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Pharmacokinetics of conjugated metabolites in rat plasma after oral administration of tectoridin

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ABSTRACT

Tectoridin is a major isoflavone found in the flowers of *Pueraria thomsonii* Benth. It possesses estrogenic, hypoglycemic, anti-oxidant, and anti-inflammatory activities. In the present study, we evaluated the plasma pharmacokinetic profile of tectoridin in rats. We isolated a new metabolite, tectorigenin-7-*O*-glucuronide-4′-*O*-sulfate (Te-7G-4′S), from the bile of rats treated orally with tectoridin and determined its chemical structure by spectral analysis. Furthermore, we developed a selective and accurate method for the simultaneous quantification of tectoridin metabolites, including Te-7G-4′S, tectorigenin-7-*O*-glucuronide (Te-7G), tectorigenin-7-*O*-sulfate (Te-7S), and tectorigenin in rat plasma, and measured their plasma concentrations in rats orally administered tectoridin (200 mg/kg). Plasma concentrations of Te-7G-4′S, Te-7G, Te-7S, and tectorigenin reached maximal values of 21.4 ± 13.8 μmol at 3.50 ± 1.87 h, 20.5 ± 9.7 μmol at 3.17 ± 1.81 h, 14.3 ± 3.3 μmol at 5.58 ± 3.07 h, and 8.67 ± 3.07 μmol at 4.92 ± 2.87 h, respectively. Enterohepatic recirculation resulted in double peaks or a flat concentration curve/time profile of the metabolites. Since plasma concentrations of tectorigenin conjugated metabolites were higher than those of the tectorigenin aglycone, it can be concluded that extensive phase II metabolism plays an important role in the pharmacokinetics of tectoridin and tectorigenin *in vivo*.

1. Introduction

Isoflavones possess numerous biological activities. The isoflavone tectoridin (**1** in Fig. 1) is present in large quantities in the flowers of *Pueraria thomsonii* Benth [1] that is used in traditional Chinese medicine for diabetes and some symptoms associated with excessive alcohol intake, such as drunkenness, headache, red face, and liver injury [2,3]. In previous studies, tectoridin was shown to have hepatoprotective [4], estrogenic [5], antihypolipidemic [6], anti-oxidative [7], and anti-inflammatory activities [8]. Recently, in China, Korea and Japan, phytochemicals containing the *P. thomsonii* flower have become one of popular herbal medicines for treatment of diseases such as alcohol intoxication, liver injury or diabetes. According to our previous study [9], the contents of tectorigenin-7-*O*-xylosylglucoside, tectoridin and tectorigenin in the *P. thomsonii* flower and in its water extracts were more than 5% and 30%, respectively. Thus, peoples consuming phytochemicals containing the *P. thomsonii* flower may be exposed to high levels of tectoridin.

Several studies have reported tectoridin metabolism. Tectoridin is metabolized to aglycone tectorigenin (**2** in Fig. 1) by human intestinal flora, and it shows more potent activities than tectoridin [5,10]. When tectoridin was orally administered to rats, six metabolites, including tectorigenin, hydrogenated tectorigenin, monohydroxylated tectorigenin, dihydroxylated tectorigenin, glucuronidated tectorigenin and sulfated tectorigenin were found in urine, and three metabolites, including tectorigenin, dihydroxylated tectorigenin and sulfated tectorigenin, were found in feces by liquid chromatography/mass spectrometry (LC/MS) analysis [11]. Zhang et al. found tectorigenin and its unknown metabolites in plasma, and tectorigenin and its two glucuronide and two sulfate conjugates in urine after oral administration of tectoridin or tectorigenin to rats by LC/MS^n analysis [12,13]. However, the conjugated sites of the metabolites could not be structurally determined due to the presence of multiple hydroxyl groups in the

Abbreviations: Te-7G-4′S, tectorigenin-7-*O*-glucuronide-4′-sulfate; Te-7G, tectorigenin-7-*O*-glucuronide; Te-7S, tectorigenin-7-*O*-sulfate; Te-4′S, tectorigenin-4′-*O*-sulfate; AUC, area under the curve; *CL*, clearance; ESI, electrospray ionization; *F*, bioavailability; C_{max}, peak plasma concentration; DAD, diode-array detector; HMBC, hetero-nuclear multiple-bond connectivity; HPLC, high performance liquid chromatography; IR, infrared radiation; IS, internal standard; LLQC, lower limit of quantitation; *MRT*, mean residence time; MS, mass spectrometry; QC, quality control; RE, relative error; RSD, relative standard deviation; T_{max}, time to maximum plasma concentration; TOF-MS, time-of-flight tandem mass spectrometry; *V*, distribution volume.

