Mechanistic Studies on the Absorption and Disposition of Scutellarin in Humans: Selective OATP2B1-Mediated Hepatic Uptake Is a Likely Key Determinant for Its Unique Pharmacokinetic Characteristics

Chunying Gao, Hongjian Zhang, Zitao Guo, Tiangeng You, Xiaoyan Chen, and Dafang Zhong

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (C.G., X.C., Z.G., D.Z.); College of Pharmaceutical Sciences, Soochow University, Suzhou, People’s Republic of China (H.Z.); and Shanghai East Hospital, Shanghai, China (T.Y.)

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ABSTRACT:

Scutellarin [scutellarein-7-O-glucuronide (S-7-G)] displayed a unique pharmacokinetic profile in humans after oral administration: the original compound was hardly detected, whereas its isomeric metabolite isoscutellarin [scutellarein-6-O-glucuronide (S-6-G)] had a markedly high exposure. Previous rat study revealed that S-7-G and S-6-G in the blood mainly originated from their aglycone in enterocytes, and that the S-7-G/S-6-G ratio declined dramatically because of a higher hepatic elimination of S-7-G. In the present study, metabolite profiling in human excreta demonstrated that the major metabolic pathway for S-6-G and S-7-G was through further glucuronidation. To further understand the cause for the exposure difference between S-7-G and S-6-G in humans, studies were conducted to uncover mechanisms underlying their formation and elimination. In vitro metabolism study suggested that S-7-G was formed more easily but metabolized more slowly in human intestinal and hepatic microsomes. Efflux transporter study showed that S-6-G and S-7-G were good substrates of breast cancer resistance protein and multidrug resistance-associated protein (MRP) 2 and possible substrates of MRP3; however, there was no preference great enough to alter the S-7-G/S-6-G ratio in the blood. Among the major hepatic anion uptake transporters, organic anion-transporting polypeptide (OATP) 2B1 played a predominant role in the hepatic uptake of S-6-G and S-7-G and showed greater preference for S-6-G with higher affinity than S-6-G (Km values were 1.77 and 43.9 μM, respectively). Considering the low intrinsic permeability of S-6-G and S-7-G and the role of OATP2B1 in the hepatic clearance of such compounds, the selective hepatic uptake of S-7-G mediated by OATP2B1 is likely a key determinant for the much lower systemic exposure of S-7-G than S-6-G in humans.

Introduction

Scutellarin or scutellarein-7-O-glucuronide (S-7-G) (Fig. 1) is an active flavonoid component in Erigeron breviscapus (Vant.) Hand-Mazz extracts. It is widely used in China for the treatment of cardiovascular diseases. Although S-7-G has been used in clinical therapy for more than 30 years, its metabolism and pharmacokinetic properties in humans remained largely unknown until the first report by our laboratory in 2006 (Chen et al., 2006). After an oral dose of 60 mg of S-7-G, the parent drug could hardly be detected in human plasma (<4 nM). Instead, its isomeric metabolite, scutellarein-6-O-glucuronide (S-6-G) (Fig. 1), exhibited markedly higher systemic exposure (Cmax was approximately 188 nM). This marked exposure difference between S-7-G and S-6-G in humans has intrigued us to design and perform a series of in vitro and in vivo studies, aiming to understand mechanisms underlying the absorption and disposition processes after oral administration of S-7-G. In an earlier study in rats from our laboratory (Gao et al., 2011), a high exposure of S-6-G was also observed after an oral dose of S-7-G, suggesting that the rat is a suitable in vivo model to investigate mechanisms associated with S-7-G absorption and disposition. The rat study also led to the following major findings (Gao et al., 2011): 1) after oral administration, S-7-G is primarily absorbed as its aglycone scutellarein (Fig. 1) after hydrolysis in the intestinal tract; 2) S-6-G and S-7-G in the blood circulation were mainly originated from their aglycone in enterocytes, and that the S-7-G/S-6-G ratio declined dramatically because of a higher hepatic elimination of S-7-G. In the present study, metabolite profiling in human excreta demonstrated that the major metabolic pathway for S-6-G and S-7-G was through further glucuronidation. To further understand the cause for the exposure difference between S-7-G and S-6-G in humans, studies were conducted to uncover mechanisms underlying their formation and elimination. In vitro metabolism study suggested that S-7-G was formed more easily but metabolized more slowly in human intestinal and hepatic microsomes. Efflux transporter study showed that S-6-G and S-7-G were good substrates of breast cancer resistance protein and multidrug resistance-associated protein (MRP) 2 and possible substrates of MRP3; however, there was no preference great enough to alter the S-7-G/S-6-G ratio in the blood. Among the major hepatic anion uptake transporters, organic anion-transporting polypeptide (OATP) 2B1 played a predominant role in the hepatic uptake of S-6-G and S-7-G and showed greater preference for S-6-G with higher affinity than S-6-G (Km values were 1.77 and 43.9 μM, respectively). Considering the low intrinsic permeability of S-6-G and S-7-G and the role of OATP2B1 in the hepatic clearance of such compounds, the selective hepatic uptake of S-7-G mediated by OATP2B1 is likely a key determinant for the much lower systemic exposure of S-7-G than S-6-G in humans.

