Evaluation of hepatic disposition of paroxetine using sandwich-cultured rat and human hepatocytes

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Hepatic disposition of paroxetine using SCH

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Abbreviations: CYP, cytochrome P450; COMT, catechol-O-methyltransferase; SCH, sandwich-cultured hepatocytes; ABC, ATP-binding cassette; BEI, biliary excretion index; UGT, UDP-glucuronosyltransferases; SULT, sulfotransferases; MRP2/Mrp2, multidrug resistance-associated protein 2; BCRP/Bcrp, breast cancer resistance protein; BSEP/Bsep, bile salt export pump
Abstract
Paroxetine, a selective serotonin reuptake inhibitor, is metabolized in the liver and excreted into bile and urine as metabolites, but species differences have been observed in hepatic disposition between rats and humans. A major metabolite in rats is M1-glucuronide, whereas M1-glucuronide and M1-sulfate are found in humans. The primary excretion route of paroxetine-derived radioactivity in rats and humans is bile and urine, respectively. The aim of this study was to examine the usefulness of sandwich-cultured hepatocytes (SCH) to evaluate in vivo species differences of the hepatic disposition of paroxetine between rats and humans. The metabolite profile of [3H]paroxetine in SCH was similar to that in hepatocytes in suspension, and the in vitro metabolite profiles were similar to the published in vivo metabolic pathways for both species. Furthermore, the biliary excretion index (BEI) of formed M1-glucuronide in rat SCH (25.8% to 50.9%) was higher than that in human SCH (15.1% to 16.7%). The BEI of formed M1-sulfate (16.4% to 29.1%) was comparable to that of M1-glucuronide in human SCH, while the BEIs of paroxetine were negligible in SCH of both species. Moreover, M1-glucuronide was demonstrated to be an MRP2/Mrp2 substrate in both species as determined by its uptake into ATP-binding cassette (ABC) transporter-expressing membrane vesicles. SCH should prove useful to evaluate the processes of hepatic uptake and metabolism of parent drugs as well as the simultaneous examination of the biliary excretion of both parent drug and liver derived metabolites.
Introduction

Paroxetine is a selective serotonin reuptake inhibitor widely used for the treatment of major depressive disorder, panic disorder, obsessive-compulsive disorder, social anxiety disorder, post-traumatic stress disorder, and generalized anxiety disorder (Marks et al., 2008). Figure 1 shows the metabolic pathway of paroxetine. Paroxetine is initially oxidized by cytochrome P450 (CYP) to an unstable catechol intermediate, and the intermediate is promptly methylated by catechol-O-methyltransferase (COMT) to M1 and M2. M1 and M2 are further conjugated by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) to glucuronide and sulfate, respectively, and eliminated from the body (Haddock et al., 1989; Kaye et al., 1989; Jornil et al., 2010). In rats, a major metabolite of paroxetine is M1-glucuronide as shown by radiolabeled studies and the radioactivity is mainly excreted into bile, up to approximately 75% of the dose after oral administration (Haddock et al., 1989). The major metabolites in humans are M1-sulfate as well as M1-glucuronide and the radioactivity after oral administration of radiolabeled paroxetine is mainly excreted into urine, up to approximately 60% of the dose (Haddock et al., 1989; Kaye et al., 1989). Thus, there are species differences in the hepatic disposition of paroxetine between rats and humans.

Liver is a key organ that functions to eliminate endogenous and exogenous compounds including drugs. Hepatic intrinsic clearance consists of four factors: basolateral uptake, basolateral efflux, intracellular metabolism, and biliary excretion. Metabolism is conventionally studied using liver microsomal fractions. The wide-spread use of liver microsomal fractions is due to their ease of preparation and hence, they are a powerful tool to predict metabolic intrinsic clearance (Obach, 1999; Naritomi et al., 2001). However, the use of hepatocytes is gaining more attention because of their increased availability and quality as a result of the improved isolation techniques. Furthermore, metabolic stability and pathway studies with hepatocytes have been recognized as useful methods for the estimation of metabolic clearance (Lau et al., 2002, Chiba et al., 2009). In addition, hepatic uptake and biliary excretion studies have generally been conducted with hepatocytes (Hoffmaster et al., 2005; Paine et al., 2008; Li et al., 2010). Therefore, hepatocytes are ideal models for estimating hepatic disposition.
Sandwich-cultured hepatocytes (SCH) that are cultivated between two layers of gelled collagen maintain polarity, morphology, and liver specific metabolic activities (Kern et al., 1997; Slaus et al., 2001; Tuschl et al., 2009). In addition, SCH develop functional canalicular networks sealed by tight junctions, and hepatic transporter proteins are expressed and localized to the correct membrane domains allowing for assessment of vectorial transport function (LeCluyse et al., 1994). These unique features make SCH useful for the prediction of biliary intrinsic clearance, drug-drug interactions, and drug-induced hepatotoxicity (Liu et al., 1999a; Marion et al., 2007; Fukuda et al., 2008; Li et al., 2010; Nakanishi et al., 2011). The use of cultured cells which only express a single ATP-binding cassette (ABC) transporter in a transcellular transport study may lead to results that are not representative of in vivo conditions. Since hydrophilic metabolites often have less membrane permeability than their lipophilic parent compound (Pang et al., 1984; de Lannoy and Pang, 1986), the use of polarized cultured cells such as MDCK cells that co-express uptake and efflux transporters has increased in studies to evaluate hepatic disposition (Ishiguro et al., 2008). However, the hepatobiliary disposition of a metabolite should be ideally assessed based on the metabolite formed in cells after adding parent compound rather than from the results of the administered preformed metabolite. For this reason, SCH are superior to double-transfected cells since they maintain intracellular metabolic activity. Indeed, some researchers have previously conducted metabolic/hepatobiliary transport studies that have proven useful to reveal the hepatic disposition of various compounds such as bilirubin, terfenadine, troglitazone, pafuramidine, and CPD-0868 (Lengyel et al., 2005; Turncliff et al, 2006; Lee et al., 2010; Yan et al., 2011). Moreover, the FDA guidance for the safety testing of drug metabolites (MIST) and the ICH-M3 (R3) have had a significant impact on drug discovery and development, and further evaluation of circulating metabolite(s) is needed in both nonclinical species and humans. In the present study, we studied species differences in the hepatic disposition of paroxetine between rats and humans using a SCH system.
Materials and Methods