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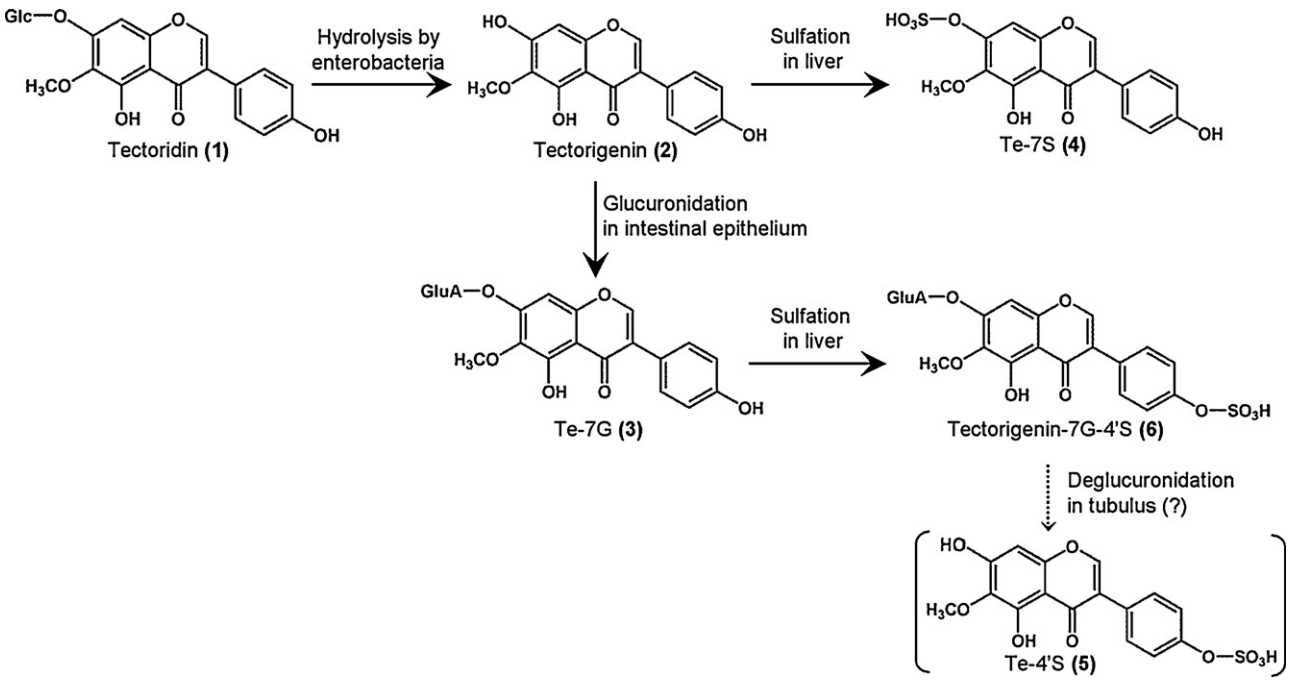

Fig. 1. Chemical structure of tectoridin and its proposed metabolic pathways in rats. Te-4′S was not found in plasma but in urine after oral administration of tectoridin to rat.

chemical structure of tectorigenin. In a recent study, we isolated and structurally identified nine metabolites including tectorigenin-7-*O*-glucuronide (Te-7G, **3** in Fig. 1), tectorigenin-7-*O*-sulfate (Te-7S, **4** in Fig. 1), tectorigenin-4′-*O*-sulfate (Te-4′S, **5** in Fig. 1), and tectorigenin [14], in urine of rats orally administered tectoridin. The above studies demonstrate the existence of phase II metabolism of tectoridin *in vivo*.