ABBREVIATIONS: S-7-G, scutellarin; scutellarein-7-O-glucuronide; S-6-G, isoscutellarin, scutellarein-6-O-glucuronide; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; HIM, human intestinal microsomes; UGT, UDP-glucuronosyltransferase; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; HBSS, Hanks’ balanced salt solution; HPLC, human embryonic kidney; OATP, organic anion-transporting polypeptide; OAT, organic anion transporter; RIM, rat intestinal microsomes; MDR, multidrug resistance protein; UPLC, ultra-performance liquid chromatography; TOF, time of flight; MS, mass spectrometry; LC-MS/MS, liquid chromatography/tandem mass spectrometry; E2-17β-G, estradiol-17β-o-glucuronide; CE, collision energy; S-6,7-diG, scutellarein-6,7-diglucuronide; GlcUA, glucuronic acid.
from the aglycone via intestinal glucuronidation; and 3) S-7-G undergoes a higher hepatic presystemic elimination than S-6-G, resulting in a dramatic decline in the S-7-G/S-6-G ratio in the rat blood.

Because metabolism and carrier-mediated transport (both efflux and uptake) are two key biochemical processes governing drug disposition, there is a possibility that a marked difference between S-6-G and S-7-G may exist with regard to their formation, metabolism, and transport in humans and rats after oral administration. Although a few metabolism or transporter studies have been reported with the focus on S-7-G (Cao et al., 2008; You et al., 2010; Wang et al., 2011), limited data are available on the disposition of S-6-G or the differences between S-6-G and S-7-G in terms of their formation and elimination mechanisms.

Therefore, the current study was performed to further explain the human pharmacokinetics of S-7-G through mechanistic studies with respect to the formation, metabolism, and transport of S-6-G and S-7-G. Experiments were conducted to accomplish the following goals: 1) identify the major metabolic route of S-7-G and S-6-G in humans by metabolite profiling of the human bile and urine after the dose; 2) compare the formation and subsequent metabolism of S-6-G and S-7-G by hepatic and intestinal microsomes, as well as by participating enzymes; and 3) identify transporters involved in the intestinal efflux and hepatic uptake of S-6-G and S-7-G and examine the potential selectivity between S-6-G and S-7-G.

Materials and Methods

Chemicals. S-7-G was purchased from Shanghai Standard Biotech Co., Ltd. (Shanghai, China), and scutellarein was purchased from Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). S-6-G [99.4% purity, as determined by high-performance liquid chromatography (HPLC)] was isolated from human urine and then purified using a method described previously (Chen et al., 2006). UDP-GlcUA, alamethicin, ATP, BCA protein assay kit, and Hanks’ buffered salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO). The following were purchased from BD Biosciences (San Jose, CA): pooled human liver microsomes (HLM), human intestinal microsomes (HIM), rat liver microsomes (RLM), recombinant UDP-glucuronosyltransferases (UGTs) (including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17), and inside-out S9 insect cell membrane vesicles expressing multidrug resistance-associated protein (MRP) 1, MRP2, MRP3, and breast cancer resistance protein (BCPFR). Rat intestinal microsomes (RIM) were provided by GenoMembrane Co., Ltd. (Yokohama, Japan). All other reagents and solvents were of either analytical or HPLC grade.

Organic anion-transporting polypeptide (OATP) 1B3- and OATP2B1-expressing human embryonic kidney 293 (HEK293) cell lines were constructed at HD Biosciences Co., Ltd. (Shanghai, China). OATP1B1-expressing HEK293 and organic anion transporter (OAT) 2-expressing S2 cell lines were provided by Dr. Xiulin Yi from the Tianjin Institute of Pharmaceutical Research (Tianjin, China).

Metabolite Profiling in Human Bile and Urine. Subjects and bile sample collection. Two patients (one female, 53 years old; one male, 60 years old), who suffered from hypertension and were surgically treated for cholelithiasis and cholecystitis, respectively, provided informed consent to participate in this project. The clinical study was conducted at Shanghai East Hospital (Shanghai, China) in accordance with the Declaration of Helsinki and State Food and Drug Administration guidelines for good clinical practice. The hospital ethics committee approved the study protocol. After the patients were administered an oral dose of scutellarin tablet (40 mg), bile was collected from their T-tubes for 20 h. The bile samples were acidified to pH 2 with 8 M phosphoric acid and stored at −80°C until analysis.

Subjects and urine sample collection. Two healthy volunteers (one male and one female, 26 years old) provided informed consent to participate in this project. Urine was collected before and within 12 h after an oral dose of scutellarin tablet (40 mg). The samples were then acidified to pH 2 with 8 M phosphoric acid and stored at −80°C until analysis.

Sample preparation. An aliquot of 100 μl of bile or 200 μl of urine was added to a 2-fold volume of acetonitrile. After vortex-mixing and centrifugation at 11,000g for 5 min, the supernatant was collected, evaporated until dry under a stream of nitrogen at 40°C, and reconstituted in 100 μl of methanol-water-formic acid (30:70:1, v/v/v).

In Vitro Glucuronidation. Formation of S-6-G and S-7-G from the aglycone. Aglycone glucuronidation in HIM, RIM, HLM, and RLM was investigated. The incubation time and microsomal protein concentrations were optimized to 5 min and 0.05 mg protein/ml, respectively, to determine accurate kinetic parameters for the reaction. The samples were incubated at 37°C in a Tris-HCl (100 mM, pH 7.4)-buffered system consisting of UDP-GlcUA (1 mM), alamethicin (50 μg/mg protein), and MgCl2 (10 mM). The reactions were terminated by addition of the same volume of ice-cold 2% formic acid in acetonitrile (v/v). Ultraperformance liquid chromatography (UPLC)-quadrupole/time-of-flight (TOF) mass spectrometry (MS) was used to screen the major metabolites, and HPLC/UVD was used to quantify S-7-G, S-6-G, and the aglycone.

