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

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Running Title
Mechanism of Absorption and Disposition of Scutellarin in Humans

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Number of text pages: 31 (Without Refs)
Number of tables: 3
Number of figures: 14
Number of references: 18
Number of words in the Abstract: 249
Number of words in the Introduction: 471
Number of words in the Discussion: 1499

ABBREVIATIONS: S-7-G, scutellarin, scutellarein-7-O-glucuronide; S-6-G, isoscutellarin, scutellarein-6-O-glucuronide; UDPGA, uridine-diphosphate-glucuronic acid; ATP, adenosine-triphosphate; UGTs, UDP-glucuronyltransferases; RIMs, rat intestinal microsomes; HIMs, human intestinal microsomes; RLMs, rat liver microsomes; HLMs, human liver microsomes; BCRP, breast cancer resistance protein; MDR1, multidrug resistance protein 1; MRP, multidrug resistance-associated protein; OATP, organic anion-transporting polypeptide; OAT, organic anion transporter; HEK293, human embryonic kidney cells; UPLC-Q/TOF MS, ultra performance liquid
chromatography-quadrupole/time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LC/UV, liquid chromatography with ultraviolet detection; Tris, tris(hydroxymethyl)aminomethane; ESI, electrospray ionization; MDF, mass defect filter.
Scutellarin (S-7-G) displayed a unique pharmacokinetic profile in humans after oral administration: the original compound was hardly detected, while its isomeric metabolite (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 S-7-G/S-6-G ratio declined dramatically due to 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 BCRP and MRP2 and possible substrates of MRP3, however there was no preference great enough to alter S-7-G/S-6-G ratio in the blood. Among the major hepatic anion uptake transporters, OATP2B1 played a predominant role in the hepatic uptake of S-6-G and S-7-G, and showed greater preference for S-7-G with higher affinity than S-6-G ($K_m$ were 1.77 and 43.9 $\mu$M, 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 cardio-cerebrovascular diseases. While 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 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 ($C_{\text{max}}$ was approximately 188 nM).

This marked exposure difference between S-7-G and S-6-G in humans has intrigued us to design and carry out a series of in vitro and in vivo studies, aiming to understand mechanisms underlying the absorption and disposition processes following 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) following hydrolysis in the intestinal tract; (2) S-6-G and S-7-G in the blood circulation were mainly originated 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.

Since 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), there are limited data 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 carried out 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 post 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 HPLC) was isolated from human urine and then purified using a previously described method (Chen et al., 2006). Uridine diphosphate glucuronic acid (UDPGA), alamethicin, adenosine-triphosphate (ATP), BCA protein assay kit, and Hank’s buffered salt solution were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The following were purchased from BD Biosciences (Woburn, MA, USA): pooled human liver microsomes (HLMs),
human intestinal microsomes (HIMs), rat liver microsomes (RLMs), recombinant UGTs (including 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17), and inside-out Sf9 insect cell membrane vesicles expressing MRP1, MRP2, MRP3, and BCPR. Rat intestinal microsomes (RIMs) was provided by Xenotech (Lenexa, KS, USA). Inside-out Sf9 insect cell membrane vesicles expressing MDR1 were purchased from GenoMembrane Co., Ltd. (Yokohama, Japan). All other reagents and solvents were of either analytical or HPLC grade.

OATP1B3- and OATP2B1-expressing HEK293 cell lines were constructed at HD Biosciences Co., Ltd. (Shanghai, China). OATP1B1-expressing HEK293 and OAT2-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 with 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 prior to 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 11,000 g 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 HIMs, RIMs, HLMs, and RLMs 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 UDPGA (1mM), 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). UPLC-Q/TOF MS was used to screen the major metabolites, and HPLC/UV was used to quantify S-7-G, S-6-G, and the aglycone.

Formation of S-6,7-diG from S-7-G and S-6-G. S-7-G and S-6-G glucuronidation was investigated in HIMs, RIMs, HLMs, and RLMs. 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 protein/mL. Incubations were carried out as described in the previous paragraph. HPLC/UV was used to quantify S-7-G and S-6-G, and to semi-quantify 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 protein/mL) in a Tris-HCl (100 mM, pH 7.4) buffered system consisting of UDPGA (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. S-6,7-diG was semi-quantified using S-7-G as the calibration standard.