Chemicals

\[^{3}\text{H}]\text{Paroxetine (22.9 Ci/mmol; greater than 97\%)}\] was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). \[^{3}\text{H}]\text{M1-glucuronide and }[^{3}\text{H}]\text{M1-sulfate were enzymatically synthesized using rat liver S9 and human liver cytosolic fractions. The purities of }[^{3}\text{H}]\text{M1-glucuronide and }[^{3}\text{H}]\text{M1-sulfate were greater than 95\%. Paroxetine maleate, S-(5'-adenosyl)-L-methionine (SAM), }\beta\text{-nicotinamide adenine dinucleotide phosphate reduced form (}\beta\text{-NADPH), uridine 5’-diphosphoglucuronic acid (UDPGA), and adenosine 3’-phosphate 5’-phosphosulfate (PAPS) were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved hepatocyte recovery medium (CHRM\textsuperscript{®}), cryopreserved hepatocyte plating medium (CHPM\textsuperscript{®}), hepatocyte maintenance supplement pack, and Geltrex\textsuperscript{™} were purchased from CellzDirect (Durham, NC) and Williams’ media E (WME) without phenol red and Hanks’ balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Sprague-Dawley (SD) rat liver S9 (pool of 400 animals, male) and human liver cytosolic fractions (pool of 50 donors, mixed gender) were purchased from XENOTECH LLC (Lenexa, KS). MRP2/Mrp2-, BCRP/Bcrp-, and BSEP/Bsep-expressing Sf9 membrane vesicles were purchased from Genomembrane Inc. (Yokohama, Japan). All other chemicals and reagents were of higher grade such as special grade or HPLC grade.

Hepatocytes

Cryopreserved SD rat and human hepatocytes were purchased from CellzDirect and Celsis In Vitro Technologies (Baltimore, MD), respectively. Characteristics of the hepatocyte lots are shown in Table 1.

Hepatocytes Sandwich Culture

Cryopreserved rat or human hepatocytes were thawed based on CellzDirect’s standard methods: hepatocytes were thawed at 37°C and decanted into CHRM\textsuperscript{®}. The hepatocytes were then centrifuged at 60g for 10 min and the supernatant was discarded. After the hepatocytes were resuspended to
4 × 10^5 viable cells/ml (rat) or 7 × 10^5 viable cells/ml (human) in CHPM®, the hepatocytes suspension were added to a collagen I-coated BioCoat™ 24-well plates (BD Biosciences, Bedford, MA) in a volume of 0.5 ml/well. After 4 h of static incubation under an atmosphere of 5% CO₂ in air at 37°C, the medium was replaced with Geltrex™ (350 μg/mL) containing WME with hepatocyte maintenance supplement. Cultures were maintained in Geltrex™-free WME with hepatocyte maintenance supplement, which was replaced every 24 h.

**Metabolite Profile of Paroxetine**

For incubation with hepatocytes in suspension, cryopreserved rat or human hepatocytes were thawed and resuspended to 6 or 10 × 10^5 viable cells/ml in WME, respectively. Two hundred and fifty microliters of the hepatocyte suspension were added into a non-coated 24-well plates, in which 250 μl of 2 μM [³H]paroxetine in WME was added in advance, and incubated statically at 37°C in a humidified chamber in a 95%/5% air/CO₂ atmosphere for 0, 0.25, 0.5, 1, 2, and 3 h. For incubation with SCH, day 4 hepatocytes were incubated statically with 0.5 ml of 1 μM [³H]paroxetine in WME in the incubator (37°C, 5% CO₂) for 0, 0.25, 0.5, 1, 3, 6, and 24 h. Each incubation was terminated by adding 0.5 ml of pretreatment solvent (acetonitrile/ethanol/methanol mixture, 1:1:1, v/v/v) and the mixture was collected in a glass tube. Each well was washed with the same pretreatment solvent twice and the wash was combined with the mixture. The resultant solution was evaporated to dryness under reduced pressure, and the residue was dissolved in a 10 mM ammonium acetate (pH 4.0)/acetonitrile mixture (9:1, v/v) and injected into a radio-HPLC.

