Absorption and Disposition of Scutellarin in Rats: a Pharmacokinetic Explanation for the High Exposure of its Isomeric Metabolite

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Running Title

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ABBREVIATIONS: S-7-G, scutellarin, scutellarein-7-O-glucuronide; S-6-G, isoscutellarin, scutellarein-6-O-glucuronide; UDPGA, uridine diphosphate glucuronic acid; UGTs, UDP-glucuronyl transferases; RLMs, rat liver microsomes; HBSS, Hanks’ balanced salt solution; 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; AUC, area under curve; Papp, apparent permeability; ATCC, American Type Culture Collection; Tris, tris(hydroxymethyl)aminomethane; CMC-Na, sodium carboxyl methyl cellulose; ESI, electrospray ionization; MDF, mass defect filter.
ABSTRACT:

Scutellarin or scutellarein-7-O-glucuronide (S-7-G) is a flavonoid used in the treatment of cardiovascular diseases. After oral administration to humans, S-7-G can hardly be detected, whereas its isomeric metabolite (scutellarein-6-O-glucuronide, S-6-G) dominates in plasma. A preliminary study in rats also revealed a low bioavailability of S-7-G, as well as a high plasma concentration of S-6-G. Therefore, the present study tried to explore the possible causes of the unusual pharmacokinetics of scutellarin in humans through investigating the absorption and disposition of S-7-G in rats. After oral administration to rats, S-7-G was largely hydrolyzed in the intestinal tract and was absorbed as aglycone. While passing through the intestinal wall, aglycone was extensively glucuronidated into S-7-G and S-6-G (about 20:1), which subsequently entered the mesenteric blood (about 15:1). However, because S-7-G exhibited more rapid uptake in hepatocytes, was glucuronidated at a 2.7-fold higher rate in the liver and was excreted in greater amounts through bile and urine than S-6-G, the S-7-G/S-6-G ratio eventually declined to around 1.5:1 in the systemic circulation. Findings revealed that S-7-G cannot be absorbed directly; S-7-G and S-6-G in the body were mostly generated from aglycone in the intestinal wall; a larger amount of S-7-G than S-6-G entered the mesenteric blood at the absorption stage, but the gap between them shrank quickly mainly due to the higher hepatic first-pass elimination of S-7-G. These findings in rats are of great value as reference for the further study to accurately interpret the pharmacokinetics of S-7-G in humans.
Introduction

Scutellarin or scutellarein-7-O-glucuronide (S-7-G, Fig. 1) is an active flavonoid component of the extract of Erigeron breviscapus (Vant.) Hand.-Mazz. It was identified from a local folk prescription more than 30 years ago and is widely used as a microcirculation promoter for the treatment of cardio-cerebrovascular diseases with a large market share in China. In addition to its vasodilator effect (Pan et al., 2008), other pharmacological activities have been reported for S-7-G, including antioxidant (Hong and Liu, 2004), anti-inflammatory (Luo et al., 2008), and neuro-protective effects (Zhu et al., 2009).

A previous study reported the unusual pharmacokinetics for S-7-G in humans (Chen et al., 2006). Following oral administration to humans, S-7-G could hardly be detected in plasma, whereas its isomeric metabolite scutellarein-6-O-glucuronide (S-6-G, Fig. 1) predominated in the circulation. Several studies that investigated the causes of the low bioavailability of S-7-G proposed that poor intestinal absorption is the main contributor (Hao et al., 2005; Cao et al., 2008; You et al., 2010). However, very few studies have focused on S-6-G, despite the fact that it is the predominant circulating metabolite in humans and might be responsible for the therapeutic effects. To date, we still do not know where and how S-6-G is formed and the mechanism by which it becomes the dominant component in the circulation instead of S-7-G.

The intestinal absorption of baicalin, a structural analog of S-7-G, has been well studied. Previous works have demonstrated that baicalin is mainly absorbed as aglycone baicalein (Akao et al., 2000; Xing et al., 2005a), which is then extensively conjugated in intestinal epithelia (Zhang et al., 2007a). Conjugates formed in enterocytes are subsequently effluxed into intestinal lumen and mesenteric blood by transporters (Zhang et al., 2007a).
Since it belongs to the same class of flavonoids, S-7-G is supposed to undergo similar absorption processes in the intestines as baicalin. In this case, plasma concentration of S-6-G would naturally be higher than S-7-G if aglycone was glucuronidated with high preference for S-6-G or if the efflux transporters in the basolateral membrane exhibited substrate selectivity for S-6-G. However, a preliminary study revealed that S-7-G formation is more than 5-fold higher than S-6-G after incubation of aglycone with either human intestinal or liver microsomes. In addition, efflux transporters in Caco-2 cell monolayers did not exhibit obvious substrate selectivity for S-6-G over S-7-G. This discrepancy observed between absorption and pharmacokinetics indicated that there must be some other differential processes of the two isomers in the body.

Due to the limitations of human models, a suitable animal model should be used for further exploration of ADME processes of S-7-G. In literature, it has been reported that the bioavailability of S-7-G was also low in rats (Zhong et al., 2003; Huang et al., 2005) and dogs (Ge et al., 2003). In a preliminary study, the plasma concentration of S-6-G was also found to be high in rats after an oral dose of S-7-G. Thus, besides higher maneuverability, rats were chosen as model organisms based on the similar pharmacokinetic phenomena as that in humans including a low bioavailability of S-7-G and a high exposure of S-6-G.

By exploring the absorption and disposition of S-7-G after oral administration, the present study was to accomplish the following objectives in rats: (1) to elucidate the mechanism of intestinal absorption of S-7-G; (2) to characterize the major metabolic and excretory pathways of S-7-G; and (3) to determine possible causes of higher S-6-G levels in circulation.

**Materials and Methods**

**Chemicals.** S-7-G was purchased from Shanghai Standard Biotech Co., Ltd (Shanghai, China),
while scutellarein was purchased from Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). S-6-G (99.4% purity by HPLC) was isolated and purified from human urine using the method described previously (Chen, et al., 2006). UDPGA, alamethicin, HBSS and Krebs-Henseleit buffer were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Pooled rat liver microsomes and cryopreserved rat hepatocytes were purchased from BD Biosciences (Woburn, MA, USA). All other reagents and solvents were of either analytical or HPLC grade.

**Animals.** Male Sprague-Dawley rats were used in the experiments (Experimental Animal Center, Shanghai Institute for Materia Medica, Chinese Academy of Sciences). All animals were housed under conventional conditions and handled according to the Guidelines for Care and Use of Laboratory Animals at Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

**Caco-2 Cell Culture.** Caco-2 cell line at passage 17 was purchased from ATCC (Rockville, MD, USA). Cells at passage 44 were seeded in 12-well transport inserts (Coring Coster, USA) in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, NY, USA) containing high glucose and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ for 21 days. Formed monolayers with transendothelial electrical resistance (TEER) values above 300 Ω·cm² were used in this study.

**Pharmacokinetic Study in Rats.** Nine rats weighing 200 to 250 g were fasted overnight and randomized into two groups. In one group (n = 6), rats were orally administered with S-7-G at a dose of 75 mg/kg, and approximate 0.3 ml of blood was sampled from the orbital sinus at 0.5, 2, 3.5, 5, 6.5, 8, 10, 12, 14, and 16 h post dose. In the other group (n = 3), rats were intravenously administered with S-7-G at a dose of 30 mg/kg, and blood was collected at 5, 15, and 30 min and at 1, 3, 5, 8, and 12 h.
after dose. Plasma samples obtained by centrifugation were immediately acidified to pH 2 with 8 M phosphoric acid. Concentrations of S-7-G and S-6-G in plasma samples were determined simultaneously by LC-MS/MS. Main pharmacokinetic parameters were calculated using WinNonlin software version 5.3 (Pharsight, MountainView, CA, USA).

