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Effect of plasma protein binding on in vitro-in vivo correlation of biliary excretion of drugs evaluated by sandwich-cultured rat hepatocytes

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Running title: In vitro-in vivo correlation of biliary excretion in rats

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Number of text pages: 34

Number of tables: 3

Number of figures: 5

Number of references: 39

Number of words in the Abstract: 250

Number of words in the Introduction: 589

Number of words in the Discussion: 1328

ABBREVIATIONS: SCRH, sandwich-cultured rat hepatocytes; NCEs, new chemical entities; Bsep, bile salt export pump; Mrp, multidrug resistance associated protein, Bcrp, breast cancer resistance protein, Mdr, multidrug resistance; Oatp, organic anion transporting polypeptide; Oat, organic anion transporter

Abstract

In the present study, we examined in vitro biliary clearance of several compounds in sandwich-cultured rat hepatocytes (SCRH) and compared it with that observed in vivo in rats, and the effect of plasma protein binding on in vitro-in vivo correlation of biliary excretion was also assessed. The in vitro biliary excretion was determined by differential cumulative uptake of compounds in SCRH preincubated in the presence and absence of $\text{Ca}^{2+}/\text{Mg}^{2+}$. The cumulative uptake study of radiolabeled substrates revealed that the function of canalicular efflux transporters such as Bsep, Mrp2, Bcrp, and Mdr1 was adequately maintained in SCRH. Unlabeled test compounds, pravastatin, rosuvastatin, valsartan, cefmetazole, and cefoperazone exhibited varying degrees of in vitro biliary excretion in the cumulative uptake study using SCRH. In vivo biliary excretions of these compounds were measured in common bile duct-cannulated rats. While their biliary excretion ratios were all more than 60 % of the dose, the in vivo intrinsic biliary clearances varied from 10.5 to 1787.2 mL/min/kg. The in vitro intrinsic biliary clearances of test compounds were well correlated with their corresponding in vivo intrinsic clearances calculated based on plasma unbound concentration ($r^2 = 0.984$), while less correlation was observed when they were calculated based on plasma total concentration ($r^2 = 0.217$). These results indicate that SCRH is a useful in vitro model for predicting in vivo intrinsic biliary clearance in rats. Additionally, for an accurate prediction, it is necessary to evaluate the in vivo intrinsic biliary clearance based on plasma unbound concentration but not total concentration.

Introduction

Biliary excretion is one of the important routes for drug elimination (Rollins et al., 1979; Levine, 1978). Therefore, elucidating biliary excretion properties of new chemical entities (NCEs) is a critical issue in the drug discovery and development stage. Several experimental methods have been used to investigate the mechanism of biliary excretion, including liver canalicular membrane vesicles, isolated and cultured hepatocytes, isolated perfused liver, and bile duct-cannulated animals (Tamai et al., 1990; Kukongviriyapan et al., 1990; Handler et al., 1994; Chandra et al., 2005; Tsuda-Tsukimoto et al., 2006), however, due to the lack of acceptable throughput, it has been desired to develop more suitable methods for evaluating hepatobiliary disposition of NCEs in the drug discovery and development stage.

In view of convenience and an advantage of accessibility, primary cultured rat hepatocytes have been expected as a potential in vitro tool for evaluating hepatobiliary disposition, however, primary cultured rat hepatocytes are well known for rapidly losing cell polarity and liver-specific functions such as albumin secretion, hepatic uptake, and enzyme activity under conventional monolayer culture conditions (Dunn et al., 1989; Foliot et al., 1985). In contrast to conventional conditions, hepatocytes cultured in a collagen-sandwich configuration were reported to maintain the liver-specific functions for several days and exhibit a three-dimensional configuration, formation of bile canaliculi, and proper localization of drug efflux transporters on the canalicular membrane (LeCluyse et al., 1994; Talamini et al., 1997).

Brouwer and colleagues reported an application of sandwich-cultured rat hepatocytes (SCRH) to evaluate biliary excretion (Liu et al., 1999a). In their technique, biliary excretion in SCRH was assessed by

cumulative uptake of a compound in the presence (a standard condition) and absence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ in the incubation medium. The cumulative uptake in the standard condition represented the amount of compound in both cytosol and bile canaliculus lumen, whereas that in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free condition represented the amount only in cytosol because the tight junction of bile canaliculi was disrupted under the condition. Therefore, the amount of compound excreted into bile canaliculus lumen was estimated from the differential uptake of compound between standard and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free conditions.

Recently, sandwich-cultured human hepatocytes have been applied to evaluate the human biliary excretion (Bi et al., 2006). Establishing an in vitro-in vivo correlation of biliary excretion is required for the prediction of human hepatobiliary excretion. Previously, the in vitro-in vivo correlation of biliary excretion for Bsep and Mrp2 substrates was evaluated using radiolabeled substrates in SCRH (Liu et al., 1999b). However, the in vivo intrinsic biliary clearance was calculated based on the plasma total concentration without consideration of the plasma unbound fraction in their correlation study. In addition, although Bcrp is also an important transporter for biliary excretion of drugs (Hirano et al., 2005; Ando et al., 2007; Zamek-Gliszczynski et al., 2005), the correlation of Bcrp substrates has not been examined. Furthermore, when the SCRH system is applied to the drug discovery process, it is of great advantage if the assay system is available for unlabeled compounds.

In the present study, we investigated the in vitro biliary clearance of several unlabeled compounds including rosuvastatin as a rat Bcrp substrate (unpublished observation) in SCRH and compared it with their corresponding in vivo intrinsic clearance. Furthermore, the influence of plasma protein binding on the in

vitro-in vivo correlation of biliary clearance was assessed. Test compounds such as pravastatin, rosuvastatin, valsartan, cefmetazole, and cefoperazone that were known to be mainly excreted into bile in rats were chosen for the correlation study (Komai et al., 1992; Nezasa et al., 2002; Yamashiro et al., 2006; Garcia-Agundez et al., 1992; Pattinson et al., 1987).

Materials and Methods

Chemicals. [^3H]Taurocholic acid (50 Ci/mmol; purity 99 %) and [^3H]salicylic acid (50 Ci/mmol; purity 99 %) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). [^3H]Estradiol 17 β -D-glucuronide (53 Ci/mmol; purity 97 %), [^3H]estrone 3-sulfate (57.3 Ci/mmol; purity 97 %), and [^3H]digoxin (23.5 Ci/mmol; purity 97 %) were obtained from PerkinElmer (Boston, MA, USA). Dulbecco's Modified Eagle Medium (DMEM), William's medium E, standard Hank's balanced salt solution (HBSS), $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS, insulin, transferrin, collagenase type I, trypsin inhibitor, TritonX-100, cefmetazole, and cefoperazone were purchased from Sigma-Aldrich, Inc. (Tokyo, Japan). Fetal bovine serum was purchased from Nichirei Bioscience (Tokyo, Japan). Sodium selenite, dexamethasone, pravastatin, and 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) diacetate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Rosuvastatin and valsartan were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Ethylene glycol tetraacetic acid (EGTA) and phenol red was purchased from Nacalai tesque, Inc. (Kyoto, Japan). Penicillin-streptomycin and phosphate buffered saline (PBS) were purchased from Invitrogen (Tokyo, Japan). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals and reagents were analytical grade.