**Calculation.** The main kinetic parameters were calculated by Prism 5 (GraphPad Software, Inc., USA) 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.

\[
V = \frac{V_{\text{max}}}{1 + \frac{K_m}{S}} \quad (\text{Eq. 1})
\]

\[
V = \frac{V_{\text{max}}}{1 + \frac{K_m}{S} + \frac{S}{K_i}} \quad (\text{Eq. 2})
\]

where \( V_{\text{max}} \) represents the maximal velocity, \( K_m \) is the substrate concentration when the velocity is half the \( V_{\text{max}} \), 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 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 glutathione. 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 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).
compound inside the vesicles was finally analyzed by 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-methylquinine (NMQ, 5 μM) for MDR1, and E2-17β-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 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% MEM NEAA (Gibco), 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 d to 4 day.

**Uptake Transporter Study.** S-6-G and S-7-G uptake by OATP1B1, OATP1B3, OATP2B1, and OAT2 was evaluated using transporter-transfected cell lines based on a previously reported method (Han et al., 2010). In brief, transfected HEK-293 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 Hank’s balanced salt solution for 10 min. The uptake was initiated by adding 0.5 mL of HBSS 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 solution. 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%
SDS. 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 E2-17β-G. Separation of analytes from matrix was achieved via an Agilent 1200
HPLC system (Agilent Technologies, Inc., USA) on a C18 column, and MS detection was conducted
by an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Inc.) signals. MRM
fragmentation transitions of \(m/z\) 463→287 (S-6-G and S-7-G), \(m/z\) 455→308 (methotrexate), and \(m/z\)
339→58 (NMQ) were monitored in positive ESI mode, and transition of \(m/z\) 447→447 (E2-17β-G) was
monitored in negative mode. Data were acquired and processed using Agilent MassHunter software
(Agilent Technologies, Inc.). Main metabolites in biological and *in vitro* metabolic samples were
identified by UPLC/UV-Q-TOF. Chromatographic separation of metabolites was achieved via an
Acquity UPLC system (Waters Corp., Milford, MA, USA) on an Agilent Poroshell 120 SB-C18 column
(2.1 mm × 75 mm i.d., 2.7 μm). The mobile phase consisted of a mixture of methanol (A) and 0.1%
formic acid in water (B). Gradient elution was initiated at 15% A, maintained for the first 3 min, and
then increased to 50% A linearly in 12 min. Afterwards, the elution was immediately increased to 100%
A, maintained for 1 min, and finally decreased to 15% A to equilibrate the column. The flow rate was
set to 0.6 mL/min. MS detection was conducted by a Synapt Q-TOF high-resolution mass
spectrometer (Waters Corp.) operated in positive ESI mode signals. An Acquity UV detector (Waters
Corp.) was used to monitor the analytes at a wavelength of 336 nm. Data were acquired from 80 Da to
1000 Da and centroided during acquisition using leucine-enkephalin as an internal reference (\(m/z\)
566.2771). A source temperature of 100°C, desolvation temperature of 400°C, and cone voltage of 40
V were applied. The transfer collision energy (CE) and trap CE were set to 2 and 3 eV, respectively;
the transfer CE was 15 eV and the trap CE was ramped from 15 to 25 eV to acquire MS/MS data. The acquired data were processed using Masslynx 4.1 software (Waters Corp.).

Results

Metabolite Profiling in Human Bile and Urine. Within 0 h to 20 h after the oral administration of 40 mg 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 the patient No. 2 than patient No. 1 (Fig. 2A). According to the medical record of the patient No. 2, the patient was suffered from cholecystitis and had been administered with cefoperazone (antibiotic), ornidazole, omeprazole and other five 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 the patient No. 2 and the potential complexity caused by drug-drug interactions, we mainly used the data from patient No. 1 for further discussion. According to the relative MS peak areas of the major metabolites in the bile from patient No. 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 that of S-6-G (M0-2).
Following oral administration of 40 mg of S-7-G, a total of six drug-related components were detected in the urine collected from healthy volunteers during the period of 0-12 h post dose (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 mass spectrometric 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 UDPGA-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, as 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 μM to 16.2 μM, whereas the $V_{max}$ values were highly different among products and microsomes. Although significant regio-selectivity for the 7-O position was observed for the aglycone glucuronidation in all of the studied catalytic systems, the extent in HIMs was not as high as that in RIMs: 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 HIMs, whereas the formation of S-6-G in RIMs was too slow for an accurate analysis.
Aglycone Glucuronidation by Human Recombinant UGT. S-6-G and S-7-G formation was studied in UDPGA-supplemented incubations of the aglycone with 12 different human UGT isoforms (i.e., 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17). 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, 1A3, 1A6, 1A7, 1A8, and 1A10, also catalyzed S-7-G formation, and other isoforms, including UGT1A1, 1A8, 1A10, and 2B7, mediated S-6-G formation. According to further kinetic studies (Fig. 5 and 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, expect 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 were 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 $V_{\text{max}}/K_m$ ratio for the glucuronidation of S-6-G was 10.8 and 5.95 times higher than that of S-7-G, respectively. Additionally, all of the human and rat hepatic and intestinal microsomes exhibited significant substrate inhibition with the two isomers (Fig. 6).