**Hydrolysis of S-7-G in Rat Intestinal Content.** After two rats were sacrificed, the whole lengths of intestine were cut out, and two segments of small and large intestine were separated at the ileocecal valve. Contents of small and large intestines were collected and mixed with the same volume of pH 7.4 PBS to make thick suspensions, respectively. About 5 μl of S-7-G DMSO-solution was immediately added into 500 μl of the intestinal content suspension to yield a final concentration of 360 μM. Incubations of S-7-G in PBS (pH 7.4) were conducted in parallel as a negative control. The reaction was allowed to proceed in closed tubes at 37°C for 5 min and terminated by adding 500 μl of 2% formic acid in methanol (v/v). Incubation was performed in triplicate. Concentrations of S-7-G and aglycone were determined by LC-MS/MS.

**Caco-2 Cell Permeability of S-7-G and Aglycone.** The permeability assay was performed in HBSS containing 0.45 mM CaCl$_2$ and 0.4 mM MgCl$_2$ at pH 6.4. A slightly acidic environment was chosen to enhance the stability of S-7-G and aglycone during the assay. Prior to the assay, Caco-2 cell monolayers were rinsed twice and equilibrated with the transport buffer for 30 min. The assay was initiated by adding HBSS containing S-7-G (25 μM) or its aglycone (25 μM) to the apical side (0.5 ml) or the basolateral side (1.5 ml) of Transwell inserts. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$ with orbital shaking at 50 rpm throughout the assay. At designated time points (15, 30, 45, 60, and 120 min for aglycone; and 30, 60, 90, and 120 min for S-7-G), 200 μl of transport buffer at the basolateral side was collected and mixed with the same volume of 2% formic
acid in methanol (v/v). To calculate recoveries of the assay, HBSS containing drug compound at the
donor side were sampled before and after the assay. Cell monolayers were rinsed thrice and lyzed with
500 μl of 2% formic acid in methanol (v/v) at the end of the experiment. The assay was performed in
triplicate. Concentrations of S-7-G and aglycone in corresponding samples were analyzed by
LC-MS/MS.

Apparent permeability coefficient (P_{app}) was calculated as follows:

\[
P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_{donor}}
\]

\(\Delta Q/\Delta t\) is the permeability rate (nmol/min), \(A\) is the surface area of the cell monolayer (cm^2), and
\(C_{donor}\) is the initial concentration at the donor side (nmol/ml).

Efflux ratio (ER) was calculated as follows:

\[
ER = \frac{P_{app(B-A)}}{P_{app(A-B)}}
\]

\(P_{app(A-B)}\) and \(P_{app(B-A)}\) represent the apparent permeability coefficient of the substrate from apical to
basolateral side and from basolateral to apical side, respectively.

**Preparation of Rat Intestinal S9 Fractions.** Four rats were sacrificed by exsanguination from the
inferior vena cava following anesthesia by ether. The whole lengths of intestine were immediately
removed and chilled in 1.15% KCl solution. Duodenum, jejunum, ileum, and colon were separated and
their corresponding S9 fractions were prepared respectively. Mucosal cells were detached by scraping
on an ice-cold surface and homogenated in chilled buffer (pH 7.4) containing 100 mM potassium
phosphate, 150 mM KCl, 250 mM sucrose, 1 mM ethylenediamine tetraacetic acid disodium salt, and
0.1 mM of 1,4-dithiothreitol. Following centrifugation of intestinal homogenate at 10,000 g and 4°C
for 30 min, supernatants were collected as the S9 fractions and stored at -70°C. Protein concentrations
of the S9 fractions were determined by the Lowry method (Waterborg and Matthews, 1996).
Glucuronidation of the Aglycone in Rat Intestinal S9 Fractions. To evaluate the glucuronidation of aglycone in rat intestine, in vitro transformation was conducted by incubating aglycone with S9 fractions of rat duodenum, jejunum, ileum, and colon. Incubations were performed at 37°C for 45 min at pH 7.4 in 100 mM Tris-HCl buffered system consisting of S9 fractions (1 mg protein/ml), UDPGA (1 mM), and MgCl2 (10 mM). Reactions were terminated by adding the same volume of 2% formic acid in methanol (v/v). Incubation was performed at three substrate concentration levels (30, 60, and 120 μM) in duplicate. Major metabolites were screened after the incubation using UPLC-Q/TOF MS. Concentrations of S-7-G, S-6-G, and aglycone in incubations were determined simultaneously by LC-MS/MS.

In situ Rat Intestinal Infusion with S-7-G and Aglycone. Six rats weighing 200 to 250 g were fasted with free access to water for 12 h and randomized into two groups: S-7-G-infused group and aglycone-infused group. Rats were anaesthetized with 20% (w/v) urethane and laparotomized on dissecting boards, with body temperature maintained by a heating lamp. During the surgery, a length of approximate 15 cm of intestine, including distal ileum and cecum, was ligated at both ends, with the least possible damage to vessels in the mesentery. The superior mesenteric vein was ligated at the proximal part and a remaining needle was inserted and stabilized at the distal end. S-7-G (10 mg) or aglycone (2.5 mg) dissolved in 2 ml of CMC-Na solution (sodium carboxyl methyl cellulose, 0.5%, w/v) was infused into the lumen of the ligated rat intestine from the two groups. Mesenteric blood was collected at time intervals of 0–10, 10–20, and 20–30 min for the S-7-G-infused group, and 0–5, 5–10, and 10–20 min for the aglycone-infused group. During the experiment, blood volume of the rat was replenished via an infusion of blood from another rat every 7 to 10 min. Collected blood was immediately centrifuged to obtain plasma, which was acidified to pH 2 with 8 M phosphoric acid.
Metabolite profiles in the mesenteric plasma were obtained by UPLC-Q/TOF MS. Concentrations of S-7-G, S-6-G, and aglycone in plasma were determined simultaneously by LC-MS/MS.

**Blood Collection from Rat Hepatic Portal Vein and Orbital Sinus after Oral Administration of S-7-G.** After fasting for 12 h, 15 rats weighing 200 to 250 g were randomly divided into 5 groups corresponding to different time points of blood collection. Rats were orally administered with S-7-G at a dose of 75 mg/kg. At time points 0.25, 1, 2, 4, and 7 h post dose, blood samples were collected almost simultaneously from the hepatic portal vein and orbital sinus of rats from the corresponding group. Before blood sampling, rats were anaesthetized with 20% (w/v) urethane. Plasma was obtained by centrifugation and acidified to pH 2 with 8 M phosphoric acid. Metabolites in both hepatic portal and systemic plasma were characterized by UPLC-Q/TOF MS. Concentrations of S-7-G and S-6-G were determined by LC-MS/MS.

**Bile Collection after Oral and Intravenous Administration of S-7-G to Rats.** After fasting for 12 h, three rats weighing 350 to 400 g were anaesthetized with 3% pentobarbital and then bile duct-cannulated. Bile prior to dose was collected as blank sample. After recovery from anesthesia, the bile duct-cannulated rats were orally dosed with S-7-G (75 mg/kg). Bile was collected at time intervals of 0–2.5, 2.5–6, 6–10, 10–20, and 20–30 h post dose into tubes containing 200 μl of 8 M phosphoric acid. Major metabolites in bile were identified by UPLC-Q/TOF MS, while biliary excretion of S-7-G was quantified by UPLC/UV at a wavelength of 336 nm, with its major metabolites semi-quantified using S-7-G as calibration standard.