Formation of Scutellarein-6,7-Digluconuride from S-7-G and S-6-G. S-7-G and S-6-G glucuronidation was investigated in HIM, RIM, HLM, and RLM. For the enzyme kinetic study, the incubation time was optimized to 7 min for S-6-G in all microsomal incubations, to 7 min for S-7-G in rat microsomes, and
to 20 min for S-7-G in human microsomes. The microsomal protein concentration was optimized to 0.2 mg of protein/ml. Incubations were performed as described in the previous paragraph. HPLC/UV was used to quantify S-7-G and S-6-G and to semiquantify the main metabolite using S-7-G as the calibration standard.

**Reaction Phenotyping.** S-7-G (8 and 40 μM), S-6-G (8 and 40 μM), and the aglycone (8 and 80 μM) were separately incubated with the 12 recombinant UGTs (0.2 mg of protein/ml) in a Tris-HCl (100 mM, pH 7.4)-buffered system consisting of UDP-GlcUA (1 mM), alamethicin (50 μg/mg protein), and MgCl₂ (10 mM). Reactions were allowed to proceed at 37°C for 25 min and then terminated by addition of the same volume of ice-cold 2% formic acid in acetonitrile (v/v). HPLC/UV was used to quantify S-7-G and S-6-G in the incubations. Scutellarein-6,7-diglucuronide (S-6,7-diG) was semiquantified using S-7-G as the calibration standard.

**Calculation.** The main kinetic parameters were calculated by Prism 5 (GraphPad Software, Inc., San Diego, CA) using a typical Michaelis-Menten equation (eq. 1). In cases where significant substrate inhibition was observed, a modified Michaelis-Menten equation (eq. 2) was adopted.

\[
V = \frac{V_{max}}{1 + \frac{K_m}{S}}
\]

\[
V = \frac{V_{max}}{1 + K_a/S}
\]

**Fig. 2.** Metabolite profiles of the human bile and urine after an oral dose of S-7-G. S-7-G was administered at a dose of 40 mg. A, mass defect filter (MDF) chromatograms of the human bile collected from two patients receiving gallbladder surgeries during the postdose period of 0 to 20 h. B, MDF chromatograms of the human urine collected from two healthy volunteers within the postdose period of 0 to 12 h.
than 10 mM. (BD Biosciences). In brief, membrane vesicles (40 g of protein) and test cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mML-glutamine, 1% modified Eagle’s medium nonessential amino acid (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere containing 5% CO2 at 37°C for HEK293 cells and at 28°C for S2 cells. Cells were split at a 1:5 ratio every 3 to 4 days.

**Studies using inside-out membrane vesicles.** The efflux of S-6-G and S-7-G was evaluated in human MRP1-, MRP2-, MRP3-, MDR1-, and BCRP-expressing membrane vesicles. Transport assays used a modified rapid filtration technique based on the manufacturer’s protocol (BD Biosciences). In brief, membrane vesicles (40 g of protein) and test compounds were incubated with or without ATP (5 mM) in the transport medium (60 μL, pH 7.4) containing 10 mM Tris-HCl, 10 mM MgCl2, 250 mM sucrose, and 2.5 mM GSH. Transport was terminated at the designated time point by addition of 200 μL of ice-cold wash buffer (10 mM Tris-HCl and 250 mM sucrose). The incubation mix was rapidly transferred to a Millipore 96-well glass fiber filter plate (Millipore Corporation, Billerica, MA) and then washed five times with ice-cold wash buffer. The compound trapped in the vesicles was retained on the filters and released by addition of 200 μL of methanol-water (70:30, v/v). The compound inside the vesicles was finally analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The study first investigated the inhibitory effects of S-6-G and S-7-G on the MRP-, MDR1-, or BCRP-mediated transport of a probe substrate. The following were used as probe substrates: methotrexate (400 μM) for BCRP, N-methyl quinine (5 μM) for MDR1, and estradiol-17β-α-glucuronide (E2-7β-G) at concentrations of 20, 100, and 10 μM for MRP1, MRP2, and MRP3, respectively. The direct transport of S-6-G and S-7-G via the four efflux transporters was evaluated by incubating the two compounds with the membrane vesicles at two concentrations around the IC50 with or without ATP. ATP-dependent transport was evaluated according to the ratios of the transport with ATP to that without ATP.

**Hepatic Uptake Transporter Study.** Cell culture. Transporter-transfected cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% modified Eagle’s medium nonessential amino acid (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere containing 5% CO2 at 37°C for HEK2923 cells and at 28°C for S2 cells. Cells were split at a 1:5 ratio every 3 to 4 days.

**Uptake Transporter Study.** S-6-G and S-7-G uptake by OATP1B1, OATP1B3, OATP2B1, and OAT2 was evaluated using transporter-transfected cell lines according to a method reported previously (Han et al., 2010). In brief, transfected HEK2923 or S2 and mock control cells were seeded on BioCoat poly-D-lysine-coated 24-well plates (BD Biosciences) at a density of 3.0 × 105 cells/well. After 36 h of culture, the cells were washed twice and equilibrated in HBSS for 10 min. The uptake was initiated by adding 0.5 mL of HBSS

\[ V = V_{max}/(1 + K_a/S + S/K_i) \]

(2) where \( V_{max} \) represents the maximal velocity, \( K_a \) is the substrate concentration when the velocity is half the \( V_{max} \), and \( K_i \) is the self-inhibition constant. The modified Michaelis-Menten equation (eq. 2) was used only when \( K_i \) was less than 10 mM.