**Accumulation of Paroxetine and Metabolites in SCH**

Modulation of Ca^{2+} in the incubation buffer was used to manipulate the tight junctions sealing the canalicular networks between cells. In Ca^{2+}-containing standard buffer (HBSS), tight junction integrity is preserved and substrates are excreted into the bile canaliculi, whereas incubation of cells in Ca^{2+}-free buffer (HBSS modified) disrupts the tight junctions, opens the canalicular spaces, and canalicular contents are released into the incubation medium (Lee et al., 2010).
Metabolic/hepatobiliary transport studies with SCH were conducted according to the method described previously with minor modifications (Lee et al., 2010). Briefly, rat or human SCH were incubated statically under an atmosphere of 5% CO₂ in air at 37°C for 0.25, 0.5, 1, 4, and 24 h with [³H]paroxetine at the initial concentration of 1 μM in WME. At each time point, the medium was removed and cells were rinsed twice and incubated statically for 5 min in the incubator (37°C, 5% CO₂), with Ca²⁺-containing buffer (HBSS(Ca/Mg⁺) containing 20 mM HEPES) or Ca²⁺-free buffer (HBSS(Ca/Mg⁻) containing 20 mM HEPES and 1 mM EGTA). The buffer was removed and cells were washed three times with the same ice-cooled buffer. Cells were lysed with 0.5 ml of pretreatment solvent and the lysate was collected in a glass tube. Each well was washed with the same pretreatment solvent twice and the wash was combined with the lysate. The resultant solution was evaporated to dryness under reduced pressure, and the residue was dissolved in a 10 mM ammonium acetate (pH 4.0)/acetonitrile mixture (9:1, v/v) and injected into a radio-HPLC.

Uptake by Membrane Vesicles

[³H]M1-glucuronide and [³H]M1-sulfate were enzymatically formed from [³H]paroxetine. [³H]M1-glucuronide was obtained from the reaction mixture consisting of 10 μM [³H]paroxetine, 2 mg/ml rat liver S9, cofactors (1 mM NADPH, 1 mM SAM, and 5 mM UDPGA), 25 μg/ml alamethicin, 5 mM MgCl₂, and 100 mM potassium phosphate buffer at pH 7.4. In the case of [³H]M1-sulfate, [³H]M1 was generated from the reaction mixture consisting of 10 μM [³H]paroxetine, 2 mg/ml rat liver S9, cofactors (1 mM NADPH and 1 mM SAM), 5 mM MgCl₂, and 100 mM potassium phosphate buffer at pH 7.4. Then, 10 μM [³H]M1 was incubated with 1 mg/ml human liver cytosol, 0.6 mM PAPS, 5 mM MgCl₂, and 100 mM potassium phosphate buffer at pH 7.4. Then, 10 μM [³H]M1 was incubated with 1 mg/ml human liver cytosol, 0.6 mM PAPS, 5 mM MgCl₂, and 100 mM potassium phosphate buffer at pH 7.4, and [³H]M1-sulfate was obtained. All incubations were done at 37°C over night. [³H]M1-glucuronide, [³H]M1, and [³H]M1-sulfate were separated by HPLC and the eluates were collected at their corresponding retention times (M1-glucuronide, 15.7 min; M1, 23.2 min; M1-sulfate, 21.7 min). Each compound was further purified by solid-phase extraction using OASIS® HLB (Waters, 6 cc, 500 mg).
Vesicular transport studies were performed by a rapid filtration technique according to the manufacturer’s protocol. Briefly, MRP2/Mrp2-expressing Sf9 membrane vesicles (1 mg/ml) were incubated with 0.5 μM [3H]M1-glucuronide or [3H]M1-sulfate at 37°C in the presence of 2 mM glutathione and co-factor (4 mM ATP or AMP) in transport buffer consisting of 70 mM KCl, 7.5 mM MgCl₂ and 50 mM MOPS-Tris (pH 7.0). In the case of BCRP/Bcrp, the composition of reaction mixture was the same as that for MRP2/Mrp2 except for the absence of glutathione. In the case of BSEP/Bsep, 0.5 μM [3H]M1-glucuronide or [3H]M1-sulfate was incubated with membrane vesicles (1 mg/ml) at 37°C in the presence of 4 mM ATP or AMP in transport buffer consisting of 100 mM KNO₃, 10 mM Mg(NO₃)₂, 50 mM sucrose, and 10 mM HEPES-Tris (pH 7.4). An aliquot of the reaction mixture was collected and added into 5-times volume of ice-cold stop buffer consisting of 70 mM KCl and 40 mM MOPS-Tris (pH 7.0) for MRP2/Mrp2 and BCRP/Bcrp, or 100 mM KNO₃, 50 mM sucrose, and 10 mM HEPES-Tris (pH 7.4) for BSEP/Bsep to terminate the reaction at 10, 30, 60, and 120 s. The terminated reaction mixture was passed through a presoaked 0.45-μm pore size membrane filter (Millipore, Bradford, MA), and then washed five times with 0.35 ml of ice-cold stop buffer. The amount of radioactivity retained on the filter was measured using a liquid scintillation counting TRI-CARB 2900TR (PerkinElmer Life and Analytical Sciences).