**Urine Collection after Oral and Intravenous Administration of S-7-G to Rats.** Three rats weighing 200-250 g were used for the urinary excretion study. After collection of blank urine, rats were orally dosed with S-7-G (75 mg/kg). Urine post dose was collected at time intervals of 0–4, 4–8,
8–12, 12–24, and 24–36 h into gatherers containing 200 μl of 8 M phosphoric acid. Major metabolites in urine were identified by UPLC-Q/TOF MS, while urinary excretion of S-7-G was quantified by UPLC/UV at a wavelength of 336 nm, with its major metabolites semi-quantified using S-7-G as calibration standard.

**Stability of the Diglucuronide of the Aglycone in Rat Intestinal Content.** After two rats were sacrificed, the whole lengths of intestine were cut out, and the intestinal contents were collected and mixed with the same volume of pH 7.4 PBS to make a thick suspension. 200 μl of the rat bile collected between 6-10 h post an oral dose of S-7-G was neutralized to pH 7.4 and mixed with 200 μl of the rat intestinal content suspension described above. Incubations of the bile in PBS (pH 7.4) were conducted in parallel as a negative control. The reaction was allowed to proceed in closed tubes at 37°C for 30 min and terminated by adding 500 μl of 2% formic acid in methanol (v/v). Incubation was performed in triplicate. S-7-G was quantified by LC/UV at a wavelength of 336 nm, and the aglycone and its diglucuronide were semi-quantified using S-7-G as calibration standard.

**Glucuronidation of S-7-G and S-6-G in Rat Liver Microsomes.** S-7-G (5 μM) and S-6-G (5 μM) were separately incubated with rat liver microsomes (0.25 mg protein/ml) in a Tris-HCl (100 mM, pH 7.4) buffered system consisting of UDPGA (1 mM), alamethicin (25 μg/ml), and MgCl₂ (10 mM). Reactions were allowed to proceed in a 96-well plate at 37°C, and terminated by adding the same volume of 2% formic acid methanol (v/v) at time points of 0, 1, 2.5, 4.5, and 6.5 min. Major metabolites in the incubations were identified by UPLC-Q/TOF MS. S-7-G and S-6-G were quantified by UPLC/UV, while the main metabolite was semi-quantified using S-7-G as calibration standard.

**Assessment of Uptake of S-7-G and S-6-G with Rat Cryopreserved Hepatocytes.** Before the uptake study, cryopreserved rat hepatocytes were thawed according to the manufacturer’s protocol and
resuspended in Krebs-Henseleit buffer. The viable cells were counted by trypan blue staining. Before
the uptake assay, a 150 μl aliquot of cell suspension was prewarmed at 37°C or precooled at 4°C for 5
min. The uptake was initiated by adding 50 μl of substrate solution into the hepatocyte suspension to
give a final volume of 200 μl. S-7-G (4 μM and 6 μM) or S-6-G (4 μM and 0.4 μM) were incubated
with hepatocytes (1.5 × 10^6 viable cells/ml) for 1.5 min. After the incubation, uptake of substrate was
stopped by being rapidly transferred into a 96-well glass A/B filter plate (Millipore, Bedford, MA,
USA) and vacuum filtering using a multiple-sample filtration manifold (Millipore, Bedford, MA, USA)
after washing the cells with ice-cold buffer for 5 times. Cells were lysed by adding 200 μl of 2%
formic acid in methanol (v/v) into each well. The medium and cell lysate were collected and analyzed
by LC/MS/MS.

Cell/medium concentration ratio (C/M ratio) was calculated as follows:

\[
\text{C/M ratio} = \frac{\Delta Q}{C_{\text{medium}} \times N_{\text{hepatocyte}}}
\]

\(\Delta Q\) is the uptake amount (pmol), \(C_{\text{medium}}\) is the concentration of the medium at the end of incubation
(nM), and \(N_{\text{hepatocyte}}\) is the number of viable cells in the incubation (10^6 cell).

Net uptake amount was obtained by subtracting the uptake amount at 4°C from that at 37°C, and the
net C/M ratio was calculated as follows:

\[
\text{Net C/M ratio} = \frac{\Delta Q_{37^\circ C} - \Delta Q_{4^\circ C}}{C_{\text{medium,37^\circ C}} \times N_{\text{hepatocyte}}}
\]

**Analytical Conditions.** For LC-MS/MS analysis, separation of analytes from matrix was achieved via
an Agilent 1200 HPLC system (Agilent Technologies, Inc., USA) on an Agilent SB-C_{18} column (4.6 mm ×
150 mm i.d., 5 μm; Agilent, USA) equipped with a Security-Guard C_{18} guard column (4 mm×3.0 mm i.d.;
Phenomenex, Torrance, CA, USA). Isocratic elution was performed at 55% (0.1% formic acid in methanol)
and 45% (0.1% formic acid in water) with a flow rate of 0.6 ml/min. MS detection was conducted by an
Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Inc., USA) operated in positive ESI mode. Baicalin was used as internal standard for the analysis. MRM fragmentation transitions of m/z 463 → m/z 287 (S-6-G and S-7-G), m/z 287 → m/z 123 (scutellarein), and m/z 447 → 271 (baicalin) were monitored. Data were acquired and processed with the Agilent MassHunter software (Agilent Technologies, Inc., USA). Identification and semi-quantification of major metabolites in biological samples were performed on an UPLC/UV-Q-TOF system. 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; USA). 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, increased to 50% A linearly in 12 min, increased to 100% immediately, maintained for 1 min, and finally decreased to 15% A to equilibrate the column. Flow rate was set to 0.6 ml/min. MS detection was conducted by a Synapt Q-TOF high-resolution mass spectrometer (Waters Corp., Milford, MA, USA) operated in positive ESI mode. Analytes were monitored at a wavelength of 336 nm using an Acquity UV detector (Waters Corp., Milford, MA, USA). Data were acquired from 80 to 1000 Da and centroided during acquisition using leucineenkephalin as internal reference (m/z 566.2771). Source temperature of 100°C, desolvation temperature of 400°C, and cone voltage of 40 V were applied. Transfer collision energy (CE) and trap CE were set at 2 and 3 eV, respectively; transfer CE was 15 eV and trap CE was ramped from 15 to 25 eV to acquire MS/MS data. Acquired data were processed using the Masslynx 4.1 software (Waters Corp., Milford, MA, USA).

Results

Pharmacokinetics of S-7-G and S-6-G in Rats Following Oral and Intravenous administration of S-7-G. After oral administration of S-7-G (75 mg/kg) to rats, both S-7-G and S-6-G
were detected in the systemic circulation. As the mean plasma concentration-time curves of both the isomers displayed in Fig. 2A, the plasma concentration of S-7-G was 1–2 times as high as that of S-6-G over almost all the collection time except the first and the last measurable time points. As the main pharmacokinetic parameters summarized in Table 1-1, AUC$_{0-\infty}$ of S-7-G was about 1.5 times that of S-6-G; $T_{max}$ values were similar for the two isomers; $C_{max}$ value of S-7-G was almost twice that of S-6-G, and exhibited much greater variation than S-6-G. Following an intravenous dose of S-7-G (30 mg/kg) to rats, S-7-G appeared immediately in plasma with a $C_{max}$ of 85.4 $\mu$g/ml 5 min post dose and was cleared rapidly with a MRT of merely 0.34 h. In contrast, plasma concentration of S-6-G remained at a low level, with an AUC less than 1/60 of that for S-7-G.