**Efflux Transporter Study.** Studies using inside-out membrane vesicles. The efflux of S-6-G and S-7-G was evaluated in human MRP1-, MRP2-, MRP3-, MDR1-, and BCRP-expressing membrane vesicles. Transport assays used a modified rapid filtration technique based on the manufacturer’s protocol (BD Biosciences). In brief, membrane vesicles (40 μg of protein) and test compounds were incubated with or without ATP (5 mM) in the transport medium (60 μL, pH 7.4) containing 10 mM Tris-HCl, 10 mM MgCl2, 250 mM sucrose, and 2.5 mM GSH. Transport was terminated at the designated time point by addition of 200 μL of ice-cold wash buffer (10 mM Tris-HCl and 250 mM sucrose). The incubation mix was rapidly transferred to a Millipore 96-well glass fiber filter plate (Millipore Corporation, Billerica, MA) and then washed five times with ice-cold wash buffer. The compound trapped in the membrane vesicles was retained on the filters and released by addition of 200 μL of methanol-water (70:30, v/v). The compound inside the vesicles was finally analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The study first investigated the inhibitory effects of S-6-G and S-7-G on the MRP-, MDR1-, or BCRP-mediated transport of a probe substrate. The

### TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M + H]+</th>
<th>Proposed Formula</th>
<th>Metabolic Pathway</th>
<th>Fragment Ions</th>
<th>Retention Time</th>
<th>Relative MS Peak Area in Bile</th>
<th>Relative MS Peak Area in Urine</th>
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<tr>
<td>M0-1</td>
<td>463.083</td>
<td>C13H11O12</td>
<td>S-7-G</td>
<td>287.051, 269.043, 169.010, 123.004</td>
<td>7.93</td>
<td>31.3</td>
<td>14.7</td>
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<tr>
<td>M0-2</td>
<td>463.087</td>
<td>C13H11O12</td>
<td>S-6-G</td>
<td>287.051, 269.043, 169.010, 123.004</td>
<td>9.19</td>
<td>8.72</td>
<td>17.4</td>
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<tr>
<td>M2</td>
<td>639.120</td>
<td>C13H11O18</td>
<td>Glucuronic conjugation</td>
<td>463.084, 287.054, 269.043, 123.005</td>
<td>6.02</td>
<td>36.8</td>
<td>20.5</td>
</tr>
<tr>
<td>M3-1</td>
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<td>C13H11O12</td>
<td>Glucoside conjugation</td>
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<td>6.05</td>
<td>1.22</td>
<td>21.3</td>
</tr>
<tr>
<td>M3-2</td>
<td>625.141</td>
<td>C13H11O12</td>
<td>Methylation</td>
<td>301.070, 286.045, 168.003</td>
<td>9.68</td>
<td>3.08</td>
<td>21.3</td>
</tr>
<tr>
<td>M4-1</td>
<td>477.013</td>
<td>C13H11O12</td>
<td>Methylation</td>
<td>301.070, 286.045, 168.003</td>
<td>9.68</td>
<td>3.08</td>
<td>21.3</td>
</tr>
<tr>
<td>M4-2</td>
<td>477.012</td>
<td>C13H11O12</td>
<td>Methylation</td>
<td>301.069, 286.045, 168.003</td>
<td>10.02</td>
<td>9.88</td>
<td>17.6</td>
</tr>
<tr>
<td>M5</td>
<td>479.081</td>
<td>C13H11O13</td>
<td>Hydroxylation</td>
<td>303.048, 285.039</td>
<td>7.46</td>
<td>5.26</td>
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Fig. 3. Formation kinetic curves of S-7-G (●) and S-6-G (■) in the intestinal and liver microsomes of humans (A and B) and of rats (C and D). Aglycone was incubated with microsomes (0.05 mg protein/ml) at various concentration levels for 5 min (n = 2).
 containing test compounds to the corresponding cell wells and then terminated at the designated time by aspirating the medium and washing the cells twice with 2 mL of ice-cold HBSS. After uptake, cells were lysed with 0.2 mL of 2% formic acid-acetonitrile (v/v). LC-MS/MS was used to determine the concentrations of the test compounds. Cell density was corrected by dissolving the cultured cells in 0.1 M NaOH and 0.1% sodium deoxycholate. Total cellular protein levels were measured using a BCA protein assay kit.

**Analytical Conditions.** For transporter studies, LC-MS/MS was used to quantify S-6-G, S-7-G, methotrexate, and E2-17\-G. Separation of analytes from matrix was achieved via an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) on a C18 column, and MS detection was conducted by Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies) signals. Multiple reaction monitoring fragmentation transitions of

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m/z 447, 463, 545, 625, 755, 308 (\text{methotrexate}), \text{ and } m/z 455, 308 (\text{enkephalin})\]

were monitored in negative mode.