Sample Analysis

An Agilent 1100 HPLC system equipped with a binary pump, column oven, and auto sampler (Agilent Technologies, La Jolla, CA) was connected to a flow scintillation analyzer 625TR (PerkinElmer Life and Analytical Sciences) and was used for radio-HPLC analysis. A SunFire™-C₁₈ 5 μm, 4.6 × 250 mm (Waters, Milford, MA), kept at 40°C, was used as the analytical column. A gradient with mobile phase A (10 mM ammonium acetate, pH 4.0) and mobile phase B (100% acetonitrile) was used at a flow rate of 1.0 ml/min. The initial composition of mobile phase B was maintained at 5% for 5 min and then increased in a linear manner as follows: 20% at 10 min, 25% at 20 min, 35% at 35 min, 50% at 55 min, 70% at 65 min, 100% at 65.1 min. It was then maintained at 100% for up to 70 min. The column was allowed to equilibrate at 5% mobile phase B for 7 min before the next
injection. Mass spectrometric analyses were performed on a QTRAP ion trap mass spectrometer (AB Sciex, Foster City, CA), which was interfaced to an Agilent HPLC system and equipped with an electrospray ionization source. Eluate from the HPLC column split at a ratio of 1:4 using a splitter was delivered to a MS detector at a flow rate of approximately 0.2 mL/min. The parameters for electrospray ionization were as follows: curtain gas, 40 psi; ionspray voltage, 5.5 kV; temperature, 550°C; ion source gas 1, 50 psi; ion source gas 2, 80 psi; declustering potential, 30 V; entrance potential, 10 V; and collision energy, 30 V. The mass spectrometer was operated in a positive ion mode. For the MS² scan, products of M1-glucuronide and M1-sulfate were set at m/z 508.2 and m/z 412.2 and the ions were monitored over the full mass ranges of m/z 100 – 550, and m/z 100 – 450, respectively. For the MS³ scan, transitions of M1-glucuronide and M1-sulfate were set at m/z 508.2 → 332.2 and m/z 412.2 → 332.2, respectively, and the ions were monitored over a full mass range of m/z 100 – 400. ChemStation LC 3D system Rev.A.10.01J software (Agilent Technologies) and FLO-ONE for Windows Version 3.65 software (PerkinElmer Life and Analytical Sciences) were used to control the radio-HPLC system and perform sample data analyses. Analyst 1.4.1 software (AB Sciex) was used to control the LC/MS system and perform sample data analyses.

Data Analysis

B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) was used to calculate the biliary excretion index (BEI), which represents the percentage of accumulated substrate that is excreted into bile canaliculi, using the following equation (Liu et al., 1999b):

\[
\text{BEI}(\%) = \frac{\text{Accumulation}_{\text{cells+bile}} - \text{Accumulation}_{\text{cells}}}{\text{Accumulation}_{\text{cells+bile}}} \times 100
\]

Statistical Analysis

For data obtained from metabolic/hepatobiliary transport studies, a paired t-test was performed for the accumulations of paroxetine, M1-glucuronide, and M1-sulfate between Ca/Mg(+)-treatment and
Ca/Mg(-)-treatment at each time point. For data obtained from vesicular transport studies, 2-sample t-test was performed for the uptakes of MPAG between ATP and AMP added. Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC), and a value of $p < 0.05$ was predetermined as the criterion for significance in each analysis.
Results

Comparison of metabolite profile between hepatocytes in suspension and SCH

The LC/MS analysis confirmed that the metabolite peaks around 15.7 min and 21.7 min in the radio-HPLC chromatogram were M1-glucuronide and M1-sulfate, respectively (Fig. 2). When 1 μM [3H]paroxetine was incubated with rat hepatocytes in suspension or SCH, M1-glucuronide was the main metabolite in the incubation mixture (Fig. 3). A time-dependent decrease of the parent form of paroxetine and an increase of metabolites were observed (Fig. 4A and Fig. 4C). The percent of paroxetine remaining after a 3-hour incubation in suspension was 29.7% and after a 24-hour incubation in SCH it was 36.6%. The percent of M1-glucuronide and M1-sulfate produced after a 3-hour incubation in suspension were 50.7% and 1.0%, and those after a 24-hour incubation in SCH were 34.5% and 0.8%, respectively. The percent of M1-sulfate and other metabolites formed were significantly lower than that of M1-glucuronide in rat hepatocytes and were essentially negligible. The total recovered amounts of paroxetine, M1-glucuronide, and M1-sulfate were more than 81.4% and 71.9% of the dose in the incubation mixture of rat suspended hepatocytes and SCH.

When 1 μM [3H]paroxetine was incubated with human hepatocytes in suspension or SCH, M1-glucuronide and M1-sulfate were produced to a similar extent and they were the main metabolites in the incubation mixture (Fig. 4B, Fig. 4D, and Fig. 5). The percent of paroxetine remaining and M1-glucuronide and M1-sulfate produced after a 3-hour incubation used in suspension were 71.4%, 8.1%, and 7.9%, respectively, and those after a 24-hour incubation in SCH were 52.8%, 12.5%, and 13.1%, respectively. The total recovered amounts of paroxetine, M1-glucuronide, and M1-sulfate were more than 87.4% and 78.4% of the dose in the incubation mixture of human suspended hepatocytes and SCH.

