Positron Emission Tomography Studies Using (15R)-16-m-[11C]tolyl-17,18,19,20-tetranorisocarbacyclin Methyl Ester for the Evaluation of Hepatobiliary Transport

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ABSTRACT

A quantitative positron emission tomography (PET) methodology was developed for in vivo kinetic analysis of hepatobiliary transport. Serial abdominal PET scans were performed on normal and multidrug resistance-associated protein 2 (Mrp2)-deficient rats after intravenous injection of (15R)-16-m-[11C]tolyl-17,18,19,20-tetranorisocarbacyclin methyl ester (15R-[11C]TIC-Me) as a radiotracer. 15R-[11C]TIC-Me was rapidly converted to its acid form in blood within 10 s. PET scans revealed that 15R-[11C]TIC was localized mainly in the liver within 5 min of injection. By 90 min, total radioactivity in bile of Mrp2-deficient rats was significantly reduced compared with controls. Metabolite analysis by thin-layer chromatography autoradiography showed that 15R-[11C]TIC is converted to at least three metabolites (M1, M2, and M3), and M2 and M3 are the major metabolites in plasma and bile, respectively. Hepatic uptake clearance of total radioactivity in normal rats was close to the hepatic blood flow rate and slightly higher than that in Mrp2-deficient rats. The intrinsic canalicular efflux clearance of M3 (CLint,bile,M3) in Mrp2-deficient rats was decreased to 12% of controls, whereas clearance of M2 was moderately decreased (54%). An in vitro transport assay detected ATP-dependent uptake of both M2 and M3 by rat Mrp2-expressing membrane vesicles. These results demonstrated that M3 is excreted primarily into the bile by Mrp2 in normal rats. We conclude that PET studies using 15R-[11C]TIC-Me could be useful for in vivo analyses of Mrp2-mediated hepatobiliary transport.

Introduction

It is now well accepted that hepatobiliary transport of anionic drugs involves multispecific transporters acting in both sinusoidal uptake and canalicular efflux. Transporters involved in hepatic uptake include organic anion transporting polypeptide (Oatp, rodents; OATP, humans) 1B1/SLCO1B1 and OATP1B3/SLCO1B3, whereas subsequent canalicular efflux transporters include multidrug resistance-associated protein (Mrp2, rodents; MRP2, humans)/ABCC2 and breast cancer resistance protein (Bcrp, rodents; BCRP, humans)/ABCG2 (Shitara et al., 2005; Maeda and Sugiyama, 2008).

Clinical studies have shown that variations in multispecific transporter activity caused by genetic polymorphisms or drug–drug interactions can alter the plasma concentration of drugs and thereby affect therapeutic efficacy and the incidence of adverse effects (Shitara et al., 2005; Maeda and Sugiyama, 2008). Pharmacokinetic analyses based on plasma concentration profiles in clinical studies provide information only on overall hepatic intrinsic clearance, but can separately evaluate the impact of variation in transporter activity on hepatic uptake and canalicular efflux. We have proposed the concept of a rate-determining process governing overall hepatic elimination of anionic drugs (Kusuhara and Sugiyama, 2009; Watanabe et al., 2009a,b, 2010). Contrary to the uptake process, variation in
canalicular efflux markedly affects the liver concentrations of drugs, whereas it has little effect on their systemic exposure. Therefore, methods for quantitative estimation of the tissue concentration of drugs in vivo are necessary for further investigation of variations in the efflux activities caused by drug–drug interactions and genetic polymorphisms.

Positron emission tomography (PET) is a powerful noninvasive method for molecular imaging in living systems. The high sensitivity and exceptional spatial–temporal resolution of PET make it a particularly useful tool for estimating the in vivo function of drug transporters in various tissues over time after intravenous administration of a radiolabeled drug. For example, PET was used to evaluate the hepatobiliary excretion of cysteinyl leukotrienes in rats and monkeys by using N-[11C]acetyl-leukotriene E4 (Guhlmann et al., 1995), whereas PET-generated overall time–radioactivity curves were used to assess impairment of Mrp2 (Nies and Keppeler, 2007; Maeda and Sugiyama, 2008). Single-photon emission computed tomography using [99mTc]Technetium-disofenec has been proposed as a useful noninvasive tool for evaluating the function of Mrp1 and Mrp2 in vivo (Hendrikse et al., 2004). In addition, Lorasso et al. (2002) reported the functional analysis of MRP2 using B22956/1, a gadolinium complex from the class of intravascular contrast agents used in magnetic resonance imaging. However, information regarding the quantification and kinetic assessment of hepatobiliary excretion in the studies described above was quite limited, because the imaging studies involved only time–radioactivity curves or elimination half-life.