**Results.** **Metabolite Profiling in Human Bile and Urine.** Within 0 to 20 h after the oral administration of 40 mg of S-7-G, nine drug-related components were detected in the bile collected from the patients that received gallbladder surgeries (Fig. 2A; Table 1). Similar to the metabolite profiles in rat bile (Gao et al., 2011), most of the detected metabolites in the human bile were formed through phase II conjugation, such as glucuronide conjugation (M2, \(m/z\ 639,120\)), methylation (M4, \(m/z\ 477,102\)), and glycoside conjugation (M3, \(m/z\ 625,142\)). In addition to the above metabolites, the isomeric metabolite S-6-G (M0-2, \(m/z\ 463,087\)) was found. Different from the findings in rats, a hydroxylated metabolite (M6, \(m/z\ 479,081\)) appeared, and the S-7-G aglycone was absent in human bile. Although the types of the major metabolites were mostly consistent in the two patients, the levels of all the drug-related metabolites were much lower in patient 2 than in patient 1 (Fig. 2A). According to the medical record of patient 2, the patient suffered from cholecirosis and had been administered cefoperazone (antibiotic), ornidazole, omeprazole, and five other drugs for days before the sample collection. The low levels of biliary metabolites might be caused by the antibiotic-induced suppression of the intestinal bacteria, which is crucial to the intestinal absorption of S-7-G. Considering the condition of patient 2 and the potential complexity caused by drug-drug interactions, we mainly used the data from patient 1 for further discussion. According to the relative MS peak areas of the major metabolites in the bile from patient 1 (Table 1), the most abundant metabolite in human bile is S-6,7-diG (M2) followed by S-7-G (M0-1), which was excreted in amounts approximately 3.6 times larger than those of S-6-G (M0-2). After oral administration of 40 mg of S-7-G, a total of six drug-related components were detected in the urine collected from healthy

**Fig. 4.** Formation of S-7-G (A) and S-6-G (B) from aglycone in incubations of human recombinant UGT. Aglycone was incubated with UGT enzymes (0.2 mg protein/ml) for 25 min at concentrations of 8 (solid) and 80 \(\mu\)M (hollow). Data points represent the means of duplicates.

**TABLE 2**

<table>
<thead>
<tr>
<th>Formation of S-7-G</th>
<th>Formation of S-6-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m)</td>
<td>(V_{max})</td>
</tr>
<tr>
<td>(\mu)M</td>
<td>mmol \cdot min(^{-1}) \cdot mg protein(^{-1})</td>
</tr>
<tr>
<td>HIM</td>
<td>5.90 ± 1.68</td>
</tr>
<tr>
<td>HLM</td>
<td>6.32 ± 1.18</td>
</tr>
<tr>
<td>RLM</td>
<td>9.00 ± 1.92</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>4.49 ± 0.89</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>7.34 ± 0.90</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>27.0 ± 4.57</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>33.3 ± 7.03</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>19.0 ± 2.39</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>5.49 ± 0.71</td>
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<tr>
<td>UGT1A10</td>
<td>5.68 ± 0.92</td>
</tr>
<tr>
<td>UGT2B7</td>
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</table>
volunteers during the period of 0 to 12 h postdose (Fig. 2B). Except for the absence of two glucose conjugates (M3-1 and M3-3) and a hydroxylated metabolite (M6), all other metabolites found in human bile were also detected in urine. The relative MS peak areas indicated that S-6,7-diG (M2) was also the most abundant metabolite in human urine. However, different from the findings from bile samples, the second most abundant metabolite in urine was S-6-G, with an excreted amount approximately 4.4 times higher than that of S-7-G. Table 1 lists the detailed chromatographic and MS information of the major metabolites in human bile and urine.

**Aglycone Glucuronidation in the Intestinal and Hepatic Microsomes.** The aglycone was rapidly glucuronidated in the UDP-GlcUA-supplemented intestinal and hepatic microsomes of both humans and rats. In each of the incubations, the aglycone was conjugated into S-6-G and, with significant preference, into S-7-G (Fig. 3). Table 2 lists the $K_m$ and $V_{max}$ values determined for the formation of S-6-G and S-7-G. The formation-substrate concentration curve and kinetic parameters were not obtained for S-6-G in rat microsomes, because its concentration in the incubation was too low for quantification. The $K_m$ values for the formation of both S-7-G and S-6-G were very low and close to each other in all four microsomes, ranging from 2.86 to 16.2 μM, whereas the $V_{max}$ values were highly different among products and microsomes. Although significant regioselectivity for the 7-O position was observed for the aglycone glucuronidation in all of the studied catalytic systems, the extent in HIM was not as high as that in RIM: the $V_{max}/K_m$ ratio for the formation of S-7-G was approximately 2.7 times higher than that of S-6-G in HIM, whereas the formation of S-6-G in RIM was too slow for an accurate analysis.

![Formation kinetic curves of S-7-G (○) and S-6-G (■) in human recombinant UGT. Aglycone was incubated with UGT enzymes (0.1 mg protein/ml) at various concentration levels for 10 min (n = 2).](https://dmd.aspetjournals.org/content/2014/11/01/Fig5.jpg)
Aglycone Glucuronidation by Human Recombinant UGT. S-6-G and S-7-G formation was studied in UDP-GlcUA-supplemented incubations of the aglycone with 12 different human UGT isoforms (i.e., UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17). In the phenotypic study, UGT1A9 exhibited the highest catalytic activity for both S-7-G and S-6-G formation (Fig. 4). Besides UGT1A9, some isoforms, including UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, and UGT1A10, also catalyzed S-7-G formation, and other isoforms, including UGT1A1, UGT1A8, UGT1A10, and UGT2B7, mediated S-6-G formation. According to further kinetic studies (Fig. 5; Table 2), S-6-G and S-7-G formation followed the typical Michaelis-Menten equation in almost all of the enzymes involved, except for UGT1A1, which exhibited significant substrate inhibition on S-7-G formation with the aglycone. Comparing S-6-G and S-7-G formation in each of the enzymes, except for UGT2B7, which only mediated S-6-G formation, all the other enzymes exhibited high preference for S-7-G formation.