Hepatobiliary disposition of paroxetine and its metabolites in SCH

When 1 μM [3H]paroxetine was incubated with rat SCH, accumulation of M1-glucuronide in cells (+ bile) declined after peaking at 30-min incubation (Fig. 6C). The BEI of M1-glucuronide at 15- and 30-min incubation was 50.9% and 25.8%, respectively, and it also declined after peaking at around
30-min incubation. The accumulation of M1-glucuronide in cells + bile (treated with Ca\(^{2+}\)-containing buffer) at 15-min incubation was not significantly higher than that in cells (treated with Ca\(^{2+}\)-free buffer), whereas the p-value of 0.107 was close to the significance. Since the amount of M1-sulfate formed in rat hepatocytes was quite small, the accumulation and BEI of M1-sulfate could not be calculated. On the other hand, accumulation of paroxetine, which was assumed not to be excreted into bile, reached the highest value between 1- to 4-hours of incubation. Although the accumulation of paroxetine in cells + bile was significantly higher than that in cells at 30-min incubation, there were no significant differences of the accumulations between cells + bile and cells at the other time point (Fig. 6A). The BEI of paroxetine in rat SCH was negligible.

When 1 \(\mu\)M \(^{3}\)Hparoxetine was incubated with human SCH, accumulation of M1-glucuronide reached a peak at 30-min incubation and then declined (Fig. 6D). The BEI of M1-glucuronide at 15- and 30-min incubation was 15.1% and 16.7%, respectively, and also declined after peaking at around 30-min incubation. The accumulations of M1-glucuronide in cells + bile were significantly higher than those in cells at 30-min and 1-h incubation. Accumulation of M1-sulfate reached a peak between 15- to 60-min incubation (Fig. 6E) and the BEI of M1-sulfate at 15-, 30-, and 60-min incubation was 19.0%, 16.4% and 29.1%, respectively. The BEI of M1-glucuronide was comparable to that of M1-sulfate in human SCH. The accumulation of M1-sulfate in cells + bile was significantly higher than that in cells at 30-min incubation. Accumulation of paroxetine in cells + bile was similar to that in cells (Fig. 6B), and the accumulation of paroxetine in cells + bile was significantly higher than that in cells at 30-min incubation. The BEI of paroxetine in human SCH was negligible. The BEIs of paroxetine, M1-glucuronide, and M1-sulfate at designated time points in both species are summarized in Table 2.

**Uptake into ABC transporter-expressing vesicles**

Since the results of hepatobiliary dispositions indicated that M1-glucuronide and/or M1-sulfate are likely to be substrates for canalicular efflux transporters, vesicular transport studies were performed to evaluate the involvement of ABC transporters. M1-sulfate was not detectable and thus its BEI was
unknown in rat SCH, however, the transport of M1-sulfate was also evaluated using rat ABC transporter-expressing membrane vesicles. A significant ATP-dependent uptake of M1-glucuronide into both rat Mrp2 and human MRP2 membrane vesicles was observed (Fig. 7A and Fig. 7B). At 1 min-incubation, a significant uptake of M1-G in both rat Bcrp and human BCRP was found in the presence of ATP, whereas it was not time-dependent (Fig. 7C and Fig. 7D). Moreover, no differences were observed in the uptake of M1-glucuronide into BSEP/Bsep-expressing vesicles regardless of the presence or absence of ATP (Fig. 7E and Fig. 7F). For M1-sulfate, an obvious involvement of the transporters was not observed in any of MRP2/Mrp2, BCRP/Bcrp, and BSEP/Bsep-expressing membrane vesicles (Fig. 8).
Discussion

According to the MIST guidance and the ICH-M3 (R2), the disposition of both the metabolite(s) and the parent drug need to be defined since the systemic exposure of metabolite(s) in non-clinical animal species has to be compared with that in humans when the metabolite(s) is observed at exposures greater than 10% of parent drug (MIST) or total drug-related exposure (ICH) in humans. Accordingly, in terms of drug development, it is necessary to develop a method to identify the major metabolite(s) and their disposition in non-clinical animal species and humans. In the present study, we evaluated the usefulness of SCH to mimic the hepatic disposition of drugs in animals and humans, especially in terms of predicting the fate of metabolites formed in hepatocytes.

Paroxetine is initially oxidized by CYP, promptly methylated by COMT, and further conjugated by UGT and SULT, indicating the involvement of multiple enzymes in its metabolism. Since UGTs are localized in the endoplasmic reticulum as well as CYPs but COMTs and SULTs are intracellular enzymes mainly located in cytosolic fractions (Jancova et al., 2010), hepatocytes are likely to be ideal enzyme sources for the evaluation of the metabolism of paroxetine. Indeed, regardless of the incubation methods, suspension or SCH, M1-glucuronide was the main metabolite from the incubation with rat hepatocytes (Fig. 3) and M1-glucuronide and M1-sulfate were those with human (Fig. 5). Additionally, the BEI of M1-glucuronide in rat SCH was higher than that in human SCH (Table 2). These results corresponded with the published in vivo disposition (Haddock et al., 1989; Kaye et al., 1989). Further studies on evaluation of down-regulation rates between Phase I and Phase II enzymes are required so as to evaluate the usefulness of SCH for quantitative assessment of the in vivo metabolite pathway.