To evaluate hepatobiliary transport quantitatively, we developed a new PET imaging method using (15R)-16-m-([11C]toly1-17,18,19,20-tetranorisoracarbacyclin (15R-[11C]TIC; Fig. 1A) as a radiotracer. We have developed 15R-TIC as a specific ligand for the central nervous system-type prostacyclin receptor (Cui et al., 1999, Satoh et al., 1999; Watanabe et al., 1999), and demonstrated that 15R-[11C]TIC-methyl ester (15R-[11C]TIC-Me; Fig. 1B), the ester produg of 15R-[11C]TIC, has a potent in vivo and in vitro neuroprotective effect against ischemic insults (Cui et al., 2006). In monkey PET studies, 15R-[11C]TIC-Me showed excellent profiles for PET imaging of prostacyclin receptors, the endogenous system for neuroprotection against ischemic neuronal cell death, hence its usefulness for neuronal cell death imaging (Suzuki et al., 2004). In addition, preliminary clinical PET studies using 15R-[11C]TIC-Me showed that 15R-[11C]TIC-Me is rapidly (<120 s) hydrolyzed to its pharmacologically active acid form, 15R-[11C]TIC, in plasma, and the radioactivity is distributed primarily in the liver, gall bladder, and intestine. Development of a quantitative PET imaging method using 15R-[11C]TIC-Me would enable the noninvasive kinetic assessment of hepatobiliary transport in vivo.

Here, we report on the development of a PET imaging analysis method to determine the intrinsic uptake, canalicular efflux, and overall hepatic elimination of 15R-[11C]TIC in rats. The method allowed for simultaneous measurement of time profiles of total radioactivities in liver, kidney, urinary bladder, and bile in a single rat. In addition, to gain insight into the transporter responsible for canalicular efflux, kinetic parameters of drug elimination were compared between normal Sprague-Dawley rats (SDRs) and Mrp2-deficient mutant rats, Eisai hyperbilirubinemic rats (EHBrs).

Materials and Methods

Materials. [11C]Labeled 15R-TIC-Me was synthesized from its deuteromethylated derivative by using a palladium-mediated rapid coupling reaction of [11C]methyl iodide (Björkman et al., 1998; Suzuki et al., 2004). The identity and concentration of 15R-[11C]TIC-Me were assessed by high-performance liquid chromatography (HPLC). Radiochemical purity was more than 95%, and the specific radioactivity was 14 to 43 GBq/μmol at the time of injection. The purified fraction was evaporated and reconstituted with approximately 4 ml of saline containing 7.5% propylene glycol and 1.3% poly-(oxyethylene) sorbitan mono-oleate. Rat Mrp2-expressing membrane vesicles were purchased from Genomemembrane, Inc. (Kanagawa, Japan). Cryopreserved rat hepatocytes were purchased from Celsis In Vitro Technologies (Chicago, IL). All other organic solvents and reagents were of HPLC grade (Nacalai Tesque, Kyoto, Japan).

Animals. Male SDRs (n = 4) and male Mrp2 hereditary-deficient EHBrs (n = 4) weighing 300 to 400 g were purchased from Japan SLc Inc. (Shizuoka, Japan). The animals were kept in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. All experimental protocols were approved by the Ethics Committee on Animal Care and Use of the Center for Molecular Imaging Science at RIKEN and performed in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985).

PET Scans. All PET scans were performed with a microPET Focus220 scanner (Siemens, Knoxville, TN) designed for laboratory animals. Rats were anesthetized and maintained with a mixture of 1.5% isoflurane and nitrous oxide/oxygen (7:3), and the femoral artery was cannulated with polyethylene tubing for collection of blood samples. Before emission scanning, animals were placed in the center of the PET camera, and a 25-min transmission scan with a rotating 57Ge–68Ga point source was performed for the abdomen positioning and attenuation correction. At the start of the emission scan, 15R-[11C]TIC-Me was administered as a single bolus via the tail vein in doses of 17 to 50 MBq. The chemical amount of 15R-TIC-Me for the bolus injection was calculated as 0.52 to 2.2 nmol/body by both specific radioactivity and administered radioactivity. An emission scan in three-dimensional list mode was performed for 90 min and sorted into 77 dynamic sinograms according to the following sequence: 28 × 5, 24 × 30, and 25 × 180 s. Arterial blood was sampled via the cannulated femoral artery for 13 times within 90 min at the following time points: 10, 20, 30, 40, and 50s and 1, 2, 5, 10, 25, 40, 60, and 90 min after administration of 15R-[11C]TIC-Me. The volume of blood sampled for each time point was within 120 μl, and the total blood volume sampled from one rat did not exceed 1.6 ml, approximately 10% of total circulating blood volume. Blood radioactivity was measured with a 1470 Wizard Automatic Gamma Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). The radioactivity in each measured sample was corrected for time decay from the point of 15R-[11C]TIC-Me administration.

Analysis of PET Data. PET images were reconstructed by using microPET manager 2.4.1.1 (Siemens) by Fourier rebinning and standard two-dimensional filtered back projection (FBP) using a ramp filter with a cutoff at the Nyquist frequency or Fourier rebinning and maximum-likelihood expectation maximization. Compared with FBP, maximum-likelihood expectation maximization reconstruction of small animal PET images provides better spatial resolution and

Fig. 1. Chemical structures of 15R-[11C]TIC (A) and 15R-[11C]TIC-Me (B).
statistical noise properties, which is advantageous for image registration. On the other hand, FBP-reconstructed images were used for quantification because FBP is superior to the other reconstruction method in quantification. Regions of interest (ROIs) representing liver, intestines, kidneys, and urinary bladder were delineated by using the ASIPro 6.3.3 program (CTI Concorde Microsystems Inc., Knoxville, TN) as follows. First, ROIs were defined on tissue images derived from the summation of time frames ranging from 1 to 90 min after 15R-[11C]TIC-Me administration, in which these tissues could easily be identified. The defined ROIs were then corrected by fitting the images of each tissue slice and time frame. All ROIs were combined and changed to volumetric ROIs. For liver and kidney images, ROIs were defined for calculating the radioactivity concentration in each tissue, and then the total amount of radioactivity in the tissue was calculated by normalizing the tissue weight of each animal. For intestine and urinary bladder images, ROIs were defined larger than the images for calculating the total amount of radioactivity distributed in the tissues. In this PET analysis, the radioactivity in intestine corresponds to that in bile excreted to intestine. Thus the radioactivity in intestine was described as “the radioactivity in bile excretion into intestine.” Time–radioactivity curves for each tissue were constructed by normalizing decay-corrected time–radioactivity measurements to the injected dose (% dose) of 15R-[11C]TIC-Me.