**Hydrolysis of S-7-G in Rat Intestinal Content.** After incubation in the rat small intestinal contents at 37°C for 5 min, about 3.3% of the initial amount of S-7-G (360 $\mu$M in 0.5 ml) was recovered as aglycone, with an average hydrolysis rate of 2.36 $\mu$mol/l/min and a total recovery of 86.2%. Meanwhile, by being incubated in the rat large intestinal contents at 37°C for 5 min, about 39% of the initial amount of S-7-G was recovered as aglycone, with an average hydrolysis rate of 28.0 $\mu$mol/l/min and a total recovery of 69.6%. Based on the fact that only neglectable decline of S-7-G and no apparent aglycone was observed after incubation of S-7-G in PBS at pH 7.0, non-microbial decomposition of S-7-G was assumed to be minor in the intestinal content with a pH of 5-7 (McConnell et al., 2008). During the incubations, mass loss can probably be attributed to some other microbial metabolic pathways.

**Caco-2 Cell Permeability of S-7-G and Aglycone.** Apical-to-basolateral permeability of both S-7-G and aglycone was determined at 25 $\mu$M. As shown in Fig. 3, no apparent transport of S-7-G was observed ($P_{app\, (A \rightarrow B)} = 0.17 \times 10^{-6}$ cm/s). In contrast, aglycone exhibited a much higher permeability
(P_{app(A-B)} = 2.4 \times 10^{-6} \text{ cm/s}) and accumulated at the basolateral side with a linear increase over time during the transport. Comparing the transport of S-7-G in the apical-to-basolateral and basolateral-to-apical directions, an efflux ratio as high as 17 was observed, indicating a significant efflux transport of S-7-G. During the assay, recoveries of S-7-G and aglycone were ~90% and ~80%, respectively. Since only trace amounts of metabolites could be detected in either transport solution or cell lysate, mass loss was mainly due to the chemical instability of the two compounds in HBSS.

**Glucuronide Conjugation of Aglycone in Rat Intestinal S9 Fractions.** To investigate whether the aglycone could be glucuronidated to form S-6-G and S-7-G in intestine epithelia, aglycone (28.5, 60.6, and 135 \mu M) was incubated with S9 fractions of duodenum, jejunum, ileum, and colon, respectively. During the transformation, glucuronidation of aglycone was observed after incubation with S9 fractions from any of the four intestinal segments. Both S-7-G and S-6-G were generated as major metabolites after incubation. S-7-G and S-6-G were formed at ratios ranging from 15.1 to 26.2, varying with substrate concentrations and S9 fractions from different intestinal sections (Table 2). UGT activities of different intestinal sections were ranked in descending order: jejunum>duodenum>ileum>colon. As the concentration of aglycone in the incubation increased, saturation of glucuronidation was first achieved in S9 fractions of ileum and colon, followed by duodenum, and then jejunum. Total concentrations of S-6-G and S-7-G produced by the four metabolic systems at various substrate concentrations are plotted in Fig. 4.

**Absorption and Metabolism in Rat Intestine Infused with S-7-G and Aglycone.** Following infusion of S-7-G (10 mg) or aglycone (2.5 mg) into a ligated segment of rat intestine (i.e. distal 10 cm of ileum and cecum), similar metabolite profiles in mesenteric plasma were observed. UV chromatograms shown in Fig. 5A and 5B revealed that S-7-G was the most abundant component in
mesenteric plasma of rats infused with either S-7-G or aglycone, whereas S-6-G, aglycone, and some methylated metabolites were detected at lower levels. Higher levels of aglycone and its methylated metabolites were observed in the aglycone-infused group, probably due to the saturation of intestinal glucuronidation by a high concentration of aglycone within a short time. Concentration ratios of S-7-G to S-6-G were found to be almost constant at about 15 in mesenteric plasma after intestinal infusion of either S-7-G or aglycone (Fig. 6), which was slightly lower than the generation ratio in intestinal S9 fractions. The similarity between S-7-G/S-6-G ratios observed in mesenteric plasma of both groups strongly proved that S-7-G cannot be absorbed directly and that S-7-G in the body is regenerated from aglycone in intestinal epithelial cells. In addition, the concentrations of all related metabolites were lower in mesenteric plasma after S-7-G infusion compared with the aglycone-infused group. This could be attributed to the fact that the hydrolysis of S-7-G in intestinal lumen is a gradual and time-consuming process. Our findings corroborate previous hypothesis that S-7-G cannot be absorbed directly.

**Plasma Concentrations of S-7-G, S-6-G and Aglycone in Rat Hepatic Portal Vein and Systemic Circulation.** Following oral administration of S-7-G to rats, S-7-G and S-6-G rapidly appeared in both hepatic portal plasma and systemic circulation, whereas aglycone could only be detected at extremely low levels throughout the entire collection period. Comparisons of plasma concentrations in the hepatic portal vein and systemic circulation revealed obvious declines in S-7-G levels, but no significant change in S-6-G levels. As shown in Fig. 7, plasma concentrations of S-7-G in hepatic portal vein were 2.65- to 10.9-fold higher than that in circulation at any given time, whereas the concentration of S-6-G in the blood was almost constant before and after passing through the liver. In hepatic portal plasma, the concentration ratio of S-7-G to S-6-G gradually declined from 5.96 to
2.88 with the prolongation of time post dose. In contrast, ratios of the two isomers in the systemic circulation remained at around 1.

**Identification of Major Metabolites in Rat Bile and Urine after Oral Administration of S-7-G.**

To investigate the primary metabolic pathways of S-7-G in rats, the major metabolites in bile and urine following oral administration of S-7-G were identified by UPLC-Q/TOF MS. Taking advantage of high-resolution MS data, the MDF method was adopted to facilitate the detection of metabolites by filtering off interferences from biological matrix. In addition to S-7-G, a total of seven major metabolites were identified in bile and urine, each corresponding to the major peaks in UV chromatograms (Fig. 8). These metabolites were formed by the following pathways: hydrolysis (M1, \( m/z \ 287.057 \)), isomerization (M0-2, \( m/z \ 463.088 \)), methylation (M4, \( m/z \ 477.106 \) and M5, \( m/z \ 301.072 \)), glucuronidation (M2, \( m/z \ 639.123 \)), and glucosidation (M3, \( m/z \ 625.147 \)).

Structures of the metabolites were characterized according to their chromatographic behaviors and high resolution MS data obtained at low and high energy (Table 3). M0-1 (\( R_t = 8.02 \) min) and M0-2 (\( R_t = 9.39 \) min) both exhibited protonated molecules [M + H]\(^+\) at \( m/z \ 463.085 \) and generated fragment ions at \( m/z \ 287.053 \), \( m/z \ 269.047 \), \( m/z \ 169.013 \), and \( m/z \ 123.009 \). Based on the identical chromatographic and mass spectral characteristics with the authentic compounds, M0-1 and M0-2 were identified to be S-7-G and S-6-G, respectively. Similarly, M1 (\( R_t = 10.6 \) min) was found to have the same retention time and mass spectral behaviors as authentic scutellarein (aglycone), with the protonated molecule at \( m/z \ 287.057 \) and fragment ion at \( m/z \ 269.050 \), \( m/z \ 169.014 \) and \( m/z \ 123.009 \). Therefore, M1 was confirmed to be the aglycone. M2 (\( R_t = 6.05 \) min) showed a precursor ion at \( m/z \ 693.123 \), which was 176 Da higher than M0. Its fragment ions were formed at \( m/z \ 463.086 \) (−176 Da) and \( m/z \ 287.056 \) (−176–176 Da), corresponding to M0 and M1, respectively. These findings indicate
that M2 is a glucuronide conjugate of M0. M3-1 (Rt = 6.16 min) and M3-2 (Rt = 6.76 min) had the
same precursor ion at m/z 625.147, which was 162 Da higher than M0. Identical fragment patterns to
produce ions at m/z 463.086 (−162 Da) and m/z 287.056 (−162–176 Da) were observed. Thus, M3-1
and M3-2 were tentatively presumed to be a pair of isomeric glucoside conjugates of M0. M4 had a
protonated ion at m/z 477.106, which was 14 Da higher than M0, with fragment ions at m/z 301.071
(−176 Da) and m/z 286.056 by a subsequent loss of ·CH3. Thus, M4 was assigned to be a methylated
metabolite of M0. M5 had a protonated molecular ion at m/z 301.072, which was 14 Da higher than
M1 and 176 Da lower than M4. All fragment ions of M4 were also observed in M5, while the retention
time of M5 was behind that of M1 (aglycone). Thus, M5 was assumed to be a methylated aglycone.