S-6-G and S-7-G Glucuronidation by Human Recombinant UGTs. The formation of S-6,7-diG was studied in UDPGA-supplemented incubations of S-6-G and S-7-G with 12 recombinant human
UGT isoforms (i.e., 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17). The results showed that S-7-G and S-6-G glucuronidation was mediated by the same UGT enzymes including UGT1A1, 1A8, and 1A10 (Fig. 7). Among these three enzymes, the most active one appeared to be UGT1A1, followed by UGT1A8. In the further kinetic studies (Fig. 8 and Table 3), UGT1A1 and UGT1A8 exhibited higher catalytic efficiency for the glucuronidation of S-6-G than that of S-7-G, agreeing with the regio-selectivity in human hepatic and intestinal microsomes. In contrast, UGT1A10 showed preference for catalyzing the glucuronidation of S-7-G. In addition, UGT1A1 exhibited substrate inhibition with S-6-G, which was also observed in human microsomes but not in UGT1A8 and 1A10. UGT1A1 thus appears to be significant in the glucuronidation of S-6-G in the human intestinal and liver microsomes, 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 (IC$_{50}$ were 50 and 20 μM for MRP2 and BCRP, respectively) and S-7-G (IC$_{50}$ 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 MRP1 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 in the order of
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 or not 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 MRP1 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 when compared to 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

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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 μM to 30 μM, while apparent substrate inhibition was observed after reaching 30 μM ($K_i = 25.9 \pm 14.8 \mu M$). 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 CLint ($V_{max}/K_m$) for S-7-G (11.1 μL/mg protein/min) was about 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 following 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 in order 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.

Based on 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 HIMs and RIMs (Table 2; Fig 3). Kinetic analysis of the total glucuronidation in HIMs resulted in V_max, K_m, and V_max/K_m values of 6.74 nmol/min/mg protein, 4.50 μM, and 1498 μL/min/mg protein, respectively. By comparing the glucuronidation efficiency in HIMs to that in RIMs (V_max/K_m = 1924 μL/min/mg protein), 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.

Regio-selectivity 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: RIMs>HLMs>HIMs>RLMs. 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 regio-selectivity between HLMs and HIMs 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 HIMs was much lower than that in RIMs, 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).

Since the formation of S-7-G from the aglycone is higher than that of S-6-G in HIMs and RIMs, its lower circulating levels after oral administration could not be explained by the
hydrolysis-reformation mechanism, we therefore 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 was also observed in rats (Gao et al., 2011). Interestingly, the glucuronidation efficiency of S-6-G was much higher than that of S-7-G in HLMs and HIMs, 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 1A8 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), due to its low catalytic efficiency, the glucuronidation of S-6-G might be still favored over S-7-G in HIMs because of high selectivity by UGT1A1 and 1A8. This selectivity for S-6-G glucuronidation was more obvious in HLMs than that in HIMs, probably due to 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 lead 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 which 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; Zhang...
et al., 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). Since 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 impact their hepatic elimination, 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, while 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. Prior to hepatic metabolism and biliary excretion, drugs need to enter the hepatocytes first, either through passive diffusion or mediated by transporters. Due to 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 vs. S-7-G by OATP2B1, a significant difference (p<0.05) was observed in term of substrate affinity, where the K_m value for the transport of S-7-G was very low, about 1/24 of that for S-6-G (1.77 ± 0.54 versus 43.0 ± 23.7 μM). 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. Since 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; (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.
Acknowledgments.