**Kinetic Analysis.** Initial 15R-[11C]TIC-Me uptake rates for rat liver and kidney were calculated by the integration plot method (Kim et al., 1988) using the portion of time–radioactivity curves encompassing approximately the first 2 min after 15R-[11C]TIC-Me administration, during which the effect of excretion from the tissue and metabolism was negligible. The uptake clearance of 15R-[11C]TIC was determined by using eq. 1:

$$
\frac{X_{t,\text{tissue}}}{C_{t,\text{blood}}} = \text{CL}_{\text{uptake, tissue}} \times AUC_{t,\text{blood}} + V_E \tag{1}
$$

where \(X_{t,\text{tissue}}\) is the amount of [11C]radioactivity in the liver or kidney at time \(t\) as determined by PET image analysis. \(C_{t,\text{blood}}\) is the concentration of 15R-[11C]TIC in blood at time \(t\), as determined by gamma counter measurement of radioactivity in blood taken from the femoral artery. \(\text{CL}_{\text{uptake, tissue}}\) is the uptake clearance for liver or kidney, and \(AUC_{t,\text{blood}}\) is the area under the blood-concentration-time curve from time 0 to time \(t\). The \(\text{CL}_{\text{uptake, tissue}}\) value can be obtained from the initial slope of the plot of \(X_{t,\text{tissue}} / C_{t,\text{blood}}\) versus \(AUC_{t,\text{blood}} / C_{t,\text{blood}}\). \(V_E\) represents the initial distribution volume in liver or kidney at time 0, calculated from the \(y\) intercept of the integration plot.

Biliary clearance was estimated by integration plot analysis using noninvasive measurements of radioactivity in the liver and the intestine (the radioactivity in bile excreted into the intestine) between 5 and 40 min after drug administration. The biliary clearance of 15R-[11C]TIC metabolites is described by the following equation:

$$
\frac{X_{t,\text{bile}}}{C_{t,\text{blood}}} = \text{CL}_{\text{int, bile}} \times AUC_{0-t,\text{Liver}} + V_E \tag{2}
$$

where \(X_{t,\text{bile}}\) is the amount of [11C]radioactivity in the intestine (the radioactivity in bile excreted into the intestine) at time \(t\), as determined by PET image analysis. \(\text{CL}_{\text{int, bile}}\) represents the intrinsic biliary clearance of each metabolite based on [11C]radioactivity in the liver, and \(AUC_{0-t,\text{Liver}}\) is the area under the hepatic concentration-time curve from time 0 to time \(t\). The \(\text{CL}_{\text{int, bile}}\) value can be obtained from the slope of the plot of \(X_{t,\text{bile}} / C_{t,\text{blood}}\) versus \(AUC_{0-t,\text{Liver}} / V_E\) was calculated as the \(y\) intercept of the integration plot.

**Radio-Metabolite Analysis of Rat Blood, Liver, and Bile Using Thin-Layer Chromatography.** Radio-metabolite analysis of rat blood, liver, and bile was performed separately from the PET study to evaluate the average values of time-metabolite composition profiles in these samples. Arterial blood samples were collected at 1, 2, 5, 10, 20, and 40 min after administration of 15R-[11C]TIC-Me. To sample bile, the bile duct in SDRs or EHBRs was cannulated before 15R-[11C]TIC-Me administration, and bile was collected over the periods 0 to 5, 5 to 10, 10 to 25, and 25 to 40 min after administration. To sample liver tissue, blood flow was terminated by transection of the abdominal aorta and vein at 10, 20, and 40 min after administration, and the liver was quickly removed and homogenized. Blood, bile, and liver homogenates were deproteinized by precipitation with acetonitrile. After centrifugation, the supernatants were applied to RP-18 thin-layer chromatography (TLC) plates (Merck Biosciences, Darmstadt, Germany). Plates were developed at room temperature with acetonitrile/water/acetic acid (50:50:0.75) as a mobile phase. After migration, plates were dried and exposed to BAS TR2040 imaging plates (Fuji Film, Tokyo, Japan) for 40 min. The distribution of radioactivity on the imaging plates was determined with digital PSL autoradiography using a Fuji FLA-7000 analyzer at 50-μm resolution, and the data were analyzed by using the MultiGauge image analysis program (Fuji Film).