This is the first report to compare multiple-enzyme metabolism in SCH with that in suspension and the in vivo metabolic pathway. Other researchers have compared individual metabolic enzyme activity between the two methods independently (Kern et al., 1997; Slaus et al., 2001; Lau et al., 2002; Treijtel et al., 2004). SCH is beneficial for the determination of the metabolic intrinsic clearance of slowly metabolizing compounds since it allows long-term incubation (Treijtel et al., 2004; Treijtel et al., 2005). The incubation period was up to 24 hours in the present study, whereas Treijtel and co-workers
conducted metabolic stability studies over 48 hours with SCH for tolbutamide and warfarin and concluded that successful prediction of metabolic intrinsic clearance of these slowly metabolizing drugs was possible. Moreover, they identified minor metabolites of tolbutamide by long-term incubation with SCH (Treijtel et al., 2004). However, liver specific functions such as metabolic activity are dependent on incubation conditions, and some metabolic enzyme activities in cultured hepatocytes were diminished during incubation (Kern et al., 1997; Slaus et al., 2001; Kienhuis et al., 2007). According to an estimate of the percent of paroxetine remaining at the maximum incubation period corrected by the number of initial viable cells, the metabolic activity of paroxetine in suspension was approximately 7- to 12-fold higher than that in SCH. Similarly, the metabolic activity of paroxetine was approximately 8- to 12-fold higher when estimated from the percent of M1-glucuronide or M1-sulfate produced (Fig. 4). These values were within the reported range, while the difference can be estimated to be smaller since the number and viability of plated cells in sandwich culture decrease slightly as the incubation time increases (Treijtel et al., 2004). The decrease of metabolic activity could not be fully prevented under the current culture conditions, however, no significant difference was observed in the comprehensive metabolite profiles between the two methods, which indicated that the metabolic activities of UGTs and SULTs decreased at a similar rate. Since the hepatocytes in suspension can be used for only several hours due to their rapidly diminishing cell viability (Berthiaume et al., 1996; Berry et al., 1997), the SCH system can be useful for providing a sufficient amount of metabolite to determine its chemical structure even if the test article is slowly metabolized.

Our findings also provide information on the possibility of performing CYP induction studies with human SCH. By administering a preformed metabolite to cultured human hepatocytes, an adequate evaluation of its inductive potential may not be possible since a hydrophilic metabolite often has lower membrane permeability than its lipophilic parent drug (Pang et al., 1984; de Lannoy and Pang, 1987). Moreover, synthesis of a sufficient amount of metabolite(s) and verification of its purity and structure are not easy, particularly in the case of minor or unstable metabolite(s). Our findings that metabolites generated in SCH corresponded to in vivo profiles suggest that comprehensive in vitro CYP inductive
potential of test article, including the effect of metabolite(s) that are exposed systemically or intrahepatically, can be assessed by the treatment of parent compound with SCH. Metabolic/hepatobiliary transport studies which provide the hepatic disposition of various compounds have been conducted (Lengyel et al., 2005; Turncliff et al., 2006; Lee et al., 2010; Yan et al., 2011). In this study, we evaluated the hepatic disposition of paroxetine and revealed the species difference of hepatobiliary disposition of M1-glucuronide between rats and humans using the SCH system could be determined. The primary excretion route of paroxetine-derived radioactivity in rats and humans is bile and urine, respectively (Haddock et al., 1989), and we assumed that this species difference regarding the fate of excreted metabolites depends on the biliary excretion of its major metabolite since paroxetine is completely eliminated by hepatic metabolism. Indeed, the BEI of M1-glucuronide, which is a major metabolite in both species, was higher in rat SCH than that in human SCH, while the BEIs of paroxetine were negligible in both species. Additionally, the BEI of M1-sulfate, which is another major metabolite in humans but not in rats, was comparable to that of M1-glucuronide in human SCH (Table 2). These results support our assumption that the species difference of paroxetine regarding the excretion fate of metabolites depends on the biliary excretion availability of its major metabolites. Furthermore, M1-glucuronide was suggested to be an MRP2/Mrp2 substrate in both species from the results of the vesicular transport studies (Fig. 7). Yet our findings indicate a possible involvement of MRP2/Mrp2 in the biliary excretion of M1-glucuronide, thus, further experiment are needed to demonstrate the effect of Mrp2 on in vivo M1-glucuronide disposition by comparison of the biliary excretion rate between normal and genetically Mrp2-deficient rats. In humans, MRP2 is encoded by the ABCC2 gene, in which several genetic variations associated with protein function or expression have been found (Suzuki and Sugiyama, 2002). However, since M1-glucuronide and M1-sulfate have markedly weaker pharmacological activities than the parent drug (Haddock et al., 1989), the alteration of effectiveness would not occur even if paroxetine was administered to patients with ABCC2 genetic variations.