We would like to thank Dr. Xiulin Yi and Mr. Zhiyu Zhang for providing the OATP1B1- and OAT2-transfected cell lines, Mr. Yanjun Bai for assisting us during the cell culture, and Ms. Cen Xie for the helpful discussion.
Authorship contributions

Participated in research design: Zhong, Zhang, and Gao

Conducted experiments: Gao, You and Guo

Contributed new reagents or analytic tools: Zhong and Chen

Performed data analysis: Zhong, Zhang and Gao

Contributed to the writing of the manuscript: Zhong, Zhang and Gao
References


Footnote

This work was supported by the National Natural Science Foundation of China [Grants 30271525 and 81173117].
Legends for figures

**Fig. 1.** Chemical structures of scutellarin (S-7-G), isoscutellarin (S-6-G), scutellarein (aglycone), and their metabolite scutellarein-6,7-diglucuronide (S-6,7-diG, M2).

**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) MDF chromatograms of the human bile collected from two patients receiving gallbladder surgeries during the post-dose period of 0 h to 20 h. (B) MDF chromatograms of the human urine collected from two healthy volunteers within the post-dose period of 0 h to 12 h.

**Fig. 3.** Formation kinetic curves of S-7-G (●) and S-6-G (■) in the intestinal and liver microsomes (A and B) of humans and (C and D) of rats. Aglycone was incubated with microsomes (0.05 mg protein/mL) at various concentration levels for 5 min (n=2).

**Fig. 4.** Formation of (A) S-7-G and (B) S-6-G 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 μM (solid) and 80 μM (hollow). Data points represent the means of duplicates.

**Fig. 5.** 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).
Fig. 6. Glucuronidation kinetic curves of S-7-G (●) and S-6-G (■) in the intestinal and liver microsomes (A and B) of humans and (C and D) of rats. 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).

Fig. 7. Formation of S-6,7-diG from (A) S-7-G and (B) S-6-G in incubations of human recombinant UGT. S-6-G or S-7-G was incubated with UGT enzymes (0.2 mg protein/mL) for 25 min at concentrations of 8 μM (solid) and 40 μM (hollow). Data points represent the means of duplicates.

Fig. 8. Formation kinetic curves of S-6,7-diG from S-7-G (●) and S-6-G (■) in human recombinant UGT. S-6-G was incubated with UGT enzymes (0.1 mg protein/mL) at various concentrations for 7 min. S-7-G was incubated for 20 min. Data points represent the means of duplicates.

Fig. 9. Inhibitory effects of (A) S-6-G and (B) S-7-G on the efflux of MRP1, MRP2, MRP3, BCRP, and MDR1. The efflux function of the transporters was evaluated according to the uptake of E2-17β-G (20 μM) for 5 min, E2-17β-G (100 μM) for 5 min, E2-17β-G (10 μM) for 1 min, methotrexate (400 μM) for 2 min, and N-methylquinine (5 μM) for 1 min into inside-out membrane vesicles expressing MRP1, MRP2, MRP3, BCRP, and MDR1, respectively. Data points represent the means of duplicates.

Fig. 10. Direct transport of S-6-G and S-7-G by (A) MRP1, (B) MRP2, (C) MRP3, and (D) BCRP. S-6-G or S-7-G was incubated with transporter-expressing membrane vesicles with (solid) or without (hollow) the presence of ATP for 5 min (n=2).
Fig. 11. Uptakes of S-6-G (solid) and S-7-G (hollow) in the OATP1B1-, OATP2B1-, OATP1B3-transfected HEK293 cells and OAT2-transfected S2 cells. The concentrations of S-6-G and S-7-G were 1 μM in the system, while the uptake time was 7 min. Data points represent the mean uptake ± S.D. (n = 3).

Fig. 12. Active uptakes of (A) S-7-G and (B) S-6-G by the OATP2B1-transfected HEK293 cells, without (white) or with the presence of 30 (grey) and 100 (black) μM estrone-3-sulfate. The concentration of S-6-G and S-7-G was at 3 μM in each incubations. The active uptake was the difference between the uptake by OATP2B1-transfected and mock cells, and was expressed as the percentage of the control group without estrone-3-sulfate. Data points represent mean ± S.D. (n = 3).