**LC/MS/MS Profiling and Identification of Metabolites in Rat Bile and Hepatocytes.** Metabolites of unlabeled 15R-TIC were identified by LC/MS/MS in in vivo experiments using normal rats and in vitro experiments using rat hepatocytes. For analysis of metabolites in bile of normal rats, the bile duct was cannulated before administration while animals were anesthetized and maintained with 1.5% isoflurane in air. A single bolus of 15R-TIC at 3 mg/kg body weight was administered via the tail vein. Bile was collected for 30 min and prepared for LC/MS/MS analysis as follows. In brief, bile samples were mixed with a 2-fold volume of acetonitrile and centrifuged at 12,000 rpm for 2 min to yield the analytical supernatant. Predose samples served as controls for determining background endogenous, nondrug-related ions observed within respective matrices or their extracts. Bile samples were mixed with 0.2 M phosphate buffer, pH 5.8, and incubated with β-glucuronidase (14 U/ml; Roche Diagnostics, Mannheim, Germany) at 37°C for 24 h. Control samples lacking β-glucuronidase were also prepared. The samples were mixed with a 2-fold volume of acetonitrile and centrifuged at 12,000 rpm for 2 min to yield the analytical supernatant.

Cryopreserved rat hepatocytes were thawed and prepared based on a previously published procedure (Li et al., 1999). A suspension of hepatocytes (2 × 10⁶ cells/ml in William’s E medium) was incubated with 15R-TIC (100 μM) at 37°C in a humidified carbon dioxide (5%) chamber for 4 h. A 2-fold volume of acetonitrile was then added to each sample to precipitate proteins, followed by centrifugation at 12,000 rpm for 2 min to yield analytical supernatants.

The LC/MS/MS analyses were performed with a LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a turbo electrospray ionization ion source, and interfaced with an HPLC system (Shimadzu, Kyoto, Japan). Chromatographic separation was carried out by using a Waters (Milford, MA) Atlantis T3 column. The flow was 0.2 ml/min at initial conditions of 90% solvent A (10 mM ammonium acetate, pH 7.2) and 10% solvent B (90% acetonitrile in 10 mM ammonium acetate, pH 7.2). Analytes were eluted by using the following gradient conditions: 0 to 1 min, 10% solvent B in solvent A; 1 to 6 min, 10% to 100% solvent B in solvent A; and 6 to 10 min: 100% solvent B. After the elution, the column was returned to 10% solvent B in solvent A over 1 min. Mass spectral data were collected by using X-caliber version 2.0.6 software (Thermo Fisher Scientific), and MS/MS data were processed by using Qual Browser version 2.0 (Thermo Fisher Scientific).

**Transport Study Using Transporter Expressing Membrane Vesicles.** Uptake of 15R-TIC or its metabolites in rat transporter-expressing membrane vesicles was measured by the rapid filtration technique as described previously (Ishikawa et al., 1990). ATP-dependent transport of 15R-TIC or its metabolites into rat Mrp2-expressing membrane vesicles was measured at 37°C in a 50-μl reaction volume containing 45 μg of vesicle protein in the reaction buffer (10 mM MOPS-Tris buffer, pH 7.0 containing 14 mM KC1, 2 mM glutathione, and 10 mM MgCl₂, 50 μM 15R-TIC or its metabolite, and 4 mM ATP or AMP). After 5 min, 200 μl of ice-cold reaction stopping buffer (40 mM MOPS-Tris buffer, pH 7.0, containing 70 mM...
KCl) was added to each sample, and the samples were filtered under vacuum through a hydrophilic PTFE membrane filter (Millipore Corporation, Billerica, MA) presoaked in reaction stopping buffer. Filters were dried, and then soaked again in 500 μl of acetonitrile containing warfarin as an internal standard for LC/MS/MS quantification.

**Statistical Analysis.** Data were calculated as the mean ± S.D. for three to four determinations. A Student’s two-tailed unpaired t test was used to identify significant differences between groups. Statistical significance was set at \( p < 0.05 \).

**Results**

**Distribution of Radioactivity in the Abdominal Region and Blood After Administration of 15R-[11C]TIC-Me.** The tissue distribution of radioactivity over time after administration of 15R-[11C]TIC-Me to normal rat, bile duct cannulated rat, and EHBR is shown in Fig. 2. Radioactivity was localized mainly in the liver and kidneys in all animals by 2 min after administration. By 60 min, radioactivity was localized primarily in the intestine in normal rats (Fig. 2A), and no signal was detected in the intestinal region of rats in which the bile duct had been cannulated (Fig. 2B). The results indicate that the radioactivity detected in the intestine corresponds to excretion of 15R-[11C]TIC-Me into the bile. In EHBRs, radioactivity was found mainly in the urinary bladder between 10 and 60 min after administration of 15R-[11C]TIC-Me (Fig. 2C). The radioactivity in the urinary bladder was higher in rats whose bile duct was cannulated, although the reason for this observation could not be explained (Fig. 2B).