The ability for M1-glucuronide transport by rat Mrp2 was higher than that by human MRP2. It is reported that biliary excretion is highly dependent on molecular mass with a certain threshold.
DMD #49817

depending on the animal species. Compounds with molecular mass higher than 325 \pm 50 \text{ g/mol} and 500 \pm 50 \text{ g/mol} are preferentially excreted into bile in rats and humans, respectively (Hirom et al., 1972; Levine, 1978), and MRP2/Mrp2 and BCRP/Bcrp are partly involved in this molecular weight-dependent biliary excretion process and species difference between rats and humans (Kato et al., 2008; Choi et al., 2009). For M1-glucuronide, its molecular mass is around 500 \text{ g/mol} (Fig. 2). Therefore, the higher biliary excretion availability of M1-glucuronide could be explained by the selectivity of MRP2/Mrp2 for compounds of a certain molecular mass.

Accumulations of M1-glucuronide and M1-sulfate in cells (+bile) reached the maximum values between 15- and 60-min of incubation and then declined (Fig. 6C to Fig. 6E). We believe this is caused by the flux from the bile canaliculi to the outer incubation medium. Lee and co-workers previously observed such a flux and reported a $T_{\text{flux}}$ (the occurring time of flux during incubation) of 16.7 min (Lee et al., 2010), which supports our findings. Moreover, the in vitro disposition of a compound is assumed to achieve equilibrium by continuous incubation of medium, cells, and bile canaliculi. Accordingly, the accumulation in the bile canicular lumen is apparently diminished due to the in vitro equilibrium as observed in the present study (Fig. 6). For adequate evaluation, the BEI of the metabolites formed in the cells should be assessed at the earliest time that the metabolite amount can be quantified.

In conclusion, we succeeded in mimicking the in vivo species difference of the hepatic disposition of paroxetine between rats and humans using SCH. SCH was found to be useful for evaluating the processes of hepatic uptake and metabolism of parent drugs as well as the biliary excretion of both parent drugs and formed metabolites in liver at the same time. Data obtained from metabolic/hepatobiliary transport studies with SCH using radiolabeled compound should allow the synthesis of metabolites to be prioritized.
Acknowledgement

We greatly thank Tamami Arai for assistance with the writing of the manuscript.
Authorship Contribution

*Participated in research design:* Matsunaga, Nunoya, Ogawa, and Tamai.

*Conducted experiments:* Matsunaga and Okada.

*Contributed new reagents or analytic tools:* None

*Performed data analysis:* Matsunaga, Nunoya, Ogawa, and Tamai.

*Wrote or contributed to the writing of the manuscript:* Matsunaga and Tamai.
References


Figure Legends

Figure 1
Metabolic pathway of paroxetine as proposed by Haddock et al. (1989), Kaye et al. (1989), and Jornil et al. (2010) CYP, cytochrome P450; COMT, catechol-O-methyltransferase; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase.

Figure 2
Product ion spectra of M1-glucuronide (A) and M1-sulfate (B). M1-glucuronide was obtained from the incubation of paroxetine with NADPH-, SAM-, and UDPGA-supplemented rat liver S9 fractions. M1-sulfate was obtained from the incubation of M1, which was generated from the reaction mixture containing paroxetine and NADPH- and SAM-supplemented rat liver S9 fractions, with PAPS-supplemented human liver cytosolic fractions. The origins of the characteristic ions of M1-glucuronide and M1-sulfate are also depicted.

Figure 3
Typical radio-chromatograms of the incubation mixture of [3H]paroxetine (1 μM) with rat hepatocytes in suspension for 3 h (A) or SCH for 24 h (B). For SCH, day 4 hepatocytes were used.
Figure 4
Disappearance of paroxetine, and appearance of M1-glucuronide and M1-sulfate over time from an incubation mixture of [3H]paroxetine with rat (A and C) or human hepatocytes (B and D). For SCH, day 4 hepatocytes were used. Metabolite profiles of radioactivity were analyzed by radio-HPLC after the incubation of [3H]paroxetine (1 μM) with rat or human hepatocytes for 0.25, 0.5, 1, 2, and 3 h in suspension (A or B, respectively) or 0.25, 0.5, 1, 3, 6, and 24 h in sandwich-culture (C or D, respectively). Data are expressed as the mean ± S.D. of 3 lots of rat or human hepatocytes. □, percent remaining paroxetine; ●, percent produced M1-glucuronide; ○, percent produced M1-sulfate

Figure 5
Typical radio-chromatograms of the incubation mixture of [3H]paroxetine (1 μM) with human hepatocytes in suspension for 3 h (A) or SCH for 24 h (B). For SCH, day 4 hepatocytes were used.