Biliary and Urinary Excretion Following Oral Administration of S-7-G to Rats. Excretion of
S-7-G in rat bile and urine were determined by UPLC/UV. The excretion of related metabolites,
including S-6-G (M0-2), aglycone (M1), glucuronide conjugate of M0 (M2), glucoside of M0 (M3),
methylated M0 (M4), and methylated aglycone (M5), were semi-quantified using S-7-G as calibration
standard. Due to the similar chromatographic retention, the peaks of M2 and M3-1 failed to
completely separate and slightly overlapped in the UV chromatogram. Thus, these two metabolites
were quantified together in this study. The excretion of main metabolites in bile (0–30 h) and urine
(0–36 h) after oral administration of S-7-G are summarized in Table 4-A and 4-B, respectively. As
shown in Table 3, MS response of M3-1 was similar to that of its isomer M3-2, whereas the UV peak
area of M3-2 was quite minor compared with that of the co-eluted peak of M2 and M3-1. In addition,
the MS response of M2 was 100- and 10-fold higher than that of M3-1 in bile and urine, respectively.
Thus, we can safely infer that the co-eluted peak could be attributed mainly to the UV response of M2.
Based on this inference, M2, the glucuronide conjugate of M0, was the most abundant metabolite,
accounting for 55.0% and 41.6% of excretion in bile and urine post dose, respectively. Next to M2, S-7-G was also excreted into bile and urine in abundance at values that were 5.46- and 9.28-fold greater than that of S-6-G, respectively. The excretion of methylated aglycone (M5) was not determined because its concentration was lower than the detection limit of UV analysis.

Compared with urinary excretion, biliary excretion was found to be the predominant route of elimination for S-7-G and its metabolites in rats, with 44.2% and 5.42% of the dose recovered from bile and urine, respectively. The total recovery in bile and urine was about 50% in this study, and two possible causes might be responsible for the low recovery. Firstly, the administered dose was not completely absorbed in the intestine, because 3-14% of the dose was recovered as the aglycone from the feces and intestinal content of the bile duct-cannulated rats. Secondly, based on the analysis on the incubation products of S-7-G in rat intestinal content and the literature (Xing et al., 2005c), a portion of the aglycone might have undergone microbial metabolism and chemical decomposition to some extent in the intestinal tract.

**Stability of the Diglucuronide of Scutellarein in Rat Intestinal Content.** Rat bile collected between 6-10 h after an oral dose of scutellarin, containing M2 (1.5 mM), S-7-G (0.92 mM) and almost no aglycone was incubated with rat intestinal content. After 30 min of incubation, 87.1% and 81.1% of the original amounts of M2 and S-7-G disappeared respectively, while the aglycone at a concentration of 1.40 mM was detected in the incubation. This result indicated that M2 is also unstable in rat intestinal content and can be hydrolyzed into the aglycone after being excreted through bile.

**Glucuronidation of S-7-G and S-6-G in Rat Liver Microsomes.** To investigate the generation of the major metabolite M2 in rats, the glucuronidation of S-7-G and S-6-G were studied by individual incubation with UDPGA-supplemented RLMs. After the incubation, both S-7-G and S-6-G were
glucuronidated, and their major metabolites were identified to be an identical diglucuronide conjugate of aglycone (m/z 639.123) with the same chromatographic retention time and mass spectral fragmentation pattern, which is supported by the co-injection of the individual incubation solution of S-7-G and S-6-G (Fig. 9). This metabolite also exhibited the same chromatographic and mass spectral behaviors as that of metabolite M2. Since S-7-G and S-6-G are a pair of isomers with a glucuronic acid conjugated at 7- O and 6- O, respectively, their common glucuronide conjugate M2 was inferred to be 6,7-O-diglucuronide scutellarein (S-6,7-diG), which concurs with previous studies (Liu et al., 2009; Xia et al., 2009). The formation of S-6,7-diG over time is illustrated in Fig. 10A. Although both S-7-G and S-6-G could be conjugated into S-6,7-diG, S-7-G exhibited a much higher glucuronidation rate than S-6-G; the formation rates of S-6,7-diG from S-7-G and S-6-G in separate incubations were 455 and 171 pmol/mg protein/min, respectively. In accordance with the higher glucuronidation rate of S-7-G, the disappearance of S-7-G was evidently faster than that of S-6-G (Fig. 10B). To further investigate the potential interactions of the two isomers in terms of their glucuronidation rate, S-6-G (5 μM) and S-7-G (5 μM) were co-incubated with UDPGA-supplemented RLMs (0.25 mg protein/ml). In the co-incubation system, S-6,7-diG was formed at a rate of 258 pmol/mg protein/min, which was between the formation rates when S-7-G and S-6-G were incubated individually (Fig. 10A). Meanwhile, depletion rates of both S-7-G and S-6-G decreased to almost the half of the rates in separate incubations (Fig. 10B).

**Assessment of Uptake of S-7-G and S-6-G with Rat Cryopreserved Hepatocytes.** As shown in Fig. 11, by comparing the uptake amounts at 37°C and 4°C (nonspecific binding), an evident uptake of S-7-G in rat hepatocytes was observed after an incubation period of 1.5 min. In contrast, the uptake of S-6-G seemed to be quite minor. By subtracting the uptake amount at 4°C from that at 37°C, the net
C/M ratios were calculated to be 53.4 ± 13.7 and 47.2 ± 10.3 μl/10⁶ cells for S-7-G at the substrate concentrations of 4 and 6 μM, and to be 3.74 ± 2.31 and 3.45 ± 0.30 μl/10⁶ cells for S-6-G at 0.4 and 4 μM, respectively. The data indicated that the uptake rate of S-7-G in rat hepatocytes is more than 10-fold higher than S-6-G even under the condition that the substrate concentration of S-7-G was 15 times as high as that of S-6-G.

Discussion

A previous study (Chen et al., 2006) reported an extremely low bioavailability and a surprisingly high exposure of an isomeric metabolite after an oral dose of S-7-G in humans. In order to understand its unusual pharmacokinetics, we explored the absorption and disposition of S-7-G in rats in this study. S-7-G is a flavonoid monoglucuronide with a pKa of 2.75 (ACD software). In the intestinal environment (pH 6–8), it is mostly dissociated and has low lipophilicity (log D≈ -4, ACD software), which most likely results in poor membrane permeability. According to literature, intestinal absorption of flavonoid glycosides generally occurs in two ways: direct absorption mediated by uptake transporters (Miyazawa et al., 1999; Hassimotto et al., 2008) or absorption in aglycone forms after hydrolysis in the intestinal tract (Setchell et al., 2002; Xing et al., 2005a; Liu and Jiang, 2006). In this study, we demonstrated that S-7-G is mainly absorbed as its aglycone after being hydrolyzed in the intestinal tract. This conclusion is supported by the following findings: (1) S-7-G could hardly pass through Caco-2 cell monolayers, whereas it was readily hydrolyzed into aglycone in fresh rat intestinal content, which exhibited 15-fold higher Caco-2 cell permeability; (2) after infusion of either S-7-G or aglycone into a ligated segment of rat intestine, similar metabolite profiles, especially, similar S-7-G/S-6-G ratios, were obtained in mesenteric plasma; and (3) in the intestinal-infusion assay, the concentrations of all drug-related components in mesenteric plasma were much lower in rats infused
with S-7-G compared with the aglycone-infused group, despite the higher dose of S-7-G (10 mg) than aglycone (2.5 mg).