The aim of the present study was to assess the pharmacokinetics of conjugated metabolites in rats orally administered tectoridin. We isolated a new tectoridin metabolite called tectorigenin-7-*O*-β-D-glucuronide-4′-*O*-sulfate (Te-7G-4′S, **6** in Fig. 1), from rat bile. Furthermore, we developed a selective and accurate method for simultaneous quantification of tectoridin metabolites in rat plasma, and characterized the pharmacokinetic profile of four of them.

2. Materials and methods

2.1. Chemicals and reagents

The standard compounds used in the qualitative and quantitative determinations were obtained as described previously. Tectoridin (**1**) and tectorigenin (**2**) were isolated from flowers of *P. thomsonii* Benth [15], and Te-7G (**3**), Te-7S (**4**), and Te-4′S (**5**) were isolated from urine of rats orally administered tectoridin [14]. The identity of these compounds was confirmed by spectoral analysis including UV, infrared radiation (IR), ^{1}H and ^{13}C NMR, and mass spectrometries (MS). The purity of tectoridin as evaluated by high-performance liquid chromatography (HPLC)/UV was 98.4%, while that of the other compounds was more than 95%. Rutin, used as an internal standard (IS), was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and had a purity of more than 98%. Other chemical reagents were of analytical or HPLC grades. Deionized water was purified by redistillation and filtered through a 0.22-μm membrane filter before use.

2.2. Animals

Male Sprague-Dawley rats (180 ± 20 g body weight about 6–8 week-old) were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. The rats were maintained in a breeding room for acclimatization for 7 days before use. They received food and water *ad libitum* and were maintained under controlled temperature (25 °C), humidity, and lighting (12 h light, 12 h dark) conditions.

2.3. Collection of bile and hepatic portal venous plasma from rats

Twenty rats were intraperitoneally injected with urethane (1.0 g/kg body weight). Under anesthesia, a polyethylene cannula was inserted into the bile duct, and tectoridin suspended in 0.5% carboxymethylcellulose solution was orally administered at a dose of 200 mg/kg body weight. Bile samples were collected for 20 h (approximately 100 mL in total). In another experiment, three rats were intraperitoneally injected with urethane, and tectoridin (200 mg/kg) was orally administered. Hepatic portal venous blood (6 mL) was collected from the hepatic portal vein using a syringe containing heparin 1–1.5 h after tactician administration. Plasma samples were prepared from the collected blood.

2.4. Isolation and identification of Te-7G-4′S from rat bile

The bile sample (100 mL) was applied to a column (5.6–48 cm) containing macroporous resin D101 (Fushun Xintai Fine Chemical Factory, Fushun, China), eluted with MeOH–H_2O (0:100–100:0, gradient condition). The water eluate was concentrated, and passed through a column (3.0–70 cm) containing Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). The column was eluted with MeOH–H_2O (30:70; v/v) .The column was eluted with MeOH–H_2O (30:70; v/v) and the eluates were grouped on the basis of TLC analysis into six major fractions (F1–F6). Fraction F4 (25 mg) was further purified by a Sephadex LH-20 column (1.5–53 cm) and eluting with

MeOH–H2O (30:70; v/v) to give a yellowish amorphous powder (5.5 mg), identified as Te-7G-4′S by NMR and MS spectra. NMR spectra were measured using the Bruker ARX-600 spectrometer (Bruker, Newark, DE, USA), and chemical shifts were given in ppm downfield relative to tetramethylsilane. The time-of-flight tandem mass (TOF-MS) spectrum was measured using Agilent 1200 series LC/MSD-TOF system (Agilent, Palo Alto, CA, USA) to acquire its exact molecular weight and product ion spectra. Parameters for analysis were set using full-scan negative ion mode with spectra acquired over a mass range from *m*/*z* 50 to 1500. The electrospray ionization (ESI) source was set to the following conditions: drying gas (N_2) flow, 12.0 L/min; nebulizer pressure, 35 psig; and drying gas temperature, 350 °C. For ESI analysis, the following parameters were considered: capillary voltage, 3500 V; fragmentor, 190 V; skimmer, 60 V; and Oct RF, 250 V.