Animals. Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) weighing 200-250 g were acclimatized for 7 days before the experiment. The temperature and a relative humidity were maintained at 23-24°C and 44-62 %, respectively. Lighting was controlled to provide a 12 h light/dark cycle.

All animals were fasted overnight before the experiment and drinking-water was supplied ad libitum. All the study protocols were reviewed and approved by the Animal Ethics Committee of Mitsubishi Tanabe Pharma Corporation.

Isolation and culture of rat hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats by a collagenase perfusion procedure (Kohira et al., 1989). Rats were anesthetized by intraperitoneal injection of pentobarbital. Rat livers were perfused with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS) containing 0.5 mM EGTA and 0.006 g/L phenol red for 10 min at a flow rate of 25 to 30 ml/min followed by perfusion with the HBSS containing 0.35 mg/mL collagenase type I, 0.56 g/L CaCl_2 and 0.05 g/L trypsin inhibitor for 10 min at a flow rate of 25 to 30 ml/min. Hepatocytes were dispersed from the digested liver in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, 1 μM dexamethasone, 6.25 $\mu\text{g/mL}$ insulin, 6.25 $\mu\text{g/mL}$ transferrin, and 6.25 ng/mL selenium. Hepatocytes were separated from connective tissue by filtering through a sterile nylon mesh and from nonparenchymal cells by repeated low speed centrifugation at 50 x g for 1 min in supplemented DMEM. Cell pellets were resuspended with supplemented DMEM and viability was determined by trypan blue exclusion. Only those cell preparations with greater than 85 % viability were used for further studies. In order to culture the hepatocytes in a collagen-sandwich configuration, the cells were plated onto 24-well collagen-coated culture plates (Asahi Technoglass Corp., Tokyo, Japan) at a density of 2×10^5 cells/well in 0.5 mL of supplemented DMEM. Cells were allowed to attach for 2 hr at 37°C in a humidified chamber with 95%/5% of air/ CO_2 atmosphere. Then, medium containing unattached cells was aspirated and William's E medium

supplemented with 0.1 μ M dexamethasone, 6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenium, and 50 μ g/ml matrigel was added to cultures. The supplemented William's E medium was changed every 24 h for 96 h.

Cumulative uptake study in SCRH. Uptake study was carried out according to the method reported previously (Liu et al., 1999a). SCRH were preincubated in 0.5 mL of standard buffer or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer at 37°C for 10 min. The standard buffer was composed of HBSS and 20 mM HEPES, and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer was composed of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS, 20mM HEPES, and 0.5 mM EGTA. After removal of the buffer by suction, an uptake reaction was initiated by adding the 0.5mL of substrate-containing standard buffer and terminated by rinsing 4 times with 0.5 mL of ice-cold standard buffer. For radiolabeled compounds, the cells were lysed with 0.5 mL of 1 % Triton X-100 in PBS by shaking for 20 min at room temperature. An aliquot of lysate was analyzed by liquid scintillation spectrometry. BCA protein assay kit was used to determine the protein concentration in the cell lysate. For unlabeled compounds, cells were lysed with 0.4 mL of acetonitrile/methanol (7:3, v/v) by shaking for 10 min at room temperature, and aliquots of cell lysate were filtered with 0.45 μ m pore size membrane filters (Nihon Millipore Ltd., Tokyo, Japan). The filtrate was dried in a centrifugal evaporator and reconstituted with an aqueous mobile phase for the LC-MS/MS analysis.

Plasma concentration and biliary excretion in rats after intravenous administration. Rats were anesthetized with diethyl ether and following studies were carried out under light anesthesia. For biliary excretion studies, a polyethylene cannula tube (0.28 mm i.d., 0.61 mm o.d., Natsume Seisakusyo Co., Ltd.,

Tokyo, Japan) was inserted into a common bile duct. The body temperature of rats was maintained by heating using a lamp. Pravastatin, cefmetazole and cefoperazone were formulated as a solution in saline, and rosuvastatin and valsartan in a mixture of saline/dimethylformamide (1:1, v/v) at a concentration of 1 mg/mL. Each compound was intravenously administered via the tail vein at a dose of 1 mg/kg. Blood and bile were collected at designated time, and the blood was centrifuged to obtain plasma. The plasma or bile samples were mixed with acetonitrile/methanol (7:3, v/v) and centrifuged. The supernatant was analyzed by LC-MS/MS.

Plasma protein binding. Plasma protein binding of test compounds was determined by an ultracentrifugation technique (Barre et al., 1985). In brief, 3 μ L of 1 mM test compound solution in 80 % acetonitrile was mixed with 300 μ L of pooled rat plasma at concentration of 10 μ M (final acetonitrile concentration, 0.8 % v/v). A 200 μ L of aliquot was centrifuged at 436,000 x g for 4 h at 37 °C. Aliquots of the unbound fraction were collected to determine the unbound concentration. The plasma samples without ultracentrifugation were diluted with 9-fold volume of saline for determination of total plasma concentration. The aliquots of each sample were added to 9-fold volume of acetonitrile and centrifuged. The supernatants were analyzed by LC-MS/MS. Plasma unbound fraction (f_p) was calculated by:

$$f_p = C_f / C_t$$

where, C_f and C_t represent the drug concentration with and without ultracentrifugation, respectively.

Blood to plasma ratio. Fresh blood was obtained in heparinized containers from Sprague-Dawley rats. The blood was spiked with each compound (final concentration at 1 μ M) and placed in a 37 °C shaking water

bath for 20 min. Aliquots were withdrawn from the incubation tubes as a blood sample. The remaining blood fraction was centrifuged to separate plasma and blood cells suspension, and aliquots of plasma fraction were collected as a plasma sample. The blood or plasma sample were mixed with acetonitrile/methanol (7:3, v/v) and centrifuged. The supernatants were analyzed by LC-MS/MS. Blood-to-plasma ratio (Rb) was calculated by:

$$Rb = C_b / C_p$$

where, C_b and C_p represent the blood and plasma concentration of compound, respectively.

Analytical procedure. The chromatography was performed on an Acquity™ UPLC system (Waters, Milford, MA, USA) equipped with Acquity™ UPLC BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μ m particle size, Waters) maintained at 40 °C. The initial mobile phase was 95% 20 mM ammonium acetate and 5% acetonitrile and the proportion of acetonitrile was linearly increased to 90% over 3 min with the flow rate of 0.3 mL/min. The autosampler was conditioned at 8 °C with the injection volume of 3 μ L.