**, P<0.01; ***, P<0.001 versus control.

Fig. 13. Uptake kinetic curves of S-6-G (black) and S-7-G (red) by OATP2B1. S-6-G or S-7-G at various concentrations was incubated with OATP2B1-transfected or mock HEK293 cells for 7 min. Data were expressed as mean ± S.D. (n = 3)

Fig. 14. Proposed scheme of the absorption and disposition processes of S-7-G and S-6-G in humans. The width of the arrows represents the relative amount of substrate undergoing a certain process.
Table 1. Major metabolites in the human bile and urine following an oral dose of 40 mg of S-7-G.

Bile was collected from two patients receiving gallbladder surgery through T-tubes over the post-dose period of 0 h to 20 h. Urine was collected from two healthy volunteers within the post-dose period of 0 h to 12 h.

<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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/z</td>
<td></td>
<td></td>
<td>m/z</td>
<td>min</td>
<td>No. 1</td>
<td>No. 2</td>
</tr>
<tr>
<td>M0-1</td>
<td>463.083</td>
<td>C₂₁H₁₈O₁₂</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>
</tr>
<tr>
<td>M0-2</td>
<td>463.087</td>
<td>C₂₁H₁₈O₁₂</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>
</tr>
<tr>
<td>M2</td>
<td>639.120</td>
<td>C₂₇H₂₆O₁₈</td>
<td>Glucuronide 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>
<td>625.142</td>
<td>C₂₇H₂₈O₁₇</td>
<td>Glucoside conjugation</td>
<td>463.084, 287.054, 269.043,123.004</td>
<td>6.05</td>
<td>1.22</td>
<td>1.12</td>
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<tr>
<td>M3-2</td>
<td>625.141</td>
<td>C₂₇H₂₈O₁₇</td>
<td>Glucoside conjugation</td>
<td>463.085, 287.054, 269.043,123.004</td>
<td>6.51</td>
<td>2.97</td>
<td>1.55</td>
</tr>
<tr>
<td>M3-3</td>
<td>625.143</td>
<td>C₂₇H₂₈O₁₇</td>
<td>Glucoside conjugation</td>
<td>463.085, 287.055, 269.043,123.005</td>
<td>5.77</td>
<td>0.81</td>
<td>5.69</td>
</tr>
<tr>
<td>M4-1</td>
<td>477.103</td>
<td>C₂₂H₂₀O₁₂</td>
<td>Methylation</td>
<td>301.070, 286.045, 168.003</td>
<td>9.68</td>
<td>3.08</td>
<td>21.3</td>
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<tr>
<td>M4-2</td>
<td>477.102</td>
<td>C₂₂H₂₀O₁₂</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>M6</td>
<td>479.081</td>
<td>C₂₁H₁₈O₁₃</td>
<td>Hydroxylation</td>
<td>303.048, 285.039</td>
<td>7.46</td>
<td>5.26</td>
<td>—</td>
</tr>
</tbody>
</table>
Table 2. Enzyme kinetic parameters for the formation of S-6-G and S-7-G in the incubations of aglycone with microsomes and human recombinant UGT.

<table>
<thead>
<tr>
<th></th>
<th>Formation of S-7-G</th>
<th></th>
<th>Formation of S-6-G</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$K_i$</td>
<td>$V_{max}/K_m$</td>
</tr>
<tr>
<td></td>
<td>$\mu M$</td>
<td>nmoi/min/mg protein</td>
<td>$\mu M$</td>
<td>$\mu l/min/mg protein$</td>
</tr>
<tr>
<td>HIM</td>
<td>5.90 ± 1.68</td>
<td>5.45 ± 0.58</td>
<td>401 ± 194</td>
<td>924</td>
</tr>
<tr>
<td>HLM</td>
<td>6.32 ± 1.18</td>
<td>8.64 ± 0.40</td>
<td>—</td>
<td>1368</td>
</tr>
<tr>
<td>RIM</td>
<td>9.00 ± 1.92</td>
<td>17.3 ± 0.9</td>
<td>—</td>
<td>1924</td>
</tr>
<tr>
<td>RLM</td>
<td>7.37 ± 1.10</td>
<td>10.0 ± 0.3</td>
<td>—</td>
<td>1361</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>4.49 ± 0.89</td>
<td>5.70 ± 0.58</td>
<td>41.3 ± 8.6</td>
<td>1269</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>7.34 ± 0.90</td>
<td>1.78 ± 0.06</td>
<td>—</td>
<td>243</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>27.0 ± 4.57</td>
<td>1.67 ± 0.11</td>
<td>—</td>
<td>62.0</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>33.3 ± 7.03</td>
<td>0.795 ± 0.068</td>
<td>—</td>
<td>23.9</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>19.0 ± 2.39</td>
<td>0.673 ± 0.029</td>
<td>—</td>
<td>35.4</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>5.49 ± 0.71</td>
<td>4.63 ± 0.16</td>
<td>—</td>
<td>843</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>5.68 ± 0.92</td>
<td>0.318 ± 0.014</td>
<td>—</td>
<td>55.9</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>
**Table 3.** Main kinetic parameters for the formation of S-6,7-diG in the respective incubations of S-6-G and S-7-G with microsomes and human recombinant UGT.