2.5. Instrumentation for chromatography

Metabolites in plasma of tectoridin-treated rats were identified by comparing retention time and MS and UV spectra with those of authentic compounds. HPLC/MS spectra were measured using the Agilent 1200 Series chromatographic system equipped with TOF-MS (Bruker Micro TOF-Q 125; Bruker). Parameters for analysis were set using a full-scan negative ion mode with spectra acquired over a mass range from *m*/*z* 50 to 1000. The ESI source was set to the following conditions: drying gas (N_2) flow rate, 4.0 L/min; drying gas temperature, 180 °C; nebulizer pressure, 0.4 bar; and capillary voltage, 3.5 kV. Chromatography was performed using the Kromasil C18 column (4.6 mm × 200 mm, 5 μm; Tianjin Scientific Instruments Co. Ltd., Tianjin, China) with a mobile phase comprising a gradient system of acetonitrile (solution A) and water containing 0.1% formic acid (solution B) at a flow rate of 0.8 mL/min at 35 °C as follows: a linear gradient of the mixture of solutions (A:B) from 12:88 (v/v) to 17:83 for 10 min, then maintained at 17:83 for 8 min, followed by a linear gradient of solutions (A:B) from 17:83 to 24:76 for 9 min, maintained at 24:76 for 5 min, and finally a linear gradient of solutions (A:B) from 24:76 to 50:50 for 28 min. The injection volume was 10 μL.

On-line UV spectra of the metabolites were obtained using Waters HPLC system (Waters Co., Milford, MA, USA) consisting of a Model 510 pump, an automated gradient controller, a model 2996 photodiode array detector, and Millennium32 software. The spectra were recorded in the range of 200–400 nm. Chromatography was performed using a Kromasil C18 column with a mobile phase comprising a gradient system of acetonitrile containing 0.05% trifluoroacetic acid (TFA; solution A) and water containing 0.05% TFA (solution B) at a flow rate of 0.8 mL/min as follows: a linear gradient of the mixture of solutions (A:B) from 14:86 to 20:80 for 19 min, followed by a linear gradient from 20:80 to 27:73 for 9 min, and from 27:73 to 42:58 for 19 min. The injection volume was 20 μL.

Plasma concentrations of metabolites were quantified using the Shimadzu LC-2010AHT HPLC system equipped with a CLASS-VP workstation (Shimadzu, Kyoto, Japan). The detection wavelength was set at 264 nm, and other HPLC conditions were identical to those used in the Waters HPLC system.

2.6. Preparation of standard solutions

Primary stock solutions of Te-7G, Te-7S, tectorigenin and tectoridin were prepared separately at a final concentration of 0.25, 0.40, 0.25 and 0.15 mg/mL, respectively, by dissolving accurately weighed reference compounds in methanol/water (5:1) or methanol. To prepare the standards for calibration, stock solutions were serially diluted with methanol to obtain the desired concentrations. A 0.10 mg/mL rutin stock solution was prepared in methanol, and diluted with methanol to obtain a working stock of 5.0 μg/mL, which was used as IS. All solutions were stored at 4 °C and brought to room temperature before use. Quality control (QC) samples were prepared by individually spiking blank rat plasma at three concentrations: low, medium, and high (0.25, 1.25, and 10.0 μg/mL for Te-7G, 0.40, 2.0, and 12.8 μg/mL for Te-7S, 0.05, 0.25, and 2.00 μg/mL for tectorigenin, and 0.30, 1.50, and 12.0 μg/mL for tectoridin, respectively). Samples were stored at −20 °C until analysis.

2.7. Sample preparation

Fifty microlitres of samples were mixed with 50 μL of rutin (5.0 μg/mL in methanol), and the mixture was de-proteinized proteinized by adding 150 μL of acetonitrile followed by vortexing (1 min) on a cyclomixer (Model TME-21; Toyo Seisakusho Company, Japan) and centrifuging (Hitachi Koki Co., Ltd., Japan) at 10,000 × *g* for 10 min. The supernatant was transferred to a glass-stoppered test tube and evaporated to dryness under a N_2 stream at room temperature. The residue was reconstituted with 50 μL of methanol. After centrifugation at 10,000 × *g* for 5 min, 20 μL or 10 μL of the solutions were injected into the analytical column separately for HPLC quantification or LC/TOF-MS identification.

2.8. Validation procedures

2.8.1. Selectivity

Selectivity of the method was evaluated by analyzing plasma samples collected from six different rats to investigate potential interferences for analytes and IS using the proposed extraction procedure and HPLC conditions.