Mass spectrometry analysis was performed on a Waters Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters) using an electrospray source in positive or negative ion mode. The ionization source parameters were: capillary voltage 1.0 kV; source temperature 120 °C; desolvation gas temperature 400 °C at a flow rate of 1000 L/h (nitrogen); cone gas flow rate 50 L/h. Nitrogen (99.9% purity) and argon (99.9999% purity) were used as cone and collision gases, respectively. The following selected ion monitoring transitions were used for analysis: 423.4 > 321.1 for pravastatin; 482.2 > 258.1 for rosuvastatin; 436.2 > 207.1 for valsartan; 472.1 > 328.0 for cefmetazole, 646.2 > 143.1 for cefoperazone at collision energy of 15,

37, 27, 15, and 41, respectively. The acid form was monitored in the measurement of pravastatin and rosuvastatin because the levels of lactone form were negligible. Data acquisition was carried out by MassLynx™ Ver. 4.1 software with a QuanLynx™ program (Waters).

Data analysis. Apparent intrinsic biliary clearance is defined as intrinsic biliary clearance in the present study. In vitro intrinsic uptake clearance (CL_{uptake,int}) and intrinsic biliary clearance (CL_{bile,int}) were estimated according to Eq.1 and Eq.2, respectively. Rat liver weight and protein content in liver tissue were assumed to be 40 g/kg of body weight and 200 mg protein/g of liver weight, respectively, in each calculation (Davies and Morris, 1993; Seglen, 1976).

$$CL_{\text{uptake,int}} = \frac{\Delta \text{Uptake (+Ca}^{2+} / \text{Mg}^{2+} \text{ }_{2-0 \text{ min}})}{\text{Incubation Time} \times \text{Concentration (medium)}} \quad \text{Eq.1}$$

where, ΔUptake represents the differences of uptake amount between 0 and 2 min, and concentration (medium) represents the concentration in the incubation medium at 0 min.

$$CL_{\text{bile,int}} = \frac{\text{Uptake (+Ca}^{2+} / \text{Mg}^{2+}) - \text{Uptake (-Ca}^{2+} / \text{Mg}^{2+})}{\text{Incubation Time} \times \text{Concentration (medium)}} \quad \text{Eq.2}$$

where, uptake (+Ca²⁺/Mg²⁺) and uptake (-Ca²⁺/Mg²⁺) represent the cumulative uptake of compound in SCRH preincubated in the presence and absence of Ca²⁺/Mg²⁺, respectively. The intrinsic biliary clearance was determined at 10 min. Biliary excretion was quantitatively assessed by the biliary excretion index (BEI) at

the time point of 10 min based on Eq.3 (Liu et al., 1999c). BEI was determined using B-CLEAR technology (Qualyst, Inc., Raleigh, NC).

$$BEI = \frac{\text{Uptake (+Ca}^{2+}/\text{Mg}^{2+}) - \text{Uptake (-Ca}^{2+}/\text{Mg}^{2+})}{\text{Uptake (+Ca}^{2+}/\text{Mg}^{2+})} \quad \text{Eq.3}$$

The in vivo kinetic parameters such as AUC_{inf} (area under the plasma concentration-time curve), V_{dss} (distribution volume), CL_{tot} (total body clearance), C_0 (initial concentration) and $T_{1/2}$ (plasma half life) were calculated by a model-independent method using the MULTI computer program (Yamaoka and Nakagawa, 1983). The in vivo biliary clearance (CL_{bile}) was calculated according to Eq.4.

$$CL_{bile} = \frac{\text{Accumulation in bile (180 min)}}{AUC(180 \text{ min})} \quad \text{Eq.4}$$

where, accumulation in bile (180 min) represents the amount of compounds excreted into bile for 180 min, and AUC (180 min) represents the area under the plasma concentration-time curve from 0 to 180 min.

The in vivo intrinsic biliary clearance was calculated by Eq.5a and Eq.5b using the well-stirred model where Q_h represents rat hepatic blood flow, 67 mL/min/kg (Nies et al., 1976), and f_p and R_b represents plasma unbound fraction and blood to plasma ratio of test compounds, respectively. In these equations, $CL_{bile,int}$ and $CL_{bile,int}'$ represent the intrinsic biliary clearance calculated based on plasma unbound concentration and plasma total concentration, respectively.

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$$CL_{bile,int} = \frac{Q_h \times CL_{bile}}{Q_h - CL_{bile}/R_b} \times \frac{1}{f_p} \quad \text{Eq.5a}$$

$$CL_{bile,int'} = \frac{Q_h \times CL_{bile}/R_b}{Q_h - CL_{bile}/R_b} \quad \text{Eq.5b}$$

Results

Cell morphology and biliary excretion of carboxydichlorofluorescein (CDF) in SCRH. Figure 1 showed a phase-contrast image of SCRH cultured for 4 days (left) and fluorescence images after 20 min preincubation with 10 μ M CDF diacetate in standard buffer (middle) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer (right). The formation of bile canalicular lumen was clearly observed in SCRH. The fluorescent Mrp2 substrate, CDF (Kitamura et al., 1990), was localized in hepatocytes and the bile canalicular lumen when it was incubated in the standard condition. In contrast, CDF localization in the bile canalicular lumen but not in hepatocytes disappeared in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free condition. These results suggest that the bile canaliculi and intact tight junctions were reestablished, and that Mrp2 functioned to accumulate the substrate into the canalicular lumen in SCRH used in this study.

Cumulative uptake of the radiolabeled substrates in SCRH. Several radiolabeled compounds were chosen to assess the function of efflux transporters in SCRH. [^3H]Taurocholic acid, [^3H]estradiol-17 β -D-glucuronide, [^3H]estrone 3-sulfate, and [^3H]digoxin were used as a substrate of Bsep, Mrp2, Bcrp, and Mdr1, respectively. Time courses of cumulative uptake of these compounds are shown in Figure 2. The cumulative uptake in standard condition was significantly higher than that in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free condition. The BEI is summarized in Table 1. The BEI of [^3H]salicylic acid showed the lowest value of 4.4 %, which is consistent with this compound not being excreted into bile in vivo.

Cumulative uptake of the unlabeled compounds in SCRH. Figure 3 shows the cumulative uptake of unlabeled compounds that are known to be mainly excreted into bile in vivo, such as pravastatin, rosuvastatin,

valsartan, cefmetazole, and cefoperazone. There were differences in cumulative uptake of compounds under standard and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free conditions except for cefmetazole. Table 2 summarizes intrinsic uptake clearance, intrinsic biliary clearance, and BEI of test compounds in SCRH. The intrinsic uptake clearance, which represents the sinusoidal uptake in hepatocytes, was high for rosuvastatin, and intermediate for pravastatin and valsartan, and low for cefmetazole and cefoperazone. BEI, which represents canalicular efflux, was high for rosuvastatin, cefoperazone, pravastatin and valsartan, and low for cefmetazole. The intrinsic biliary clearance, which represents the net biliary excretion, was high for rosuvastatin, intermediate for pravastatin and valsartan, and low for cefmetazole and cefoperazone.