While passing through intestinal epithelia, the aglycone was believed to undergo extensive intestinal first-pass metabolism, since no apparent aglycone was detected in hepatic portal plasma during the entire collection period after oral administration of S-7-G (Fig. 5C). Moreover, after infusing S-7-G or aglycone into the intestinal tract, free aglycone was only detected at low levels in the mesenteric plasma, whereas its monoglucuronides predominated. Considering the poor membrane permeability of glucuronides and their rapid appearance in the mesenteric plasma, efflux transporters at the basolateral membrane of enterocytes were supposed to be involved in the import of S-6-G and S-7-G formed inside enterocytes into mesenteric blood. Findings demonstrate that the absorption of S-7-G is a complex process that could be influenced by several factors, such as microbial hydrolysis, intestinal phase II conjugation, and efflux transporters. Therefore, it is not surprising that following oral administration of S-7-G great individual variation in the pharmacokinetics was observed in rats and humans (Chen et al., 2006).

In the intestinal epithelia, aglycone was mostly glucuronidated into monoglucuronides (S-7-G and S-6-G), which entered the liver and were further glucuronidated into S-6,7-diG. This conclusion was based on findings that mesenteric plasma predominantly contained monoglucuronides after an infusion of S-7-G or aglycone into rat intestine, whereas only trace amounts of S-6,7-diG, which was the most abundant component in bile, urine, and systemic plasma, were found in mesenteric plasma.

Additionally, there is little chance for the S-6-G and S-7-G formed in the intestine to be hydrolyzed and re-conjugated in the liver, although both β-glucuronidase (O'Leary et al., 2001) and UGTs (King et al., 2000) have been found in the liver. One reason for this is that β-glucuronidase is a type of
lysosomal enzyme. Under the normal physiological conditions, lysosomal membrane is stable and β-glucuronidase is mostly trapped inside (George, 2008), whereas, UGTs were primarily localized on the endoplasmic reticulum, thus, glucuronidation rather than hydrolysis will play the dominant role in the liver. Moreover, according to the rat pharmacokinetics results (Table 1), there is tremendous difference in AUC ratios of S-7-G/S-6-G after oral and intravenous administration of S-7-G. This suggests that hydrolysis of S-7-G and formation of S-6-G cannot take place simultaneously in the liver; otherwise, no such difference would have been observed.

In vitro glucuronidation of aglycone in rat intestinal S9 revealed that S-7-G and S-6-G were formed at a ratio of 20:1. However, they were detected at similar levels in the systemic circulation after oral administration of S-7-G. This discrepancy could be attributed to the differences of the two isomers in either absorption or disposition. Results imply that selectivity for S-6-G and S-7-G was minor during intestinal absorption, since the S-7-G/S-6-G ratio (~15:1) in mesenteric plasma following intestinal-infusion of S-7-G or aglycone was only slightly lower than their generation ratio (~20:1).

After oral administration of S-7-G, plasma concentration of S-7-G in the hepatic portal vein was found to be 10.9 to 2.65 times higher than that in systemic circulation, whereas no significant change in concentration of S-6-G was observed as the blood passed through the liver (Fig. 7). Findings suggest that there must be great differences between S-7-G and S-6-G in disposition, and that these differences might be largely responsible for the decline of S-7-G/S-6-G ratio in the blood.

S-6,7-diG was the most abundant metabolite in rats following oral administration of S-7-G. Both S-7-G and S-6-G could be glucuronidated into S-6,7-diG, but at different rates. The formation rate of S-6,7-diG from S-7-G was about 2.7-fold higher than that from S-6-G when they were incubated individually, while in the co-incubation of S-6-G and S-7-G, the formation rate of S-6,7-diG fell
between the rates in separate incubations (Fig. 10). To roughly discriminate whether the two reactions were catalyzed by the same enzyme, a “mixed-substrate” method was used. If the total reaction rate of mixed substrates is less than the sum of the rates in separate reaction systems (at saturated concentrations of substrates), then the two reactions are probably mediated by the same enzymes (Rao et al., 1970; Lucier et al., 1971). During the initial 6.5 min of incubation, UGTs were supposed to be saturated in both co-incubation and individual incubations because the formation of S-6,7-diG was a zero-order reaction. Thus, according to the criteria of the “mixed-substrate” method, we can infer that the glucuronidation of S-6-G and S-7-G are mediated, at least partly, by the same UGTs. Furthermore, since the total glucuronidation rate in the co-incubation of S-7-G and S-6-G was almost half the sum of the rates in their individual incubations, the higher glucuronidation rate of S-7-G could be attributed to higher reaction speed instead of higher affinity to enzymes; otherwise, the total rate in the co-incubation might have approximated that in individual incubation of S-7-G.

Hepatic uptake is the first and a critical step for the further hepatic metabolism and biliary excretion. In this study, by comparing the hepatic uptake using rat hepatocytes, a more than 10-fold higher cell/medium concentration ratio was observed for S-7-G (Fig. 11). This difference in hepatic uptake could well explain why the plasma concentration of S-7-G declined sharply as the blood passed through the liver, while the concentration of S-6-G almost remained unchanged (Fig. 7). Furthermore, by taking all the factors including the drug concentration in hepatic portal blood, hepatic uptake and glucuronidation rate into account, we can infer that the majority of S-6,7-diG, which accounted for about 50% of the drug-related components in excreta, was formed from S-7-G. In this case, at least 83% of the drug-related components in excreta were derived from S-7-G, while only 5% of them could be attributed to S-6-G, with a total excretion ratio between S-7-G and S-6-G as high as 16.4. Thus, it can
be explained why the concentration ratio of S-7-G to S-6-G in blood declined from 15:1 at the absorption stage to 1.5:1 in the systemic circulation, and the fact that S-7-G entered into rat hepatocytes and was transformed to M2 more readily than S-6-G should be responsible.

The absorption and elimination of S-7-G have much in common with baicalin: 1) intestinal bacterial hydrolysis is indispensable to their absorption (Xing et al., 2005b); 2) both of them go through extensive conjugative metabolism with the preference for 7-O position, and the 6,7-O-diglucuronide conjugate of the aglycone is the predominant metabolite (Abe et al., 1990; Xing et al., 2005b; Zhang et al., 2007b); 3) after an oral dose, their parent forms and metabolites are mainly excreted through bile (Abe et al., 1990). However, because the data on human pharmacokinetics and metabolism after oral administration of baicalin are not available, it is unknown whether the similar pharmacokinetic phenomenon as S-7-G can be observed for baicalin.

Comparing the results in rats and those obtained from human in vitro models in the preliminary study, similarities in intestinal absorption were observed: the aglycone was glucuronidated with a high regio-selectivity for S-7-G; and S-7-G formed in enterocytes was the predominant form of absorption. These observations were discrepant with the fact that the exposure of S-6-G was similar with S-7-G in rats and was much higher than S-7-G in humans. Therefore, in humans the differences of S-6-G and S-7-G in disposition could also be the key to explaining the high exposure of S-6-G. Nevertheless, only the above similarities are not yet sufficient for the direct extrapolation of the rat results to human situations, and further study is still needed to reveal the exact causes in humans. According to the results in rats, more attention should be paid to the substrate selectivity in terms of the hepatic uptake and glucuronidation in humans.