2.8.2. Calibration curves and linearity

Calibration curves were prepared by spiking pooled control rat plasma with standard working solutions (50 μL each) to produce the calibration curve points equivalent to 0.125, 0.250, 0.625, 1.25, 2.50, 6.25, and 12.5 μg/mL for Te-7G, 0.20, 0.40, 1.0, 2.0, 3.2, 8.0, and 16.0 μg/mL for Te-7S, 0.025, 0.050, 0.125, 0.250, 0.500, 1.25, and 2.50 μg/mL for tectorigenin, and 0.15, 0.30, 0.75, 1.5, 3.0, 7.5, and 15.0 μg/mL for tectoridin, respectively. Samples were quantified using the ratio of the peak area of the analytes to that of IS. Peak area ratios were plotted against analyte concentrations, and standard curves were calculated using weighted ($1/X^2$) least squares linear regression.

2.8.3. Precision and accuracy of the assay

Accuracy and precision were assessed by determination of QC samples at three concentrations in five replicates (0.25, 1.25, and 10.0 μg/mL for Te-7G; 0.40, 2.0, and 12.8 μg/mL for Te-7S; and 0.05, 0.25, and 2.00 μg/mL for tectorigenin) on three validation days. Precision was expressed by relative standard deviation (RSD) and accuracy by relative error (RE). The intra- and inter-day precisions were required to be less than 15%, and the accuracy to be within ±15%. The lower limit of quantitation (LLOQ) was determined quantitatively by an analytical method with a precision of not more than 20% and an accuracy within ±20%. LLOQ was evaluated by analyzing samples prepared in five replicates on three separate days.

2.8.4. Recovery

Extraction recovery evaluated in three replicates of each QC sample (0.25, 1.25, and 10.0 μg/mL for Te-7G; 0.40, 2.0, and 12.8 μg/mL for Te-7S; and 0.05, 0.25, and 2.00 μg/mL for tectorigenin) was determined by comparing the peak area of extracted plasma (pre-spiked) standard QC samples to that of post-spiked standards at equivalent concentrations. Similarly, extraction

recovery of IS was determined at a single concentration of 5.0 μg/mL using QC samples at medium concentration.

2.8.5. Stability experiments

The stability experiments on Te-7G, Te-7S and tectorigenin were performed at three concentrations of the QC samples, using three replicates at each concentration (0.25, 1.25, and 10.0 μg/mL for Te-7G; 0.40, 2.0, and 12.8 μg/mL for Te-7S; and 0.05, 0.25 and 2.00 μg/mL for tectorigenin). Because the parent drug tectoridin was not determined in all the plasma samples, the stability experiment on tectoridin was also carried out at three concentrations of the QC samples (0.30, 1.50, and 12.0 μg/mL) following the same analytical procedure to prove its stability in plasma sample.

Short-term stability was determined after exposure of the spiked samples at room temperature for 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to −25 °C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 20 days. Post-extraction stability was determined after the extracted samples had been stored in the autosampler pending analysis at the temperature of the autoinjector (22–25 °C) for 24 h. The analyte was considered stable in the biological matrix when 85–115% (RE) of the initial concentration was found. To estimate the stability of IS before HPLC analysis, the extracted QC samples (at the medium concentration) that contained IS were repeatedly injected at 4-h intervals for 24 h at ambient temperature (22–25 °C).

2.9. Pharmacokinetic study

Tectoridin was suspended in 0.5% carboxymethylcellulose solution and administered orally to six conscious rats at a dose of 200 mg/kg bw. Blood samples were collected from the retro-orbital plexus of rats under light anesthesia with ether. Blood samples were collected in microfuge tubes containing heparin as an anticoagulant at 0, 0.125, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48, 60, and 72 h after treatment. Plasma was harvested by centrifuging blood samples at 3500 × *g* for 10 min and stored at −20 °C until analysis. Plasma (50 μL) samples were spiked with IS and the concentrations of tectoridin metabolites were measured as described above. QC samples were distributed among calibrators and samples in the analytical run to adjust the error.