Plasma concentration and cumulative biliary excretion of unlabeled compounds in rats.

Pravastatin, rosuvastatin, valsartan, cefmetazole, and cefoperazone were intravenously administered to common bile-duct cannulated rats at the dose of 1 mg/kg. The plasma concentration-time curve and the cumulative biliary excretion-time curve of test compounds are shown in Figure 4. Pharmacokinetic and biliary excretion parameters of test compounds are summarized in Table 3. All compounds were rapidly eliminated with a plasma half-life within 40 min. Biliary excretions of these compounds were all over 60 % of dose.

As shown in Table 3, the plasma protein binding ratio was extremely high for valsartan, modestly high for rosuvastatin, and low for others. Especially large differences were observed between $\text{CL}_{\text{bile,int}}$ and $\text{CL}_{\text{bile,int}}'$ of valsartan and rosuvastatin because of their high protein binding. The blood to plasma ratio of pravastatin, rosuvastatin, valsartan, cefmetazole, cefoperazone, and taurocholic acid was 0.65, 0.67, 0.60,

0.66, 0.57, and 0.63, respectively. All test compounds were less distributed to the blood cells.

Comparison of biliary excretion between in vitro and in vivo. The parameters of in vitro and in vivo biliary excretion of test compounds were compared by several approaches. The following in vivo parameters of taurocholic acid were obtained from the literature: biliary excretion ratio, biliary clearance, and plasma unbound fraction were 100 %, 29.8 mL/min/kg, and 0.15, respectively (Inoue et al., 1985). Based on these parameters, the in vivo intrinsic biliary clearance calculated from Eq.5a and Eq.5b were 675.7, 160.9 mL/min/kg, respectively. Figure 5A shows a comparison of the in vivo biliary excretion ratio and the BEI obtained from in vitro study. No clear correlation was observed between these parameters ($r^2 = 0.638$). Furthermore, the in vitro intrinsic biliary clearances of test compounds were compared with their corresponding in vivo clearances calculated based on plasma total or unbound concentration (Figure 5B, 5C). There was poor in vitro-in vivo correlation ($r^2 = 0.217$) in the case that the in vivo intrinsic biliary clearances were calculated based on plasma total concentration (CL_{bile,int'}, Figure 5B). In contrast, the in vitro intrinsic biliary clearances were well correlated ($r^2 = 0.984$) when the in vivo intrinsic biliary clearances were calculated based on plasma unbound concentration (CL_{bile,int}, Figure 5C).

Discussion

In the present study, the *in vitro* biliary excretion was evaluated using SCRH to predict biliary excretion *in vivo*. Firstly, it was investigated whether SCRH prepared in our study was capable of investigating *in vitro* biliary excretion or not. The phase-contrast microscopy study revealed that the SCRH exhibited liver-like cell morphology and the formation of bile canaliculi (Figure 1, left). When the transporters are expressed and tight junctions are formed adequately, the accumulation of substrates of hepatic efflux transporters can be detected in the bile canalicular lumen, however, the accumulation of substrates in the bile canalicular lumen is not observed under the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free condition, because the integrity of tight junction is $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent. As an example, the fluorescent Mrp2 substrate, CDF, was accumulated in bile canalicular lumen under the standard condition (Figure 1, middle). However, the accumulation of fluorescence was not observed under the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free condition (Figure 1, right). These results demonstrated that our SCRH reestablished bile canaliculi and intact tight junctions, and that it maintained the functional activity of Mrp2.

Polarized excretion by canalicular membrane transporters is essential for biliary excretion of compounds. Bsep, Mrp2, Bcrp, and Mdr1 are dominant transporters responsible for biliary excretion of endogenous and exogenous substances (Chandra and Brouwer, 2004). As shown in Figure 2 and Table 1, taurocholic acid, estradiol-17 β -D-glucuronide, estrone 3-sulfate and digoxin, which are substrates for Bsep, Mrp2, Bcrp, and Mdr1, respectively, were excreted into bile canalicular lumen in SCRH. These observations indicated that our SCRH expressed the functional canalicular transporters.

Next, SCRH were applied for the in vitro biliary excretions of unlabeled compounds. The biliary excretion of pravastatin, rosuvastatin, valsartan, and cefoperazone was clearly observed in SCRH, while it was negligible in case of cefmetazole (Figure 3). The following three parameters were determined in the present study using SCRH: 1) intrinsic uptake clearance for sinusoidal uptake from incubation medium into the cells, 2) BEI for translocation from hepatic intracellular space to bile canaliculus lumen, and 3) intrinsic biliary clearance for net biliary excretion from incubation medium to bile canaliculus lumen (Table 2). Rosuvastatin showed high biliary excretion, sinusoidal uptake and canaliculus efflux. Pravastatin and valsartan exhibited the intermediate biliary excretion, sinusoidal uptake and canaliculus efflux. The higher uptake of rosuvastatin compared with pravastatin is consistent with the results of previous report (Nezasa et al., 2003). Biliary excretions of cefmetazole and cefoperazone were low compared with those of rosuvastatin, pravastatin and valsartan as were their sinusoidal uptake. While the sinusoidal uptake of cefoperazone into hepatocytes was lower than that of cefmetazole, the canaliculus efflux of cefoperazone was fairly higher than that of cefmetazole as reported previously (Nakakariya et al., 2007; Kato et al., 2008). Consequently, net biliary excretion of cefoperazone was higher than that of cefmetazole. These results suggest that the canaliculus membrane transport as well as sinusoidal uptake greatly affects the intrinsic biliary clearance of drugs.

It is important to note that the actual intrinsic biliary clearance should be determined by the transport across the canaliculus membrane based on the intracellular unbound concentration of compounds. However, it is practically impossible to determine experimentally the intracellular unbound concentration of

compounds in both in vitro and in vivo studies, because many compounds could bind to various intracellular components such as proteins, lipids, DNAs, and others. Accordingly, in vitro biliary intrinsic clearance was calculated based on the unbound concentration in incubation medium instead of the intracellular unbound concentration. Similarly, in vivo biliary intrinsic clearance was calculated based on the plasma unbound concentration. Therefore, the in vitro and in vivo intrinsic biliary clearance in this study should be considered as an 'apparent' intrinsic biliary clearance.