Figure 6
Accumulation – time profiles of paroxetine, M1-glucuronide, and M1-sulfate in rat (A and C) or human SCH (B, D, and E). Day 4 hepatocytes were used. Metabolite profiles of radioactivity were analyzed by radio-HPLC after the incubation of [3H]paroxetine (1 μM) with rat or human SCH for 0.25, 0.5, 1, 4, and 24 h. Data are expressed as the mean ± S.D. of 3 lots of rat or human hepatocytes.
●, Ca/Mg(+)-treatment (cells + bile); ○, Ca/Mg(-)-treatment (cells) *, p < 0.05; **, p < 0.01 significant for Ca/Mg(+) versus Ca/Mg(-)-treatment by paired t-test
Figure 7

Transport – time profiles of M1-glucuronide into rat or human ABC transporter-expressing membrane vesicles. Radioactivity in membrane vesicles was determined by liquid scintillation counting after the incubation of $[^3]$H]M1-glucuronide (0.5 μM) with 1 mg/ml rat Mrp2 (A), human MRP2 (B), rat Bcrp (C), human BCRP (D), rat Bsep (E), or human BSEP (F) transporter-expressing membrane vesicles. Data are expressed as the mean ± S.D. of independent 3 determinations. ●, 4 mM ATP; ○, 4 mM AMP

*, p < 0.05; **, p < 0.01; ***, p < 0.001 significant for ATP versus AMP added by 2-sample t-test

Figure 8

Transport – time profiles of M1-sulfate into rat or human ABC transporter-expressing membrane vesicles. Radioactivity in membrane vesicles was determined by liquid scintillation counting after the incubation of $[^3]$H]M1-sulfate (0.5 μM) with 1 mg/ml rat Mrp2 (A), human MRP2 (B), rat Bcrp (C), human BCRP (D), rat Bsep (E), or human BSEP (F) transporter-expressing membrane vesicles. Data are expressed as the mean ± S.D. of independent 3 determinations. ●, 4 mM ATP; ○, 4 mM AMP

*, p < 0.05; **, p < 0.01; ***, p < 0.001 significant for ATP versus AMP added by 2-sample t-test.
## Table 1  Characteristics of cryopreserved hepatocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Lot number</th>
<th>Sex</th>
<th>Age</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD Rat</td>
<td>Rs587</td>
<td>M</td>
<td>7 weeks</td>
<td>Pool of 4 animals</td>
</tr>
<tr>
<td></td>
<td>Rs609</td>
<td>M</td>
<td>7 weeks</td>
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<tr>
<td></td>
<td>Rs616</td>
<td>M</td>
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</tr>
<tr>
<td>Human</td>
<td>KQG</td>
<td>F</td>
<td>38 years old</td>
<td>Single donor, C</td>
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<tr>
<td></td>
<td>GHA</td>
<td>F</td>
<td>1 year old</td>
<td>Single donor, C</td>
</tr>
<tr>
<td></td>
<td>RTM</td>
<td>F</td>
<td>61 years old</td>
<td>Single donor, C</td>
</tr>
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</table>

M, male; F, female; C, Caucasian
Table 2  BEIs of paroxetine, M1-glucuronide and M1-sulfate in rat and human SCH

<table>
<thead>
<tr>
<th>Species</th>
<th>Incubation time (h)</th>
<th>BEI (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Paroxetine</td>
</tr>
<tr>
<td>SD Rat</td>
<td>0.25</td>
<td>5.1 ± 6.7</td>
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<tr>
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<td>0.5</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.6 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.9 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.5 ± 7.8</td>
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<tr>
<td>Human</td>
<td>0.25</td>
<td>2.4 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

BEI was calculated as described in *Materials and Methods* after $[^3]$Hparoxetine (1 μM) was incubated with rat or human SCH. Data are expressed as mean ± S.D. of 3 lots of rat or human hepatocytes. Data were assumed to be zero in calculating mean and S.D. when the individual BEI was a negative value. NC, not calculated.
Fig. 1

Paroxetine → CYP → Paroxetine catechol

Paroxetine catechol → COMT → M2
Paroxetine catechol → COMT → M1

Paroxetine catechol → UGT → M1-Glucuronide

M1 → COMT → M1-Sulfate

M1 → SULT → M3
Fig. 2

A M1-Glucuronide

MH+ = 508.3

B M1-Sulfate

MH+ = 412.3
Fig. 3

A Suspension (Rat)

M1-Glucuronide

Paroxetine

M1-Sulfate

Radioactivity (cpm)

Time (min)

B Sandwich (Rat)

M1-Glucuronide

Paroxetine

M1-Sulfate

Radioactivity (cpm)

Time (min)
Fig. 4

A  Suspension (Rat)

B  Suspension (Human)

C  Sandwich (Rat)

D  Sandwich (Human)
Fig. 5

A Suspension (Human)

B Sandwich (Human)
Fig. 6

A  Paroxetine (Rat)

B  Paroxetine (Human)

C  M1-Glucuronide (Rat)

D  M1-Glucuronide (Human)

E  M1-Sulfate (Human)
Fig. 7

A  Rat Mrp2

B  Human MRP2

C  Rat Bcrp

D  Human BCRP

E  Rat Bsep

F  Human BSEP

Incubation time (min)

Uptake of M1-glucuronide (pmol/mg protein)
Fig. 8

A  Rat Mrp2

B  Human MRP2

C  Rat Bcrp

D  Human BCRP

E  Rat Bsep

F  Human BSEP

Uptake of M1-sulfate (pmol/mg protein)

Incubation time (min)