2.10. Data analysis

A two-compartment pharmacokinetic model was proposed and validated by DAS 2.1.1 (Drug and Statistics version 2.1.1, Mathematical Pharmacology Professional Committee of China, Shanghai, China). Plasma Te-7G, Te-7S, and tectorigenin concentrations were calculated according to the respective calibration curves constructed using the ratio of the peak area of Te-7G, Te-7S, or tectorigenin to that of IS obtained from a single injection. Due to lack of sufficient Te-7G-4′S standards to complete the validation procedure, the calibration curve for this metabolite was not established. Because both Te-7G-4′S and Te-7S showed the same maximum absorption at 264 nm, rather than at 262 nm where the maximum absorption of Te-7G is, the plasma Te-7G-4′S concentration could be calculated on the basis of the calibration curve of Te-7S with correction in molecular mass of Te-7G-4′S to Te-7S. It is reasonable to assume that the molar absorptivity of Te-7G-4′S and Te-7S could be considered the same based on the fact that Te-7G-4′S has the same structural unit as Te-7S. UV absorption data of both metabolites were similar, as shown in Fig. 2A and Supplemental Fig. 1. As a consequence, the mass absorptivity ratio of Te-7G-4′S to Te-7S could be calculated from the molar absorptivity ratio of Te-7G-4′S to Te-7S with a correction in molecular mass of Te-7G-4′S to Te-7S. A similar assumption was made by Rüfer et al. [16]. Finally,

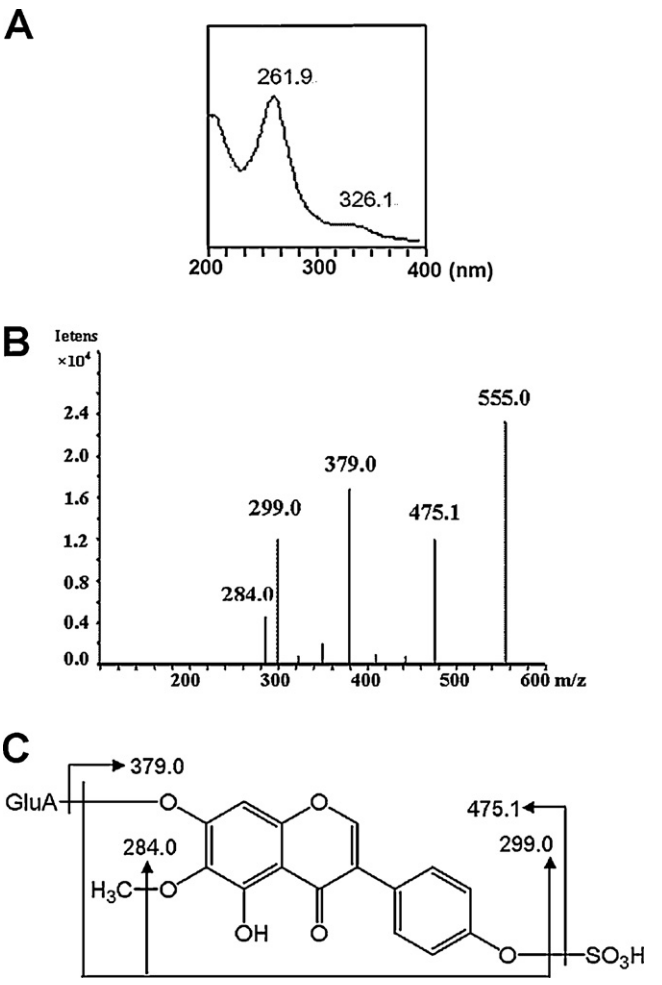

Fig. 2. Spectral characteristics of Te-7G-4′S (**6**). UV spectrum (A), ESI-TOF-MS fragmentation pattern of the $[M-H]^-$ ions at *m/z* 555.0 (B), and proposed dissociation of the compound (C).

plasma Te-7G-4′S concentrations were calculated using Eqs. (1) and (2):

$$C_{\text{Te-7G-4'S}} = \frac{kA_{\text{Te-7G-4'S}}/A_{\text{IS}} - b}{a} \quad (1)$$

$$k = \frac{E_{\text{Te-7S}}}{E_{\text{Te-7G-4'S}}} = \frac{\varepsilon_{\text{Te-7S}}/M_{\text{Te-7S}}}{\varepsilon_{\text{Te-7G-4'S}}/M_{\text{Te-7G-4'S}}} = \frac{M_{\text{Te-7G-4'S}}}{M_{\text{Te-7S}}} \quad (2)$$