As shown in Figure 4 and Table 3, pravastatin, rosuvastatin, valsartan, cefmetazole, and cefoperazone were mainly excreted into bile with comparable values of their biliary excretion ratios that were ranging from 62.7 to 86.3 %. In contrast, their biliary intrinsic clearances showed more variability ranging from 10.5 to 1787.2 mL/min/kg. Even though biliary intrinsic clearance was low, compounds could exhibit high biliary excretion ratios as observed in cefmetazole and cefoperazone. Thus, biliary excretion ratio was determined not only by biliary intrinsic clearance but also by the other factors, such as plasma protein binding, hepatic blood flow, metabolic and renal clearances.

In the present study, the in vitro and in vivo biliary excretions were compared by several approaches. When the in vivo biliary excretion ratio was compared with BEI, there was no good relationship between them (Figure 5A). As described previously, BEI reflected the translocation of compound from intracellular space to canalicular lumen across canalicular membrane. At present, it is difficult to predict the in vivo biliary excretion ratio directly from the parameters of SCR_H. Subsequently, we also compared the in vitro intrinsic biliary clearance with that of in vivo. The in vitro intrinsic biliary clearances of test compounds

were well correlated with their corresponding in vivo clearances when the in vivo clearances were calculated based on plasma unbound concentrations ($r^2 = 0.984$), while less correlation were observed when they were calculated based on plasma total concentrations ($r^2 = 0.217$). It has been well recognized that only protein-unbound substrates but not protein-bound ones are transported and metabolized (Obach, 1999; Takahashi, 1999; Takeda et al., 2002). The in vitro biliary excretion was determined under protein-free conditions in the present SCRH experiments. Therefore, in vitro results should be compared with in vivo biliary excretion profiles based on plasma unbound fraction. These results indicated that it was essential for a good in vitro-in vivo correlation to consider the plasma unbound fraction, and that the in vitro intrinsic biliary clearance was the best parameter for the prediction of in vivo biliary excretion profile.

Our preliminary study using rat Bcrp-expressing membrane vesicles, it was shown that rosuvastatin could be a substrate of rat Bcrp (unpublished observation). This is the first report which successfully demonstrated the good in vitro-in vivo correlation of intrinsic biliary clearance using a Bcrp substrate. Our SCRH system turned out to be applicable to a diverse range of compounds in drug discovery and development stages.

In order to apply the SCRH assay system to drug discovery, we made some improvement in the present study. Instead of 60-mm plastic culture dishes with a thick gelled collagen substratum and an overlaid thick gelled collagen, we used 24-well plates with a rigid collagen substratum (collagen-coated plate) and an overlaid thin collagen layer according to previous report (Sidhu et al., 1993). The application of 24-well plates reduced the amount of hepatocytes and test compounds. In addition, it was easier to keep the

configuration of the collagen layer in a thin form than in a thick one. Additionally, LC-MS/MS enabled us to quantify the uptake amount of unlabeled compounds at low nanomolar concentrations and to carry out the assay with acceptable throughput. The present modified experimental methods of SCRH help us apply the in vitro system to an early drug discovery stage which requires high-throughput and generality.

In a drug discovery and development stage, it is also important to elucidate whether the elimination pathway of NCEs is single or multiple. It is undesirable that NCEs have a single elimination pathway from the viewpoint of the alteration of elimination clearance caused by drug-drug interactions and/or genetic polymorphisms on drug transporters and/or metabolizing enzymes. By comparing the present prediction of in vivo biliary clearance with in vivo metabolic clearance predicted from conventional experiments, the contribution of biliary excretion to total clearance can be estimated. Therefore, the sandwich culture system is a useful tool for not only the prediction of hepatobiliary disposition of NCEs but also estimating the possible alteration of hepatic clearances.

In summary, the present study demonstrates that SCRH is a useful in vitro model to predict in vivo biliary excretion of a diverse range of compounds including Bcrp substrates. Plasma protein binding has a large impact on in vivo biliary intrinsic clearance, especially for highly bound compounds. Additionally, in order to achieve an accurate prediction, it is necessary to calculate the in vivo intrinsic biliary clearance based on plasma unbound concentration.

Acknowledgments

We gratefully acknowledge Dr. Hisako Fujimura and Naoko Murakami for supporting the isolation of rat hepatocytes and cell culture; Dr. Akira Saito for valuable advice; and Masaya Fukuda and Masao Yamanouchi for their expert technical assistance of in vivo studies.

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Figure legends

Fig. 1. Phase-contrast images (left) of SCRH after 4 days culture. Fluorescence images (middle and right) after 20-min treatment with 10 μ M CDF diacetate incubated in standard (middle) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free (right) buffers.

Fig. 2. Cumulative uptake of radiolabeled substrates of bile canalicular membrane transporters in SCRH. Each substrate (1 μ M) was incubated in standard buffer (closed circles) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer (open circles). Data are shown as the mean \pm S.E.M. (N=6). *, significantly different between standard and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer when assessed by Student's t test ($P < 0.05$).

Fig. 3. Cumulative uptake of unlabeled compounds in SCRH. Each compound was incubated in standard buffer (closed circles) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer (open circles). Data are shown as the mean \pm S.E.M. (N=6). *, significantly different between standard and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer when assessed by Student's t test ($P < 0.05$).

Fig. 4. Plasma concentration-time profile (A) and cumulative biliary excretion (B) of pravastatin (open circle), rosuvastatin (open triangle), valsartan (open square), cefmetazole (closed circle), and cefoperazone (closed triangle) after intravenous administration to rat at the dose of 1 mg/kg. Data are shown as the mean \pm S.E.M. of three animals.

Fig. 5. Comparison of in vitro and in vivo kinetic parameters for biliary excretion. The correlation was assessed between biliary excretion index (BEI) in vitro and in vivo biliary excretion ratio (A), and intrinsic biliary clearances between in vitro and in vivo with pravastatin (open circle), rosuvastatin (open triangle), valsartan (open square), cefmetazole (closed circle), cefoperazone (closed triangle), and taurocholic acid (closed rhombus). In vivo intrinsic biliary clearance was calculated based on plasma total (B) or unbound concentrations (C). Data are shown as the mean \pm S.E.M. (N=6 and 3 for in vitro and vivo experiments, respectively).

Table 1

BEI of radiolabeled substrates in SCRH

Data are taken from Figure 2. All compounds were tested at 1 μ M as described under Materials and Methods. BEI was determined at 10 min. Each value represents the mean \pm S.E.M. (N=6). The suggested major transporters responsible for the hepatobiliary disposition of each substrate were listed in parentheses.