Glucuronidation of S-6-G and S-7-G in the Intestinal and Hepatic Microsomes. The metabolic clearance of S-6-G and S-7-G in the body was compared by studying their further glucuronidation in the intestinal and hepatic microsomes of both humans and rats. The kinetic curves and corresponding parameters observed for the S-6,7-diG (M2) formation are shown in Fig. 6 and Table 3, respectively.

Although S-6-G and S-7-G glucuronidation could be catalyzed by both human and rat microsomes, a significant species difference was observed: in humans, S-6-G > S-7-G; in rats, S-7-G > S-6-G. In human hepatic and intestinal microsomes, the $V_{\text{max}}/K_{\text{m}}$ ratio for the glucuronidation of S-6-G was 10.8 and 5.95 times higher than that of S-7-G, respectively. In addition, all of the human and rat hepatic and intestinal microsomes exhibited significant substrate inhibition with the two isomers (Fig. 6).

### TABLE 3

<table>
<thead>
<tr>
<th>Incubation of S-6-G</th>
<th>Incubation of S-6-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>HIM</td>
<td>643 ± 69</td>
</tr>
<tr>
<td>HLM</td>
<td>62.4 ± 10.0</td>
</tr>
<tr>
<td>RIM</td>
<td>349 ± 125</td>
</tr>
<tr>
<td>RLM</td>
<td>15.2 ± 2.3</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>17.4 ± 52</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>198 ± 21</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>225 ± 105</td>
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<td>198 ± 21</td>
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<tr>
<td>UGT1A10</td>
<td>225 ± 105</td>
</tr>
</tbody>
</table>

**FIG. 6.** Glucuronidation kinetic curves of S-7-G (●) and S-6-G (■) in the intestinal and liver microsomes of humans (A and B) and of rats (C and D). S-6-G or S-7-G was incubated with microsomes (0.2 mg protein/ml) at various concentration levels for 5 min, except for S-7-G, which was incubated with liver microsomes for 20 min ($n = 2$).
was also observed in human microsomes but not in UGT1A8 and UGT1A10. Thus, UGT1A1 appears to be significant in the glucuronidation of S-6-G in the HIM and HLM, considering the similar catalytic potency, substrate selectivity, and extent of substrate inhibition.

**Efflux Transporter Study.** To investigate the efflux mechanisms of S-6-G and S-7-G in the human intestine, the major intestinal efflux transporters including MRP1, MRP2, MRP3, MDR1, and BCRP were studied using the transporter-expressing membrane vesicle systems. Indirect assays were first conducted by determining the inhibitory effects of S-6-G and S-7-G on substrate transport to obtain a general understanding on the affinity of S-6-G and S-7-G to the transporter proteins. In these indirect assays, the transport of E2-17β-G mediated by MRP2 and that of methotrexate by BCRP were markedly inhibited by both S-6-G (IC₅₀ values were 50 and 20 μM for MRP2 and BCRP, respectively) and S-7-G (IC₅₀ values were 75 and 10 μM for MRP2 and BCRP, respectively); the transport of MRP3 was inhibited by S-6-G, but not S-7-G; and slight inhibition of S-7-G and S-6-G was observed on MRPl and MDR1 (Fig. 9). According to their inhibitory capability on the functions of transporters, the affinities of S-6-G and S-7-G to transporter proteins were on the order of BCRP > MRP2 > MRP1 > MRP3 > MDR1 for S-7-G and BCRP > MRP3 > MRP2 > MRP1 > MDR1 for S-6-G. Further studies were conducted by measuring the uptakes of S-6-G and S-7-G into the BCRP-, MRP1-, MRP2-, and MRP3-expressing membrane vesicles to determine whether S-6-G and S-7-G are substrates of transporters that can be inhibited in the indirect assays. As shown in Fig. 10, the ratio of ATP-dependent uptake to nonspecific adsorption (+ATP−/−ATP ratio) of S-6-G and S-7-G was highest in BCRP-expressing vesicles (57.5–65.1), followed by MRP2 (3.07–4.32) and MRP3 (1.61–2.85). No significant ATP-dependent transport was observed in MRPl for either S-6-G or S-7-G. Comparing the effluxes of S-6-G and S-7-G by the same transporter, no significant difference was observed for MRP2 and BCRP in terms of the +ATP−/−ATP ratio, except that MRP3 showed a slight preference for S-6-G.

**Uptake Transporter Study.** A phenotypic study was performed using S2 cells that express OAT2 and HEK293 cells that individually express OATP1B1, OATP1B3, and OATP2B1 to identify transporters that mediate the hepatic uptake of S-6-G and S-7-G in humans. As shown in Fig. 11, the highest uptake was observed in OATP2B1-transfected cells for both S-7-G and S-6-G, which resulted in OATP2B1/mock ratios of 65.0 and 20.4, respectively. Unlike OATP2B1, only slight or negligible active uptake was observed for OATP1B1, OATP1B3, and OAT2 compared with values in the mock cells. In the presence of OATP2B1 substrate estrone-3-sulfate (30 and 100 μM), OATP2B1-mediated S-6-G (3 μM) and S-7-G (3 μM) uptakes were significantly inhibited (p < 0.01), and such inhibition was estrone-3-sulfate concentration-dependent (Fig. 12).