In summary, this study systemically investigated the absorption and disposition of S-7-G in rats,
with particular attention on the generation of S-6-G and the changes in relative concentrations of S-7-G and S-6-G in the bloodstream. As shown in Fig. 12, after oral administration, S-7-G was mainly absorbed as aglycone after hydrolysis in the intestinal tract. S-6-G and S-7-G in the body were mostly formed in enterocytes by the glucuronidation of aglycone. Although S-7-G entered the mesenteric blood in a much greater amount than S-6-G, their concentration ratio ultimately declined to ~1.5:1 in systemic circulation, mainly due to the fact that S-7-G entered into rat hepatocytes and underwent glucuronidation more readily than S-6-G. The results in this study are of great value as reference for the interpretation of pharmacokinetic data of S-7-G in humans, nevertheless, further study is still needed to reveal the exact causes in humans.

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Authorship contributions

Participated in research design: Zhong, Chen, and Gao.

Conducted experiments: Gao.

Contributed new reagents or analytic tools: Zhong, Chen and Gao.

Performed data analysis: Zhong, Chen and Gao.

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


Huang JM, Weng WY, Huang XB, Ji YH and Chen E (2005) Pharmacokinetics of scutellarin and its aglycone...


Legends for figures

Fig. 1. Chemical structures of scutellarin (S-7-G), isoscutellarin (S-6-G), and scutellarein (aglycone).

Fig. 2. Plasma concentration-time curves of S-7-G (●) and S-6-G (□) in rats following oral and intravenous administration of S-7-G. A, rats were orally administered with S-7-G at a dose of 75 mg/kg (n = 6); B, rats were intravenously administered with S-7-G at a dose of 30 mg/kg (n = 3). Data points are expressed as plasma concentrations over time (mean + S.D.).

Fig. 3. Accumulated amounts% of S-7-G and aglycone that permeated across Caco-2 monolayers from the apical to the basolateral side. Loading concentration of both S-7-G (●) and aglycone (●) was 25 μM. Data are expressed as means ± S.D. (n = 3).

Fig. 4. Glucuronidation of aglycone in the S9 fractions of different rat intestinal sections. Aglycone (28.5, 60.6, and 135 μM) was incubated with UDPGA-supplemented S9 fractions (1 mg protein/ml) of rat duodenum (●), jejunum (■), ileum (▲), and colon (●), respectively. Incubation lasted for 45 min, and data points represent the total concentrations of S-7-G and S-6-G generated against substrate concentrations (means of duplicates).

Fig. 5. Representative UPLC/UV chromatograms of rat plasma. A, mesenteric plasma collected within 10–20 min after infusion of S-7-G (10 mg) into a ligated segment of rat intestine. B, mesenteric vein plasma collected within 5–10 min after infusion of aglycone (2.5 mg) into a ligated segment of rat intestine. C, hepatic portal plasma obtained at 4 h after oral administration of S-7-G at a dose of 75
mg/kg. D, systemic plasma obtained at 4 h after oral administration of S-7-G at a dose of 75 mg/kg. E, blank plasma.

**Fig. 6.** Concentrations of S-7-G (□), S-6-G (■), and aglycone (▨) in mesenteric plasma after infusion of S-7-G or aglycone into a ligated segment of rat intestine. A, S-7-G (10 mg) was infused. B, aglycone (2.5 mg) was infused. Numbers between the columns of S-7-G and S-6-G represent their concentration ratios in mesenteric plasma. Data are expressed as means + S.D. (n = 3).

**Fig. 7.** Plasma concentrations of S-7-G and S-6-G in rat hepatic portal vein and systemic circulation after oral administration of S-7-G at 75 mg/kg. A, plasma concentration of S-7-G in rat hepatic portal vein (■) and circulation (●). B, plasma concentration of S-6-G in rat hepatic portal vein (■) and systemic circulation (●). Data points are expressed as mean ± S.D. (n = 3).

**Fig. 8.** Metabolite profiles of S-7-G in rat bile and urine after oral administration. S-7-G was administered at a dose of 75 mg/kg. A, MDF chromatogram of rat bile collected within 6–10 h post dose. B, UV chromatogram of rat bile collected within 6–10 h post dose. C, MDF chromatogram of rat urine collected within 4–8 h post dose. D, UV chromatogram of rat urine collected within 4–8 h post dose.

**Fig. 9.** UPLC/UV chromatograms of the glucuronide metabolite of S-7-G and S-6-G in UDPGA-supplemented RLMs. A, S-7-G (20 μM) was incubated with pooled RLMs (2 mg protein/ml) in the presence of 25 μg/ml alamethicin and 2 mM UDPGA for 45 min. B, S-6-G (20 μM) was
incubated with pooled RLMs (2 mg protein/ml) in the presence of 25 μg/ml alamethicin and 2 mM UDPGA for 45 min. C, Co-injection of the incubation solutions of A and B.

**Fig. 10.** Glucuronidation of S-7-G and S-6-G in UDPGA-supplemented RLMs. S-7-G (5 μM) and S-6-G (5 μM) were separately incubated and co-incubated with pooled RLMs (0.25 mg protein/ml) in the presence of 25 μg/ml alamethicin and 2 mM UDPGA. A, formation of S-6,7-diG over time when S-7-G and S-6-G were incubated separately (◆ and ■) and together (○). B, percentage of remaining S-7-G and S-6-G over time when they were incubated separately (◆ and ■) and together (◇ and □). Data points represent mean ± S.D. (n = 3).

**Fig. 11.** Uptake of S-7-G and S-6-G in cryopreserved rat hepatocytes. S-7-G (4 and 6 μM) or S-6-G (4 and 0.4 μM) were incubated with rat hepatocytes (1.5 × 10⁶ cell/ml) at 37°C (hollow) and 4°C (solid) for 1.5 min. Each data point is expressed as the mean cell/medium concentration ratio + S.D. (n=3).

**Fig. 12.** Proposed scheme of intestinal absorption and disposition of S-7-G and S-6-G in rats. The width of arrows indicate the amount of substrate: a wider arrow represents more substrate undergoing the process.
### Tables

#### Table 1-1. Pharmacokinetic parameters of S-7-G and S-6-G in rats after oral administration of S-7-G.

Rats were orally administered with S-7-G at a dose of 75 mg/kg. The values are expressed as means ± S.D. (n = 6).

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<th>AUC_{(0-∞)}</th>
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<th>T_max</th>
<th>MRT_{(0-t)}</th>
<th>T_{1/2}</th>
<th>Bioavailability</th>
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<tr>
<td></td>
<td>mg/L*h</td>
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<td>mg/L</td>
<td>h</td>
<td>h</td>
<td>h</td>
<td>%</td>
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<tr>
<td>S-7-G</td>
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<td>5.84 ± 3.05</td>
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<td>S-6-G</td>
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<td>3.84 ± 1.65</td>
<td>0.668 ± 0.137</td>
<td>5.50 ± 0.76</td>
<td>5.67 ± 1.16</td>
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Table I-2. Pharmacokinetic parameters of S-7-G and S-6-G in rats after intravenous administration of S-7-G.

Rats were intravenously administered with S-7-G at a dose of 30 mg/kg. The values are expressed as means ± S.D. (n = 3).