Substrates (influx / efflux transporter)	BEI (%)
Taurocholic acid (Ntcp / Bsep)	76.8 \pm 2.3
Estradiol 17 β -D-glucuronide (Oatps / Mrp2)	28.9 \pm 5.9
Estrone 3-sulfate (Oatps / Bcrp)	18.4 \pm 0.9
Digoxin (Oatps / Mdr1)	49.3 \pm 2.7
Salicylic acid (Oats / -)	4.4 \pm 6.6

Table 2

Kinetic parameters for in vitro biliary excretion of unlabeled compounds in SCRH

Data are taken from Figure 3. All compounds were tested at 1 μ M except for pravastatin of 10 μ M. Intrinsic uptake clearance ($CL_{\text{uptake,int}}$), intrinsic biliary clearance ($CL_{\text{bile,int}}$), and biliary excretion index (BEI) of test compounds were calculated from Eq.1, Eq.2, and Eq.3, respectively, described in Materials and Methods. $CL_{\text{bile,int}}$ and BEI were determined at 10 min. Each value represents the mean \pm S.E.M. (N=6).

	$CL_{\text{uptake,int}}$		$CL_{\text{bile,int}}$		BEI
	$\mu\text{L}/\text{min}/\text{mg Protein}$	$\text{mL}/\text{min}/\text{kg}$	$\mu\text{L}/\text{min}/\text{mg Protein}$	$\text{mL}/\text{min}/\text{kg}$	%
Pravastatin	8.11 ± 0.69	64.9 ± 5.5	1.08 ± 0.48	8.7 ± 3.8	27.1 ± 11.7
Rosuvastatin	62.49 ± 11.03	499.9 ± 88.2	15.56 ± 5.79	124.5 ± 46.3	39.5 ± 11.1
Valsartan	17.85 ± 0.98	142.8 ± 7.8	3.50 ± 1.98	28.0 ± 15.9	15.5 ± 11.2
Cefmetazole	1.50 ± 0.06	12.0 ± 0.5	0.005 ± 0.019	0.04 ± 0.16	0.1 ± 4.5
Cefoperazone	0.39 ± 0.02	3.1 ± 0.1	0.07 ± 0.01	0.5 ± 0.1	30.8 ± 5.7
Taurocholic acid	N.C.	N.C.	6.37 ± 0.81	50.9 ± 6.5	76.8 ± 2.3

N.C., not calculated

Table 3

Pharmacokinetic and biliary excretion parameters after intravenous administration (1 mg/kg) of unlabeled compounds to rat

Data are taken from Figure 4. Kinetic parameters were determined by a model-independent method using the MULTI computer program. Plasma protein binding ratio (fp) was determined by an ultracentrifugation method as described in Materials and Methods. Each value represents the mean \pm S.E.M. of three animals.

	Pravastatin	Rosuvastatin	Valsartan	Cefmetazole	Cefoperazone
Pharmacokinetic Parameters					
C ₀ (μg/mL)	2.9 \pm 0.3	5.3 \pm 1.1	20.2 \pm 5.5	3.4 \pm 0.8	7.5 \pm 1.1
T _{1/2} (min)	13.6 \pm 1.1	36.7 \pm 5.3	39.0 \pm 7.6	41.2 \pm 2.1	30.9 \pm 2.5
AUC ₁₈₀ (μg/mL x min)	22.1 \pm 2.2	36.6 \pm 6.0	258.0 \pm 50.2	97.5 \pm 9.6	84.4 \pm 26.9
Vdss (mL/kg)	421.2 \pm 30.7	353.4 \pm 65.3	99.6 \pm 10.3	440.9 \pm 10.3	236.7 \pm 25.2
CL _{tot} (mL/min/kg)	43.7 \pm 3.9	28.0 \pm 5.5	4.2 \pm 1.0	9.6 \pm 1.0	13.4 \pm 3.3
fp	0.672	0.039	0.004	0.738	0.429
Biliary Excretion Parameters					
Excretion Ratio (% of Dose)	62.7 \pm 9.0	86.3 \pm 7.5	83.2 \pm 4.8	64.7 \pm 5.1	75.0 \pm 14.3
CL _{bile} (mL/min/kg)	27.9 \pm 5.8	24.3 \pm 5.2	3.5 \pm 0.6	6.5 \pm 1.0	11.0 \pm 4.0
CL _{bile,int} (mL/min/kg)	229.6 \pm 153.3	104.8 \pm 52.1	6.3 \pm 1.2	11.8 \pm 2.1	31.2 \pm 13.6
CL _{bile,int} (mL/min/kg)	222.0 \pm 148.3	1787.2 \pm 888.2	865.5 \pm 165.7	10.5 \pm 1.9	41.5 \pm 18.0