The time-dependent uptakes of S-6-G and S-7-G by OATP2B1 were measured at a substrate concentration of 2 μM and found to be linear against time within the first 20 min of incubation. OATP2B1-mediated uptakes of S-6-G and S-7-G were further determined at...
various substrate concentrations after 7 min of incubation. As shown in Fig. 13, the OATP2B1-mediated uptake of S-7-G rapidly increased as the substrate concentration increased from 0.3 to 3 μM and displayed a plateau at approximately 10 μM S-7-G without apparent substrate inhibition. Unlike S-7-G, the OATP2B1-mediated uptake of S-6-G increased over a much wider substrate concentration range from 0.3 to 30 μM, whereas apparent substrate inhibition was observed after reaching 30 μM (K_i = 25.9 ± 14.8 μM). Comparing the kinetic parameters of the two isomers, S-7-G exhibited much higher affinity for OATP2B1 than S-6-G, with K_m values of 1.77 ± 0.54 and 43.0 ± 23.7 μM, respectively, and the CL_int (V_max/K_m) for S-7-G (11.1 μl/mg protein/min) was approximately 4.3 times higher than that for S-6-G (2.6 μl/mg protein/min).

Discussion

Our previous study revealed a unique pharmacokinetic characteristic of S-7-G after an oral dose (60 mg) in humans: the level of S-7-G was extremely low (C_max was approximately 4 nM) in the plasma, whereas its isomeric metabolite S-6-G was present at much higher concentrations (C_max was approximately 188 nM) (Chen et al., 2006). A subsequent mechanistic study in rats not only confirmed the finding but also provided evidence suggesting that 1) the circulating S-7-G and S-6-G were originated from the glucuronidation of the aglycone, which was derived from the hydrolysis of S-7-G in the gut after oral administration and 2) hepatic clearance played an important role in maintaining the ratio of S-7-G/S-6-G in the blood (Gao et al., 2011). The current study focused on metabolic enzymes and drug transporters to uncover mechanisms underlying the formation, metabolism, and transport of S-6-G and S-7-G, with an expectation to identify key determinants that govern S-7-G human pharmacokinetics.

On the basis of the finding that S-7-G and S-6-G in the circulation mostly originated from the intestinal glucuronidation of the aglycone (Gao et al., 2011), we first examined the formation of S-7-G and S-6-G in intestinal microsomes. The results indicated that the aglycone was glucuronidated extensively in both HIM and RIM (Table 2; Fig. 3). Kinetic analysis of the total glucuronidation in HIM resulted in V_max, K_m, and V_max/K_m values of 6.74 nmol · min⁻¹ · mg protein⁻¹, 4.50 μM, and 1498 μl · min⁻¹ · mg protein⁻¹, respectively. By comparing the glucuronidation efficiency in HIM with that in RIM (V_max/K_m = 1924 μl · min⁻¹ · mg protein⁻¹), the catalytic capability was similar between the two species for the formation of S-7-G and S-6-G. In light of the previous finding (Gao et al., 2011) that almost...
no free aglycone was present in the mesenteric blood after injecting 10 mg of S-7-G into a rat intestinal segment (15 cm), the aglycone generated in the human intestinal tract (7.1 m) after oral administration of 60 mg S-7-G should undergo similar intestinal first-pass metabolism. Therefore, the intestine is believed to be the primary site for the formation of S-6-G and S-7-G in humans.

Regioselectivity for the 7-O position was observed during the glucuronidation of the aglycone (Table 2; Fig. 3), but the degree of selectivity varied in different species and tissues with the following order: RIM > HLM > HIM > RLM. Kinetic studies on the participating enzymes indicated that all of the UGT enzymes tested, except UGT2B7, exhibited higher catalytic efficiency for the formation of S-7-G than S-6-G (Table 3; Fig. 5). The difference in the regioselectivity between HLM and HIM might be due to differential expression and distribution of UGT enzymes in these tissues (Strassburg et al., 2000; Ohno and Nakajin, 2009). Another observation was that S-7-G formation in HIM was much lower than that in RIM, consistent with the findings that the systemic exposure ratio of S-7-G/S-6-G in humans (Chen et al., 2006) was lower than that in rats (Gao et al., 2011).

Because the formation of S-7-G from the aglycone is higher than that of S-6-G in HIM and RIM, its lower circulating levels after oral administration could not be explained by the hydrolysis-reformation mechanism; therefore, we examined metabolic clearance of S-7-G and S-6-G after their formation. The metabolite profiling of human bile and urine samples indicated that S-6-G and S-7-G were mainly metabolized to S-6,7-diG through subsequent glucuronidation in humans, a major metabolic pathway also observed in rats (Gao et al., 2011). The glucuronidation efficiency of S-6-G was much higher than that of S-7-G in HIM and RIM, suggesting that metabolic clearance of S-7-G via secondary glucuronidation was not a determinant in plasma S-7-G/S-6-G ratio in humans. Kinetic studies on the participating enzymes indicated that UGT1A1 and UGT1A8 exhibited a higher degree of selectivity toward S-6-G with high catalytic efficiency, whereas UGT1A10 showed selectivity for S-7-G yet with lower catalytic efficiency. Even though UGT1A10 is reported to be expressed highly and primarily in the intestine (Ohno and Nakajin, 2009) because of its low catalytic efficiency, the glucuronidation of S-6-G might still be favored over S-7-G in HIM because of high selectivity by UGT1A1 and UGT1A8. This selectivity for S-6-G glucuronidation was more obvious in HLM than in HIM, probably because of the absence of UGT1A10 in the human liver (Ohno and Nakajin, 2009).