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<th>T_{max}</th>
<th>MRT(0-4)</th>
<th>T_{1/2}</th>
<th>CL</th>
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<tr>
<td></td>
<td>mg/L*h</td>
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<td>h</td>
<td>h</td>
<td>h</td>
<td>L/h/kg</td>
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<tr>
<td>S-7-G</td>
<td>28.5 ± 3.33</td>
<td>28.6 ± 3.36</td>
<td>85.4 ± 13.9</td>
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<td>S-6-G</td>
<td>0.465 ± 0.144</td>
<td>0.566 ± 0.142</td>
<td>0.148 ± 0.021</td>
<td>0.250 ± 0.000</td>
<td>2.88 ± 0.95</td>
<td>3.29 ± 1.11</td>
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Table 2. Glucuronidation of aglycone in the S9 fractions of duodenum, jejunum, ileum, and colon.

S9 concentrations were 1 mg/ml in incubations, and values represent means of duplicates.

<table>
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<tr>
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<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>pmol/mg protein/min</td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>28.5</td>
<td>17.4</td>
<td>0.662</td>
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<td>1149</td>
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<td>Jejunum</td>
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<td>15.9</td>
<td>0.708</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>60.6</td>
<td>36.1</td>
<td>1.67</td>
<td>840</td>
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<tr>
<td></td>
<td>135</td>
<td>61.4</td>
<td>2.48</td>
<td>1420</td>
</tr>
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<td>Ileum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>28.5</td>
<td>13.6</td>
<td>0.701</td>
<td>317</td>
</tr>
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<td>60.6</td>
<td>15.8</td>
<td>0.739</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>135</td>
<td>13.9</td>
<td>0.751</td>
<td>326</td>
</tr>
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<td>Colon</td>
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<td></td>
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<td>10.1</td>
<td>0.417</td>
<td>234</td>
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<td>60.6</td>
<td>13.1</td>
<td>0.628</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td>135</td>
<td>9.43</td>
<td>0.624</td>
<td>223</td>
</tr>
</tbody>
</table>
Table 3. Identification of the major metabolites in rat bile and urine by UPLC-Q/TOF MS after oral administration of S-7-G.

Rats were orally administered with S-7-G at 75 mg/kg. Bile and urine were collected over the 30- and 36-h period post dose, respectively.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M+H]+ m/z</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>M0-1</td>
<td>463.085</td>
<td>C_{21}H_{18}O_{12}</td>
<td>S-7-G</td>
<td>287.053, 269.047, 169.013, 123.009</td>
<td>8.02</td>
<td>20.7</td>
<td>30.9</td>
</tr>
<tr>
<td>M0-2</td>
<td>463.088</td>
<td>C_{21}H_{18}O_{12}</td>
<td>S-6-G</td>
<td>287.055, 269.046, 169.012, 123.009</td>
<td>9.39</td>
<td>4.74</td>
<td>9.01</td>
</tr>
<tr>
<td>M1</td>
<td>287.057</td>
<td>C_{17}H_{10}O_{6}</td>
<td>Hydrolysis to aglycone</td>
<td>269.050, 169.014, 123.009</td>
<td>10.6</td>
<td>1.57</td>
<td>3.80</td>
</tr>
<tr>
<td>M2</td>
<td>639.123</td>
<td>C_{27}H_{26}O_{18}</td>
<td>Glucuronide conjugation</td>
<td>463.086, 287.056, 269.046,123.009</td>
<td>6.05</td>
<td>58.6</td>
<td>37.3</td>
</tr>
<tr>
<td>M3-1</td>
<td>625.147</td>
<td>C_{27}H_{28}O_{17}</td>
<td>Glucose conjugation</td>
<td>463.086, 287.056, 269.046,123.010</td>
<td>6.16</td>
<td>0.520</td>
<td>3.16</td>
</tr>
<tr>
<td>M3-2</td>
<td>625.144</td>
<td>C_{27}H_{28}O_{18}</td>
<td>Glucose conjugation</td>
<td>463.086, 287.054, 269.046,123.010</td>
<td>6.67</td>
<td>5.00</td>
<td>3.53</td>
</tr>
<tr>
<td>M4</td>
<td>477.106</td>
<td>C_{22}H_{19}O_{12}</td>
<td>Methylation</td>
<td>301.071, 286.049, 168.006</td>
<td>10.1</td>
<td>8.37</td>
<td>11.8</td>
</tr>
<tr>
<td>M5</td>
<td>301.072</td>
<td>C_{16}H_{10}O_{6}</td>
<td>Methylation of M1</td>
<td>286.051, 168.009</td>
<td>13.7</td>
<td>0.570</td>
<td>0.510</td>
</tr>
</tbody>
</table>

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Table 4-1. Excretion of major metabolites in rat bile after oral administration of S-7-G.

Three rats were orally administered with S-7-G at a dose of 75 mg/kg, and bile was collected over the 30-h period post dose.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Excretion Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 1</td>
<td>No. 2</td>
</tr>
<tr>
<td>Glucuronide conjugate (M2)</td>
<td>7285</td>
<td>7536</td>
</tr>
<tr>
<td>S-7-G (M0-1)</td>
<td>3679</td>
<td>3731</td>
</tr>
<tr>
<td>Methylated M0 (M4)</td>
<td>611</td>
<td>634</td>
</tr>
<tr>
<td>S-6-G (M0-2)</td>
<td>607</td>
<td>701</td>
</tr>
<tr>
<td>Glucose conjugate (M3)</td>
<td>595</td>
<td>749</td>
</tr>
<tr>
<td>S (M1)</td>
<td>162</td>
<td>340</td>
</tr>
<tr>
<td>Methylated aglycone (M5)</td>
<td>27.8</td>
<td>31.9</td>
</tr>
<tr>
<td>Total excretion</td>
<td>12968</td>
<td>13723</td>
</tr>
<tr>
<td>Recovery of dose (%)</td>
<td>45.1</td>
<td>44.6</td>
</tr>
</tbody>
</table>
**Table 4-2. Excretion of major metabolites in rat urine after oral administration of S-7-G.**

Three rats were orally administered with S-7-G at a dose of 75 mg/kg, and urine was collected over the 36-h period post dose.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Excretion Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 1</td>
<td>No. 2</td>
</tr>
<tr>
<td>Glucuronide conjugate (M2)</td>
<td>414</td>
<td>488</td>
</tr>
<tr>
<td>S-7-G (M0-1)</td>
<td>377</td>
<td>440</td>
</tr>
<tr>
<td>S (M1)</td>
<td>75.8</td>
<td>102</td>
</tr>
<tr>
<td>S-6-G (M0-2)</td>
<td>44.0</td>
<td>50.9</td>
</tr>
<tr>
<td>Methylated M0 (M4)</td>
<td>35.9</td>
<td>18.4</td>
</tr>
<tr>
<td>Glucose conjugate (M3)</td>
<td>27.5</td>
<td>23.6</td>
</tr>
<tr>
<td>Total excretion</td>
<td>974</td>
<td>1123</td>
</tr>
<tr>
<td>Recovery of dose (%)</td>
<td>5.96</td>
<td>6.24</td>
</tr>
</tbody>
</table>
Figure 1

Scutellarin (S-7-G)

Isoscutellarin (S-6-G)

Scutellarein (aglycone)
Figure 8

Combined Metabolite Peaks (Found Expected Peaks only) [Analyte] 4.36e4

A

B

Combined Metabolite Peaks (Found Expected Peaks only) [Analyte] 1.11e4

C

D

i.g._bile_6-10h

Range: 3.217e-1

Range: 8.754e-2
Figure 11

[S-7-G (4 μM) S-7-G (6 μM) S-6-G (4 μM) S-6-G (0.4 μM)]

C/M ratio (μl/10^6 cells)

- 37°C
- 4°C