Figure 1

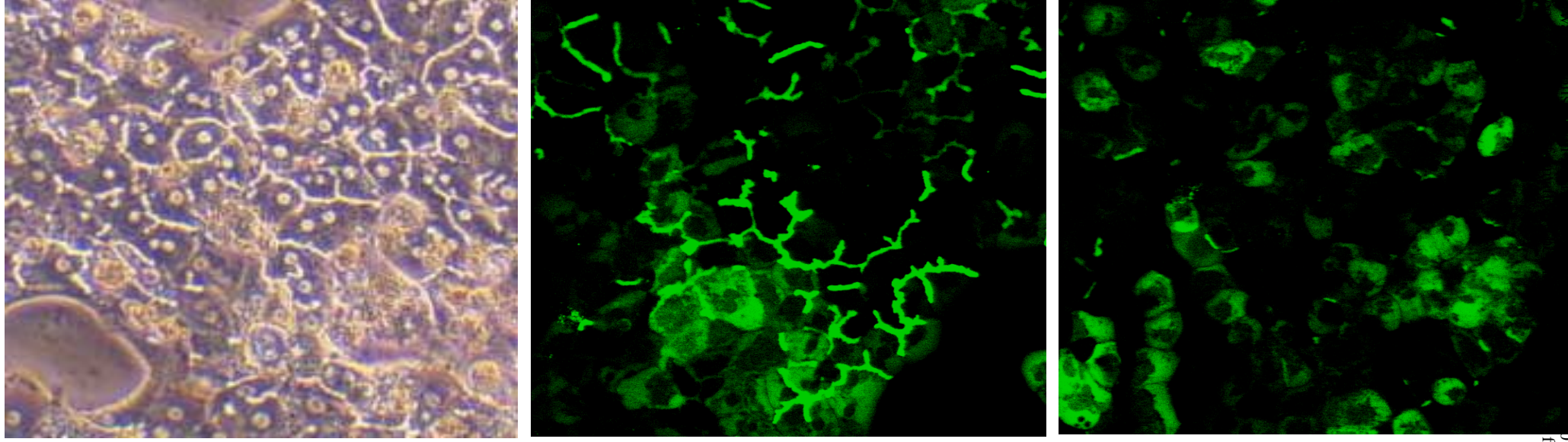


Figure 2

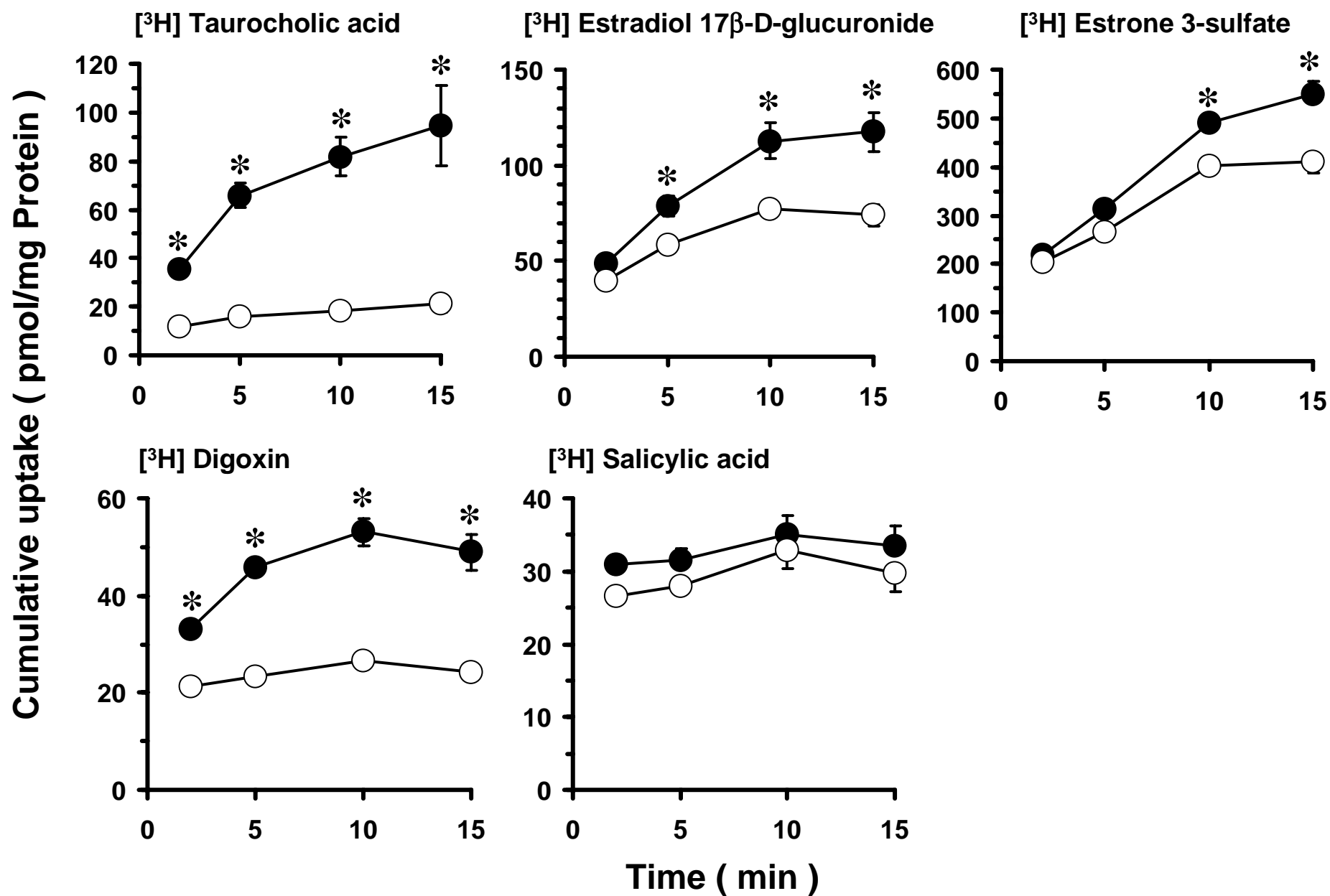


Figure 3