where $C_{\text{Te-7G-4'S}}$ is the concentration of Te-7G-4′S in the plasma sample, $A_{\text{Te-7G-4'S}}$ and A_{IS} represents the peak areas of Te-7G-4′S and IS, *a* and *b* are the slope and intercept, respectively, of the calibration curve of Te-7S, *k* is the conversion factor of the peak area of Te-7G-4′S relative to peak area of Te-7S, $E_{\text{Te-7G-4'S}}$ and $E_{\text{Te-7S}}$ are the mass absorptivity of Te-7G-4′S and Te-7S, $\varepsilon_{\text{Te-7G-4'S}}$ and $\varepsilon_{\text{Te-7S}}$ are the molar absorptivity of Te-7G-4′S and Te-7S, and $M_{\text{Te-7G-4'S}}$ and $M_{\text{Te-7S}}$ are the molecular mass of Te-7G-4′S and Te-7S.

3. Results

3.1. Isolation and structural determination of Te-7G-4′S

By repeated chromatography using macroporous resin D101 and Sephadex LH-20 columns, an unknown metabolite was isolated from the rat bile sample, and its chemical structure was determined using UV, MS, and ^{1}H and ^{13}C NMR analyses. It had a maximal absorption at 262 nm in the UV spectra, indicating that it had an isoflavone skeleton. Its molecular formula was

Table 1
NMR data of new tectoridin metabolite (Te-7G-4′S, **6**).

Position	δ_C [a]	δ_H [a] (*J* in Hz)	HMBC[b]
2	155.3	8.50, s	3, 4, 9, 1′
3	125.3		
4	180.7		
5	153.0		
6	132.7		
7	156.9		
8	94.4	6.91, s	6, 7, 9, 10
9	152.6		
10	106.5		
1′	121.9		
2′, 6′	129.6	7.49, d (8.4)	1′, 6′/2′
3′, 5′	120.2	7.23, d (8.4)	3, 4′, 5′/3′
4′	153.6		
1″	100.2	5.09, d (7.2)	7, 2″, 3″
2″	73.1		
3″	76.8		
4″	72.0		
5″	73.6		
6″	172.2		
6-OMe	60.4	3.78, s	6

[a] All spectra were recorded on a Bruker ARX-600 spectrometer, in dimethylsulfoxide-d_6. 1″–6″ refer to numbering of glucuronic acid moiety.
[b] HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon. All chemical shift assignments were carried out on the basis of HMBC–NMR techniques.

determined to be $C_{22}H_{20}O_{15}S$ from the quasi-molecular ion peak at *m/z* 555.0458 (calculated as 555.0445) in high resolution ESI-TOF-MS. Three characteristic fragment ions at *m/z* 475, 379 (base ion), and 299 were also displayed in the MS spectrum, indicating the loss of a sulfate group (80 amu), a glucuronyl group (176 amu), or a glucuronyl plus a sulfate group (256 amu) from the quasi-molecular ion, respectively, as summarized in Fig. 2. Thus, this compound should be a glucuronide-sulfate bisconjugate. The conjugation sites were determined by NMR data. The resonance for protons and carbons of the aglycone moiety in the ^1H and ^{13}C NMR spectrum (Table 1) closely resembled those of known tectorigenin assigned according to the values of ^1H and ^{13}C NMR data in the literature [10,17]. The signals of an anomeric proton (δ 5.09, 1H, *d*, *J* = 7.2 Hz, H-1″) and a carboxylic group at C-6″ (δ_C 172.2) indicated the presence of a β-D-glucuronic acid moiety [18]. It could be attached to C-7 position according to the downfield shift of C-7 (+3.7 ppm) and the downfield shifts of C-6 (+1.3 ppm) and C-8 (+0.6 ppm) relative to the corresponding signals of tectorigenin, as evidenced by heteronuclear multiple-bond correlation (HMBC) between H-1″ (δ_H 5.09) and C-7 (δ_C 156.9) (Table 1). The sulfate moiety could be attached to the C-4′ position according to an upfield shift of C-4′ (−3.8 ppm) and downfield shifts of C-3′ and C-5′ (+5.2 ppm) relative to the corresponding signals of tectorigenin [10,14,17–20]. Thus, this compound was determined to be tectorigenin-7-*O*-β-D-glucuronide-4′-*O*-sulfate (Te-7G-4′S, **6** in Fig. 1). A bisconjugate Te-G-S was reported to be identified in rat bile after oral administration of tectorigenin by LC–MS^n techniques [21]. However, the proposed MS or MS^n data could not provide sufficient evidence to confirm the conjugated positions of the glucuronic acid and sulfate moieties. In our study, the conjugated positions of the bisconjugate were clearly elucidated by using NMR as well as MS data.