<table>
<thead>
<tr>
<th>Incubation of S-7-G</th>
<th>Incubation of S-6-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ μM</td>
<td>$V_{max}$ nmol/min/mg protein</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>174 ± 52</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>198 ± 21</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>225 ± 105</td>
</tr>
</tbody>
</table>
Fig. 1

Scutellarin (S-7-G)

Scutellarein (aglycone)

Isoscutellarin (S-6-G)

Scutellarein-6,7-diglucononide (S-6,7-diG, M2)
Fig. 2

Combined Metabolite Peaks (All Found Peaks) [Analyte]

A-1. Bile (Patient-1)

Combined Metabolite Peaks (Found Expected Peaks only) [Analyte]

A-2. Bile (Patient-2)

Combined Metabolite Peaks (Found Expected Peaks only) [Analyte]

B-1. Urine (Volunteer-1)

Combined Metabolite Peaks (Found Expected Peaks only) [Analyte]

B-2. Urine (Volunteer-2)
Fig. 3

A. HIM-aglycone

B. HLM-aglycone

C. RIM-aglycone

D. RLM-aglycone
Fig. 5

**UGT1A1**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G
  - S-6-G

**UGT1A3**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G

**UGT1A6**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G

**UGT1A7**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G

**UGT1A8**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G

**UGT1A9**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G
  - S-6-G

**UGT1A10**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-7-G
  - S-6-G

**UGT2B7**

- Formation rate (pmol/min/mg protein)
- Aglycone (μM)
- Symbols:
  - S-6-G
Fig. 6

A

HIM

Formation of S-6,7-diG (pmol/mg protein/min)

from S-7-G

from S-6-G

Substrate (µM)

B

HLM

Formation of S-6,7-diG (pmol/mg protein/min)

from S-7-G

from S-6-G

Substrate (µM)

C

RIM

Formation of S-6,7-diG (pmol/mg protein/min)

from S-7-G

from S-6-G

Substrate (µM)

D

RLM

Formation of S-6,7-diG (pmol/mg protein/min)

from S-7-G

from S-6-G

Substrate (µM)
**Fig. 7**

**A** Formation of S-6,7-diG from S-7-G

```
Formation rate (pmol/min/mg protein)

1A1  1A3  1A4  1A6  1A7  1A8  1A9  1A10  2B4  2B7  2B15  2B17

- 8 µM
- 40 µM
```

**B** Formation of S-6,7-diG from S-6-G

```
Formation rate (pmol/min/mg protein)

1A1  1A3  1A4  1A6  1A7  1A8  1A9  1A10  2B4  2B7  2B15  2B17

- 8 µM
- 40 µM
```
Fig. 9

A

B

Relative Transport (%)

MRP1  MRP2  MRP3  BCRP  MDR1

S-6-G (µM)

S-7-G (µM)
Fig. 12

The bar chart shows the uptake (percentage of control) of S7G and S6G in different conditions:

- **Control**
- **with 30 μM E3S**
- **with 100 μM E3S**

Significant differences are indicated by asterisks: *** (p < 0.001), ** (p < 0.01), * (p < 0.05).
Fig. 13

\[ K_m = 43.0 \pm 23.7 \ \mu M \quad K_i = 25.9 \pm 14.8 \ \mu M \]

\[ V_{max} = 113 \pm 49 \text{ pmol/mg protein/min} \]

\[ K_m = 1.77 \pm 0.54 \ \mu M \quad V_{max} = 19.7 \pm 1.3 \text{ pmol/mg protein/min} \]