Time–radioactivity curves for tissues in the abdominal regions are shown in Fig. 3. In normal rats, a maximum of 40 ± 2% of the dose was distributed in the liver by 5 min after administration, at which point the radioactivity began to fall quickly until 90 min (Fig. 3A). The time–radioactivity curve for EHBR liver was similar to that for the normal rats.
In contrast, the radioactivity profile of the kidney in normal rats differed from that in EHBRs (Fig. 3B). The radioactivity found in the kidneys in normal rats reached 8.3 ± 3.4% of the dose by 26 min and remained constant, whereas radiation in EHBR kidneys peaked at 4.3 min and decreased rapidly after that point until 90 min. The radioactivity in intestine (the radioactivity in bile excreted into the intestine) was 3.4-fold lower in EHBRs (15 ± 1% of the dose) than normal rats (51 ± 3% of the dose) by 90 min after 15R-[11C]TIC-Me administration (Fig. 3C). On the other hand, the radioactivity found in the urinary bladder of normal and EHBRs gradually increased until the end of the PET scan and was 7-fold higher in EHBRs (49 ± 3% of the dose) than normal rats (7.3 ± 4.3% of the dose) by 90 min after administration (Fig. 3D).

The time–radioactivity curve for the blood of normal rats revealed a two-phase elimination, in which radioactivity fell quickly up to 5 min with elimination constant \( (k_{fast}) \) of 1.1 ± 0.1 min\(^{-1} \) and then decreased slightly until 90 min with elimination constant \( (k_{slow}) \) of 0.25 ± 0.004 min\(^{-1} \) (Fig. 3E). Up to 5 min after administration, the time–radioactivity curve for EHBR blood dipped with \( k_{fast} \) of 0.94 ± 0.1 min\(^{-1} \) as it did for normal rat blood. However, after the 5-min point the EHB curve remained essentially constant until 60 min, at which point it decreased slightly \( (k_{slow} 0.0044 ± 0.0033 \text{ min}^{-1}) \). The AUC\(_{0–90\text{min}}\) of EHB blood (22 ± 9% of the dose \( \times \text{min/ml} \)) was 2.7-fold higher than the AUC\(_{0–90\text{min}}\) of normal rat blood (8.0 ± 0.7% of the dose \( \times \text{min/ml} \)).

**Radio-Metabolite Analysis of 15R-[11C]TIC in Rat Blood, Bile, and Liver.** Figure 4, A–F shows the distribution of radioactivity on TLC plates applied with extracts of blood, bile, and liver prepared after administration of 15R-[11C]TIC-Me to normal rats and EHBRs. Analysis of the TLC autoradiograms showed that at least three metabolites (M1–M3) and 15R-[11C]TIC were present in the blood of both normal rats and EHBRs (Fig. 4, A and B). The spot corresponding to intact 15R-[11C]TIC-Me was not detectable in blood even by 1 min after administration, whereas a spot corresponding to 15R-[11C]TIC was detected in samples of blood in both normal rats and EHBRs up to 5 min. Metabolite M2 was the predominant metabolite in the blood of normal rats by 10 min after administration, whereas M3 was most abundant in the blood of EHBRs.

Figure 4, C and D shows radiochromatograms of bile extracts from normal rats and EHBRs, respectively. Metabolites M1, M2, and M3 all were detected in the bile from both groups, with M3 being the most abundant. The radiochromatograms of liver extracts show that metabolite M2 was...
most abundant in the liver of both groups (Fig. 4, E and 4F). PET image analysis data on the time profile tissue distribution of total radioactivity combined with radiometabolite analyses enabled us to determine the time–radioactivity curves of the major metabolites in the tissues. Figure 4, G and H shows the time–radioactivity curves of major metabolites M2 and M3 in liver and bile. The curves for M2 in the liver and M3 in the bile were almost similar to those of total radioactivity in normal rats and EHBRs (Fig. 3, A and C).

Profiles of 15R-TIC and Its Metabolites in Rat Bile and Hepatocytes. Figure 5 shows mass spectrometric analyses of normal rat hepatocyte extracts incubated with 15R-TIC and bile extracts prepared after administration of 15R-TIC. Three major deprotonated ions (m/z 355, 531, and 559) representing the major metabolites were detected in extracts of rat hepatocytes incubated with 15R-TIC, as determined by subtraction of the ion chromatogram representing time 0 from that at 240 min (Fig. 5A). The peaks of major metabolites in the HPLC chromatograms eluting with retention times of 8.5 min (m/z 355, M1), 8.1 min (m/z 559, M2), and 7.7 min (m/z 531, M3) increased in abundance after hepatocytes were incubated with 15R-TIC.

Figure 5B shows ion chromatograms for the bile extracts from normal rats, as determined from the difference between chromatograms representing before and after administration of 15R-TIC. Ions representing major metabolites eluted with retention times of 8.4 min (M1) and 7.7 min (M3). Ions with retention times of 9.1 min (15R-TIC; data not shown) and 8.1 min (M2) were nearly undetectable in rat bile.

The major metabolites were isolated by HPLC and analyzed by TLC with phosphomolybdic acid staining. The identity of each metabolite was confirmed by the Rf value of the major TLC spots. Thin-layer chromatography analysis of the m/z 355, 531, and 559 ions observed by LC/MS produced major spots with Rf values of 0.4, 0.7, and 0.5, which corresponded to the expected Rf values for M1, M2, and M3, respectively (data not shown). When bile samples were incubated with β-glucuronidase, M3 disappeared with a concomitant increase in M1, whereas there was no change in the intensity of ions representing M3 and M1 in control bile samples incubated for 24 h without β-glucuronidase (Fig. 5C).

