2008). However, the significance of these transporters on the sinusoidal efflux in humans has not been clarified. Sandwich-cultured hepatocytes enable us to evaluate the sinusoidal efflux and biliary excretion separately, so sandwich-cultured human hepatocytes might provide us with information regarding the relative contribution of backflux and sequestration of drugs (Bi et al., 2006). For reabsorption in the kidney, candidate transporters on the brush border membrane in proximal renal tubular cells have been identified. In rats, Oatp1a1 is reported to be involved in the reabsorption of organic anions such as estradiol-17β-glucuronide and dibromosulfophthalein, and their renal clearance was smaller in male rats than in female rats because of its gender-specific expression (Gotot et al., 2002; Kato et al., 2002). However, in this study, the possible involvement of reabsorption was observed even in female rats, implying the existence of other mechanisms. There is no information regarding the backflux in the kidney to date. Future studies will be needed to clarify the molecular mechanisms of backflux and reabsorption in the kidney. More recently, it has been demonstrated that vectorial transport of drugs could be observed across the monolayer of epithelial cells from the proximal renal tubule seeded onto the culture insert, and the expression of several transporters was also confirmed (Lash et al., 2006, 2008). This kind of experimental system may help us investigate bidirectional drug transport in the kidney.

The Oatp family transporters and Oat1 and Oat3 are mainly involved in the uptake of organic anions in the liver and kidney, respectively. The substrate specificity of Oatp transporters overlaps that of Oat3 such as pravastatin, olmesartan, temocaprilat, and PCG. Thus, the fraction excreted into bile and urine of organic anions may be determined by the relative transport activity of Oatp transporters and Oat3. By comparing the transport activity of transporter-specific ligands or relative expression level in organ samples and transporter expression systems, the major elimination route of drugs can be estimated from the relative transport activity in each expression system. In this study, we selected minimally metabolized compounds for analysis. Recent reports have suggested the importance of uptake transporters in the hepatic clearance of some drugs such as repaglinide, bosentan, and atorvastatin, which are extensively metabolized by cytochrome P450 enzymes (Shitara et al., 2006). Even in the case of extensively metabolized drugs, if the uptake process is mediated by transporters, this prediction method may be applied for these substrates because the hepatic clearance may be solely determined by the uptake clearance.

To predict the human pharmacokinetics, human cryopreserved hepatocytes and kidney slices are now available and have been used in the characterization of the contribution of uptake transporters to the overall uptake of drugs and drug-drug interactions (Hirano et al., 2004; Nozaki et al., 2004). Using the same approach, we will be able...
to predict the elimination route of drugs in humans using in vitro uptake assays.

In summary, absolute values of hepatic and renal clearances and the fraction excreted in urine can be predicted for minimize metabolized drugs from in vitro uptake studies using isolated hepatocytes and kidney slices, except for drugs whose renal clearance was relatively small. Although further studies are needed for more accurate prediction, this model will be applicable to drug screening for the prediction of organ clearance and the distribution of compounds in the liver and kidney.

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References

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