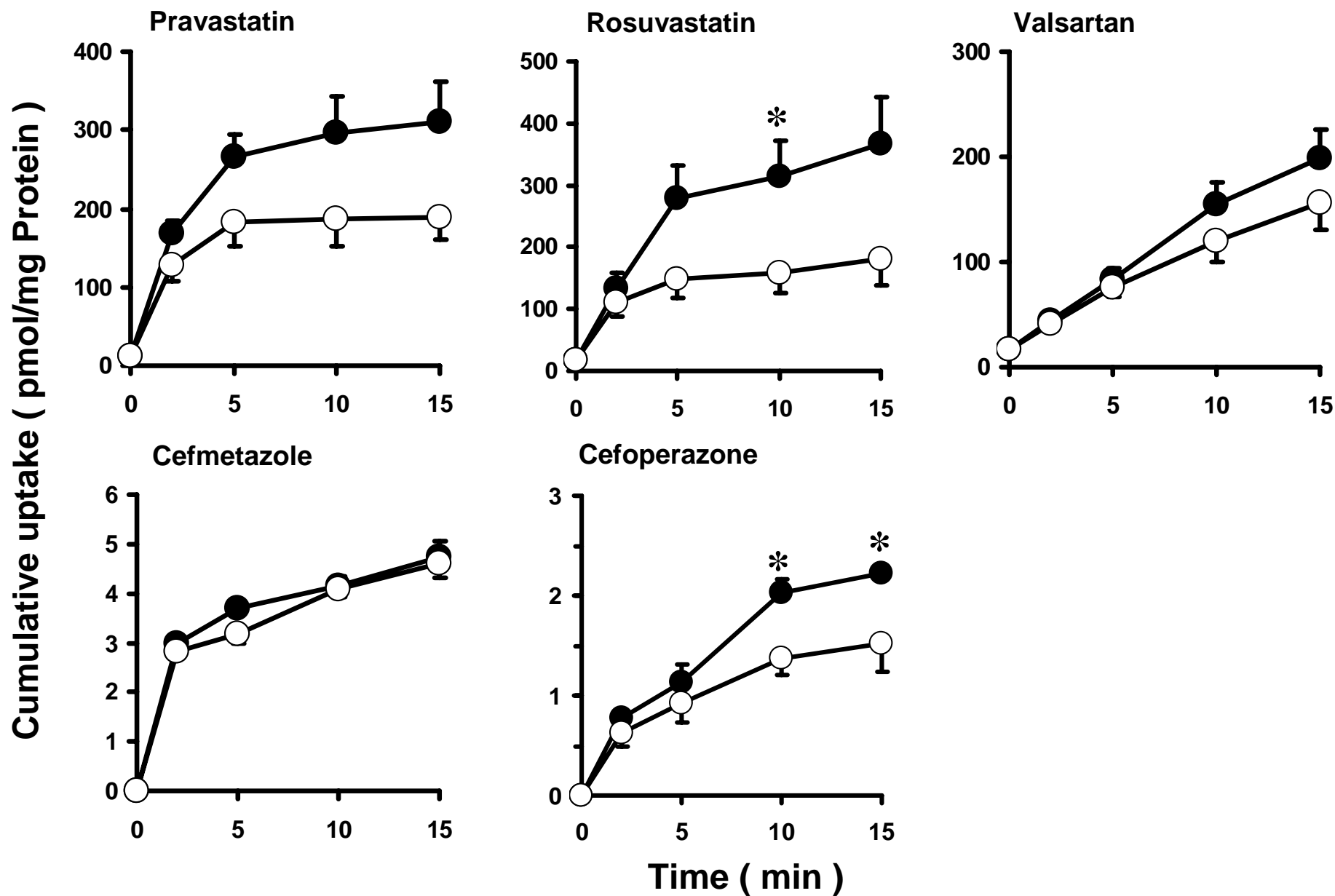


Figure 4

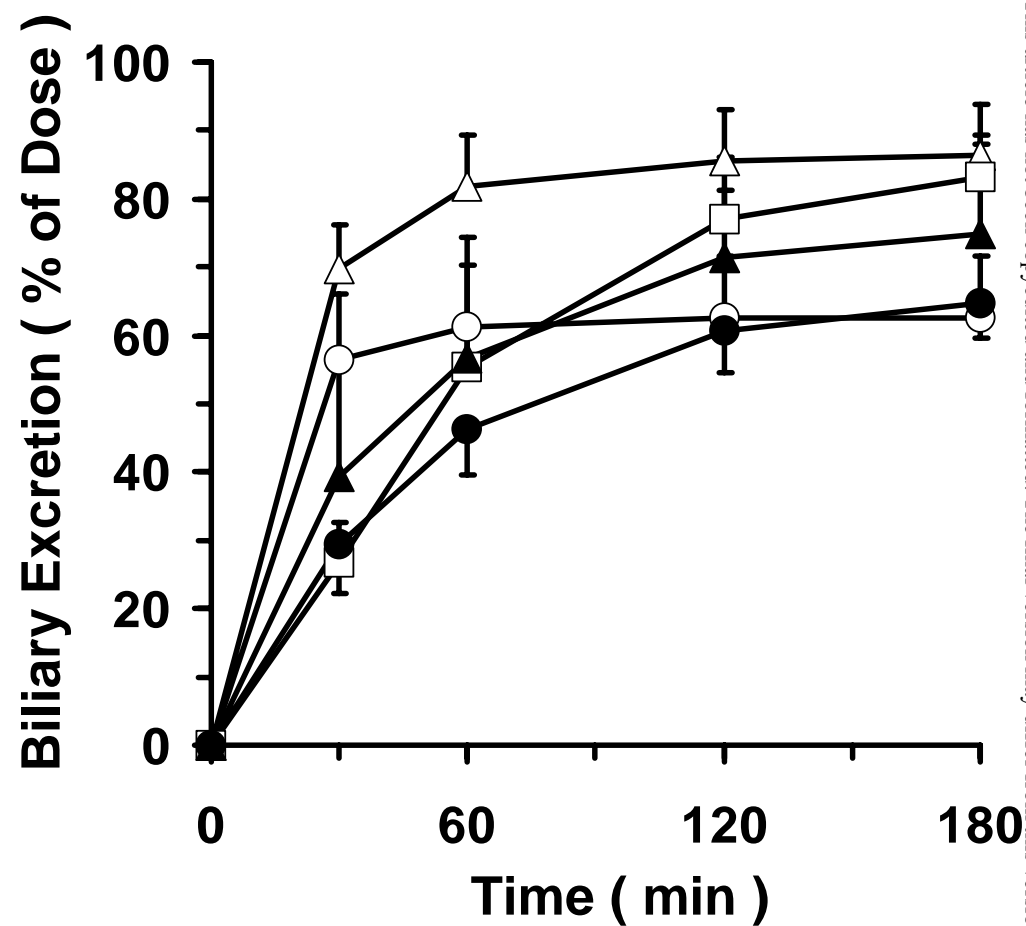
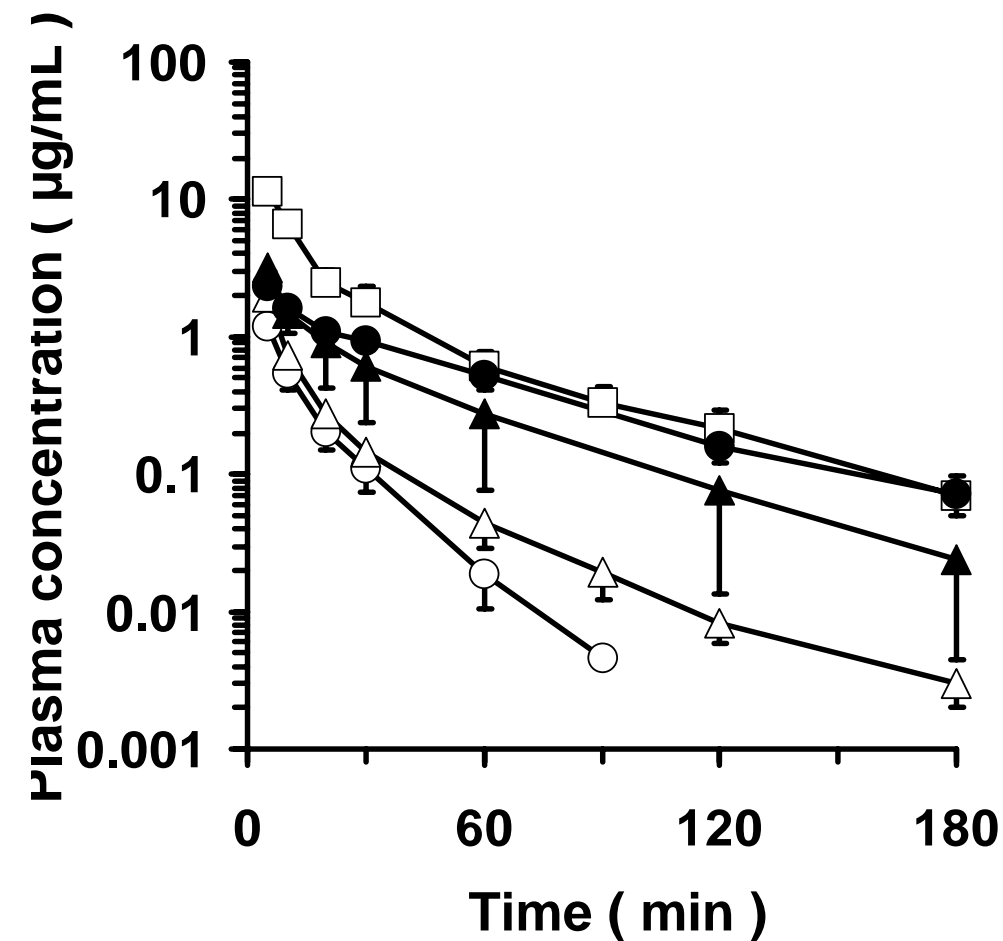


Figure 5