Formation kinetics and metabolic clearance studies indicated that S-7-G was formed more easily but metabolized more slowly than S-6-G in human microsomes. Such metabolic selectivity would have led to a higher systemic exposure of S-7-G in humans. However, human pharmacokinetic data indicated otherwise where the plasma concentration of S-7-G was much lower than that of S-6-G over the entire sampling period (Chen et al., 2006). These conflicting yet intriguing results suggested that factors other than metabolism must exist that may be responsible for higher first-pass elimination of S-7-G and its low circulating concentrations.

Flavonoids are frequently reported as substrates (Walgren et al., 2000; Zhang et al., 2007, 2011) or modulators (Zhang et al., 2004; Wang et al., 2005; Morris and Zhang, 2006) of efflux and uptake transporters. Because of the poor passive permeability of S-6-G and S-7-G caused by their low lipophilicity (log P = −0.37), transporters may play crucial roles in their absorption, distribution, metabolism, and excretion (Wu and Benet, 2005; Shitara et al., 2006). Because S-6-G and S-7-G are primarily formed in enterocytes from aglycone, their transport into the blood or back into the intestinal tract could be mediated by some intestinal efflux transporters. As S-6-G and S-7-G
pass through the liver, the hepatic uptake mediated by the uptake transporters on the sinusoidal hepatocyte membrane may greatly affect their hepatic elimination, and so do the efflux transporters localized on the canalicular side of the hepatocyte membrane. Therefore, intestinal efflux and hepatic uptake transporters were investigated with special attention on their substrate selectivity toward S-6-G and S-7-G.

According to the literature (Giacomini et al., 2010), efflux transporters that are expressed on the apical enterocyte membrane mainly include MRP2, BCRP, and MDR1, whereas MRP3 and MRP1 (Berggren et al., 2007) are reportedly expressed on the basolateral membrane. The present study revealed that both S-6-G and S-7-G are strong substrates for MRP2 and BCRP, and weak substrates for MRP3. Taken together, however, none of the efflux transporters tested exhibited adequate selectivity between S-7-G and S-6-G, and therefore, the efflux transport could not be the primary cause for the observed human pharmacokinetic phenomenon.

The liver is regarded as the most important organ for the disposition of various endogenous and exogenous substances in the body. Before hepatic metabolism and biliary excretion, drugs need to enter the hepatocytes first, either through passive diffusion or mediated by transporters. Because of their poor passive permeability, the hepatic uptake of S-6-G and S-7-G mediated by transporters could be a key determinant in maintaining S-7-G/S-6-G ratio in the circulation. On the sinusoidal hepatocyte membrane, OATP1B1, OATP1B3, OATP2B1, and OAT2 are reported to be major uptake transporters for exogenous anions (Giacomini et al., 2010). Among these four transporters, OATP2B1 was found to be primarily responsible for the hepatic uptakes of S-6-G and S-7-G in the present study. A similar finding was also reported for baicalin, a structural analog of S-7-G (Zhang et al., 2011). By comparing the uptake kinetics of S-6-G versus S-7-G by OATP2B1, a significant difference \( (p < 0.05) \) was observed in terms of substrate affinity, where the \( K_m \) value for the transport of S-7-G was very low, approximately 1/24 of that for S-6-G \( (1.77 \pm 0.54 \text{ vs } 43.0 \pm 23.7 \text{ mM}) \). The \( V_{max}/K_m \) ratio of S-7-G was 4.3 times higher than that of S-6-G (Fig. 13) and the uptake rate of S-7-G was markedly higher than that of S-6-G. These results indicate that OATP2B1 is a high-affinity and high-capacity uptake transporter of S-7-G. Because S-7-G and S-6-G concentrations were below 200 nM in the human blood after an oral dose of S-7-G (Chen et al., 2006), it thus can be hypothesized that the OATP2B1-mediated hepatic uptake plays a key role in the hepatic elimination of S-7-G and S-6-G.

In summary, the present study examined mechanisms underlying the formation, metabolism, and transport of S-6-G and S-7-G and explored kinetic differences and/or selectivity by key participating enzymes and transporters. As illustrated in Fig. 14, the proposed absorption and disposition processes of S-7-G after oral administration are composed of the following key events: 1) after the hydrolysis of S-7-G in the intestinal tract, S-6-G and S-7-G were primarily formed through the intestinal glucuronidation of aglycone with a
S-7-G/S-6-G ratio of 2.7:1; 2) S-6-G and S-7-G formed within enterocytes can be excreted into the intestinal tract by MRP2 and BCRP or pumped into the mesenteric blood by MRP3 with similar selectivity; and 3) as S-6-G and S-7-G pass through the liver, OATP2B1 exhibits approximately 24-fold higher affinity and 4.3-fold higher uptake efficiency for S-7-G. Considering the crucial role played by uptake transporters in the hepatic elimination of low-permeability compounds, selective OATP2B1-mediated hepatic uptake of S-7-G is likely a key determinant for the much lower systemic exposure of S-7-G than S-6-G in humans.

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Authorship Contributions

Participated in research design: Gao, Zhang, and Zhong.
Conducted experiments: Gao, Guo, and You.
Contributed new reagents or analytic tools: Chen and Zhong.
Performed data analysis: Gao, Zhang, and Zhong.
Wrote or contributed to the writing of the manuscript: Gao, Zhang, and Zhong.

References


Address correspondence to: Dafang Zhong, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haisi Rd., Shanghai 201203, China.
E-mail: dfzhong@mail.shcnc.ac.cn