Figure 5D illustrates the results of electrospray ionization MS/MS of the major metabolites. The m/z 559 (M2) ion generated a number of fragment ions also found in fragmentation of 15R-TIC (e.g., m/z 541, 383, 365, 347, 259, and 231). On the other hand, MS/MS fragmentation of the m/z 531

Fig. 5. Extracted chromatograms for [M-H]- ions and product ion MS/MS spectra for the major 15R-TIC metabolites. A, ion chromatograms of deprotonated ions (m/z 355, 531, and 559) in rat hepatocyte extracts as determined by subtraction of the ion chromatogram at 0 min from that at 240 min after hepatocytes were incubated with 15R-TIC. B, ion chromatograms of rat bile extracts as determined by subtraction of the before 15R-TIC administration from the after 15R-TIC administration ion chromatograms. C, ion chromatograms of metabolites M1 and M3 in rat bile extract treated with β-glucuronidase. NL, neutral loss; RT, retention time. D, product ion MS/MS spectra of the major metabolites of 15R-TIC in bile of normal rats.
TABLE 1
Pharmacokinetic parameters of 15\[11C\]TIC-Me after intravenous administration in normal rats and EHBRS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Rats</th>
<th>EHBRS</th>
<th>Ratio (EHBRS/Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL_{uptake,liver} (ml/min/kg)</td>
<td>45 ± 9</td>
<td>31 ± 15</td>
<td>0.69 ± 0.36</td>
</tr>
<tr>
<td>V_{E,liver} (ml/kg)</td>
<td>16 ± 10</td>
<td>11 ± 3</td>
<td>0.69 ± 0.47</td>
</tr>
<tr>
<td>CL_{uptake,kidney} (ml/min/kg)</td>
<td>6.3 ± 0.5</td>
<td>5.0 ± 1.8</td>
<td>0.79 ± 0.29</td>
</tr>
<tr>
<td>V_{E,kidney} (ml/kg)</td>
<td>3.6 ± 0.8</td>
<td>3.2 ± 0.9</td>
<td>0.89 ± 0.32</td>
</tr>
<tr>
<td>CL_{int,bile, RA} (ml/min/kg)</td>
<td>1.4 ± 0.5</td>
<td>0.20 ± 0.06</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>CL_{int, bile, M2} (ml/min/kg)</td>
<td>5.6 ± 1.9</td>
<td>0.67 ± 0.30</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>CL_{int, bile, M3} (ml/min/kg)</td>
<td>0.41 ± 0.15</td>
<td>0.22 ± 0.01</td>
<td>0.54 ± 0.20</td>
</tr>
<tr>
<td>CL_{int, bile, TIC-A} (ml/min/kg)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CL_{int, bile, TIC-C} (ml/min/kg)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected.

(M3) ion generated a number of ions also generated upon fragmentation of M1 (e.g., m/z 355, 337, 187, 175, and 161).

Uptake Clearance of 15\[11C\]TIC in Rat Liver and Kidney. The pharmacokinetic parameters calculated by integration plots for liver and kidney in normal rats and EHBRS are shown in Table 1. The integration plots were linear in the liver and kidney for a short period, which varied from 0.3 to 2 min after administration of 15\[11C\]TIC (Fig. 6, A and B). During this period, the \[^{11}\text{C}\] radioactivity in the blood was derived primarily from 15\[11C\]TIC, and the amounts of 15\[11C\]TIC-Me and the other metabolites were negligible. The uptake of radioactivity in the tissue corresponds to that of 15\[11C\]TIC. The liver uptake clearance (CL_{uptake,liver}) in normal rats and EHBRS was 45 ± 9 and 31 ± 15 ml/min/kg, respectively. The V_{E} of liver (V_{E,liver}) in normal rats and EHBRS was 16 ± 10 and 11 ± 3 ml/kg, respectively. The initial kidney uptake clearance (CL_{uptake,kidney}) in normal rats (6.3 ± 0.5 ml/min/kg) was almost the same as that in EHBRS (5.0 ± 1.8 ml/min/kg). The V_{E} of kidney (V_{E,kidney}) in normal rats and EHBRS was 3.6 ± 0.8 and 3.2 ± 0.9 ml/kg, respectively.

Biliary Excretion of 15\[11C\]TIC Metabolites. The integration plots using AUC_{liver} and X_{bile} (the radioactivity in bile excreted into the intestine) as determined by PET quantification are shown in Fig. 6C. The plots were linear between 5 and 40 min after administration of 15\[11C\]TIC-Me. A large difference in the plot of total radioactivity was observed between normal rats and EHBRS. The intrinsic biliary clearance (CL_{int,bile}) of the total radioactivity was calculated as 1.4 ± 0.5 and 0.20 ± 0.06 ml/min/kg in normal rats and EHBRS, respectively (Table 1). Using time–radioactivity profile data for M2 and M3 in the liver and intestine (the radioactivity in bile excreted into the intestine) (Fig. 4, G and H), the intrinsic clearance for biliary excretion of M2 and M3 (CL_{int,bile,M2} and CL_{int,bile,M3}) was calculated by integration plot analysis (Fig. 6C). The CL_{int,bile,M2} in EHBRS (0.22 ± 0.01 ml/min/kg) was only 54% of the CL_{int,bile,M2} value in normal rats (0.41 ± 0.15 ml/min/kg). There were also large differences in the CL_{int,bile,M3} value between normal rats and EHBRS (5.6 ± 1.9 and 0.67 ± 0.30 ml/min/kg, respectively).

ATP-Dependent Transport of Unlabeled 15\[11C\]TIC and Its Metabolites in Rat Transporter-Expressing Membrane Vesicles. The ATP-dependent accumulation of 15\[11C\]TIC and its metabolites into rat MRP2-expressing membrane vesicles is shown in Fig. 7. The uptake of metabolites M2 and M3 into the vesicles was 6640 ± 890 and 2570 ± 310

![Fig. 6](image-url) Integration plots of tissue uptake and biliary excretion of total radioactivity and major metabolites in normal rats and EHBRS. A and B, hepatic (A) and renal (B) uptake of 15\[11C\]TIC in normal rats and EHBRS. C, biliary excretion of total radioactivity and major 15\[11C\]TIC metabolites M2 and M3 in normal rats and EHBRS. The data represent the mean ± S.D. (n = 4).
pmol/mg protein/5 min, respectively, which is significantly higher than that of estradiol-17β-glucuronide (1050 ± 430 pmol/mg protein/5 min). The uptake of M1 in the presence of ATP and the uptake of M1, M2, and M3 in the absence of ATP were less than the lower limit of detection by LC/MS/MS. The presence or absence of ATP apparently had no significant effect on the uptake of 15R-TIC (711 ± 364 and 754 ± 841 pmol/mg protein/5 min, respectively).

**Discussion**

In the present study, kinetic analyses of hepatobiliary transport and renal excretion of 15R-[11C]TIC were carried out in rats to demonstrate that PET imaging enables simultaneous determination of tissue uptake and canalicular efflux in a single animal. We also sought to demonstrate that PET imaging is applicable to the functional characterization of the transporters responsible for tissue uptake and efflux.

After intravenous administration, 15R-[11C]TIC-Me-associated radioactivity was excreted primarily by the liver and to the lesser degree by the kidney. In EHBRS, which have a hereditary deficiency of Mrp2, the blood AUC0–90min was 2.7-fold higher than in normal rats. In addition, in EHBRS renal excretion compensated for the decreased transport of radioactivity from the liver into the bile (Fig. 3). These results agreed with previous reports showing that biliary excretion of several organic anions was drastically lower in EHBRS compared with normal rats (Chu et al., 1997; Jedlitschky et al., 1997; Sasabe et al., 1998).

Hepatic uptake clearance (CLuptake,liver; eq. 1) was determined by integration plot analysis. Because only 15R-[11C]TIC was detected even as early as 10 s after administration (data not shown), tissue uptake clearance with regard to total radioactivity could be regarded as being caused by 15R-[11C]TIC. The CL uptake, liver of 15R-[11C]TIC in normal rats was comparable with the hepatic blood flow rate (55 ml/min/kg; Davies and Morris, 1993), indicating very efficient hepatic uptake of 15R-[11C]TIC. The VE,liver in normal rats (16 ± 10 ml/kg) was comparable with the vascular space in liver (11 ml/kg; Everett et al., 1956), validating the determination of uptake clearance from the linear region of the integration plots. The fact that the CL uptake,kidney value was 7-fold lower than the CL uptake,liver indicates tissue uptake capability is the determining factor for the elimination pathway of 15R-[11C]TIC in rats.

Metabolite analysis indicated that 15R-[11C]TIC is extensively metabolized in rats to at least three radiometabolites.
(M1, M2, and M3) (Fig. 4). Metabolite M2 accounts for most of the radioactivity associated with the liver, whereas M3 accounts for most of the radioactivity found in the bile of normal rats (Fig. 4, G and H). In EHBRs, biliary excretion of M3 was markedly reduced, whereas that of M2 was only slightly reduced. In vitro metabolism assays using rat hepatocytes incubated with unlabeled 15R-[11C]TIC demonstrated that the major metabolite is M3 (Fig. 5A). However, M3 was not prominent in the liver, presumably because M3 is rapidly excreted into the bile (6.6% of the dose was present in bile at 40 min) and urine (7.3% of the dose was present in urine at 40 min). Furthermore, to confirm whether the radioactivity underwent enterohepatic recirculation, we administered the bile sample from rats injected with 15R-[11C]TIC-Me to the other rats via the intraduodenal route. The radioactivity in the blood was low level in the rats (less than 0.08% dose/ml), and almost all the radioactivity administered intraduodenally remained in intestine (more than 86% of dose) at 60 min (data not shown), indicating no significant enterohepatic circulation existed in this experiment.

The absolute chemical structures of the major 15R-TIC metabolites have not been elucidated. However, it is likely that metabolite M1, which is 28 Da smaller than 15R-TIC, is produced by de-ethylation of 15R-TIC. The similarities between the electrospray ionization mass spectra of the m/z 559 ion (M2) and 15R-TIC were confirmed by their MS/MS product ion spectra, which suggested that M2 is the acyl-glucuronide of 15R-TIC. The similarities between the MS/MS spectra of the m/z 355 (M1) and 531 ions (M3) suggest that M3 could be the acyl-glucuronide of M1. The fact that incubation with β-glucuronidase converted M3 to M1 (Fig. 5D) also indicates that M3 is the glucuronide conjugate of M1.

The intrinsic biliary clearance (CLint,bile; eq. 2) of the major metabolite M3 was decreased to 14% in EHBRs whereas the 50% reduction of the CLint,bile of M2 in EHBR was not statistically significant compared with normal rats. To support in vivo data, we examined the uptake of 15R-TIC and M1, M2, and M3 in rat recombinant Mrp2-expressing membrane vesicles. Both M2 and M3 were transported in the membrane vesicles in an ATP-dependent manner, and the uptake of both metabolites over the first 5 min was higher than that of estradiol-17β-glucuronide (Fig. 7), suggesting that M2 and M3 are good substrates of Mrp2. On the other hand, the uptake of M3 by Bcrp-expressing vesicles was approximately 2.5-fold greater in the presence of ATP than in the absence of ATP. However, a significant ATP-dependent uptake of M3 could not be detected in the vesicles (data not shown). Taken together, it is likely that Mrp2 is the major transporter for the canalicular efflux of M3. However, unlike M3, the effect of Mrp2 dysfunction on intrinsic canalicular efflux of M2 was not remarkable (Table 1). We speculate that the contribution of Mrp2 to canalicular efflux differs for M2 and M3. In addition to Mrp2, Bcrp and P-glycoprotein are expressed in hepatocyte canalicular membranes and accept some Mrp2 substrates (Hirano et al., 2005; Matsumura et al., 2005). These transporters may mediate the canalicular efflux of M2 together with Mrp2. In addition, the ATP-dependent uptake of M2 in rat recombinant Mrp2-expressing membrane vesicles was greater than uptake of M3, whereas the intrinsic biliary clearance (EHBR/normal rat; Table 1) of M3 was more than that of M2. We believe that this discrepancy can be accounted for by the difference in the unbound fraction in the liver.

PET image analyses of hepatobiliary transport in EHBRs provided some interesting findings. For example, the hepatic distribution of radioactivity in EHBRs was similar to that in normal rats in our study, whereas Chu et al. (1997) reported that the hepatic concentration of Mrp2 substrates was increased in Mrp2-deficient rats. Moreover, the decrease in the renal distribution of radioactivity was also observed in EHBRs. Mrp3 mediates transport of conjugated organic anions across the basolateral membrane from hepatocytes to the sinusoidal blood. It has been shown that Mrp3 expression in the liver and kidney is up-regulated in EHBRs, but its expression is very low in normal rats (Hirohashi et al., 1998; Kuroda et al., 2004). Therefore, in EHBRs major metabolite M3 presumably is transported across the sinusoidal membrane from hepatocytes and renal tubular epithelial cells into the circulating blood by up-regulated Mrp3.

In this study, the CLuptake,liver of 15R-[11C]TIC was slightly decreased in EHBRs. Expression of Oatp1a1 and Oat1a4 is down-regulated in terms of both mRNA and protein level in EHBRs (Kuroda et al., 2004). In addition, the high concentration of bilirubin glucuronide typically found in EHBRs might inhibit the uptake of ligands (Kurisu et al., 1991), which, together with decreased transporter activity caused by down-regulation of Oat expression, may explain the observed decrease in the CLuptake,liver of 15R-[11C]TIC. Determining whether 15R-[11C]TIC-Me is useful as a radiotracer for the functional analysis of Oatp1a1 and Oat1a4 will require further investigation.

In terms of clinical applications of PET studies, the use of 15R-[11C]TIC-Me may be preferable to 15R-[11C]TIC, because 15R-[11C]TIC-Me has a history of clinical use (Suzuki et al., 2004). Quantitative PET image analysis using 15R-[11C]TIC-Me provides a tool for noninvasive in vivo kinetic assessment of hepatobiliary excretion and possibly renal excretion in humans and may enable researchers to uncover the metabolic impact of genetic polymorphisms or drug–drug interactions involving MRP2. We preliminarily succeeded in quantitative PET imaging of the hepatobiliary excretion in human by using 15R-[11C]TIC-Me with lower radioactive dose (less than 60 MBq/human body), in which radiation exposure (less than 0.3 mSv) is considered to be a safe level (Virta et al., 2008). For instance, Hirouchi et al. (2004) characterized the expression level, localization, and function of single-nucleotide polymorphism variants of the MRP2 protein by quantitative PET imaging. Clinical studies revealed the association of a genetic polymorphism in MRP2 with docetaxel-induced leucopenia (Kiyotani et al., 2008) and an association between MRP2 polymorphisms/haplotype and irinotecan disposition and diarrhea (de Jong et al., 2007). Horikawa et al. (2002) suggested that clinical doses of probenecid, uricosuric, and renal tubular blocking agent would be able to also inhibit MRP2. Considering the results of our in vitro transport experiments, quantitative measurement of MRP2 activity in human liver necessitates that 15R-[11C]TIC be converted to M2 or M3. Elucidating the metabolism of 15R-[11C]TIC in the human liver is therefore a prerequisite for its use in human clinical studies involving PET imaging.

In conclusion, we demonstrated the utility of noninvasive
PET imaging by using 15R-[11C]TIC-Me as a tool for kinetic analysis of hepatobiliary transport and renal excretion. We demonstrated that PET can be used in the same animal for the separate quantitative evaluation of hepatic uptake and biliary excretion. Our in vivo evaluation of the role of Mrp2 in the biliary excretion process using 15R-[11C]TIC-Me as a radiotracer indicates that it is a good probe for studying the function of other transporter proteins in a clinical setting.

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References


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