3.2. Identification of main metabolites in rat plasma and hepatic portal venous plasma by HPLC/UV and LC/TOF-MS

HPLC/UV chromatograms of rat plasma samples collected 0.5–1.5 h after oral tectoridin administration revealed four specific peaks characterized by typical isoflavone UV absorptions at 262–264 nm (Fig. 2A and Supplemental Fig. 1) at retention times of 17.4, 27.5, 29.8, and 42.4 min (Fig. 3). Direct comparison of retention time and UV and MS spectra (Fig. 2A and Supplemental Fig. 1) with those of authentic standards isolated from urine or bile of rats orally administered tectoridin [14] revealed Te-7G-4′S (**6**), Te-7G (**3**), Te-7S (**4**), and tectorigenin (**2**) as the metabolites. However, tectoridin (**1**) and Te-4′S (**5**) were not detected (Fig. 3D). Fig. 3E shows representative HPLC/UV chromatograms of rat hepatic portal venous plasma. Te-7G-4′S (**6**) and three other metabolites (**2**, **3**, **4**) were detected in the hepatic portal venous plasma of three rats orally administered tectoridin (200 mg/kg).

3.3. Method validation

3.3.1. Assay selectivity

Selectivity was assessed by comparing the chromatograms of six separate batches of control rat plasma with corresponding spiked plasma samples. Representative chromatograms of control rat plasma (Fig. 3B) and control rat plasma spiked with Te-7G, Te-4′S, Te-7S, tectoridin, tectorigenin, and IS (Fig. 3C) are shown. No interfering peaks were found for the analytes.

3.3.2. Calibration curve

The plasma calibration curve was constructed using seven calibration standards (0.125–12.5 μg/mL for Te-7G, 0.20–16.0 μg/mL for Te-7S, 0.025–2.5 μg/mL for tectorigenin, and 0.15–15.0 μg/mL for tectoridin). The calibration standard curve had a reliable reproducibility as determined by the best fit of peak-area ratios (peak area analyte/peak area IS) *versus* concentration, and fitted to $Y = aX + b$ using weighing factor ($1/X^2$). The average regression ($n = 2$) was found to be ≥0.990. The lowest concentration with an RSD <20% was considered as LLOQs and those for Te-7G, Te-7S and tectorigenin, and tectoridin were found to be 125, 200, 25 and 150 ng/mL, respectively (Table 2).

3.3.3. Precision and accuracy

The precision of the method was determined by calculating RSD for QC samples at three concentrations over three validation days. The intra-day precision was 18.2% or less, and the inter-day precision was 15.3% or less at each QC sample concentration. The accuracy of the method, expressed in terms of RE, ranged from −10.5% to 14.5% at the three QC sample concentrations. The above results (Table 3) demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.3.4. Extraction recovery

Mean extraction recoveries of Te-7G, Te-7S, and tectorigenin ranged from 78.8% to 80.7%, 70.3% to 88.2%, and 87.0% to 94.6%, respectively, as shown in Table 4. The extraction recovery of IS was 105.8%.

3.3.5. Stability

The stability results showed that Te-7G, Te-7S, and tectorigenin spiked into rat plasma were stable when kept at room temperature (22–25 °C) for 24 h, at ·20 °C for 20 days, during three freeze/thaw cycles, and in extracted samples pending analysis for 24 h at 22–25 °C, as shown in Table 5. The RSD value of the peak area of IS in the extracted QC samples from six repeated injections at 4-h intervals at room temperature (22–25 °C) was within 2%, suggesting that it is fairly stable within 24 h of post-extraction period.

Also, the stabilities of tectoridin in plasma were good enough at −20 °C for 20 days, after three freeze–thaw cycles, at room temperature (22–25 °C) for 24 h, and prepared plasma samples at room temperature for 24 h after preparation. Therefore, the reason that tectoridin was not detectable in plasma samples should be due to

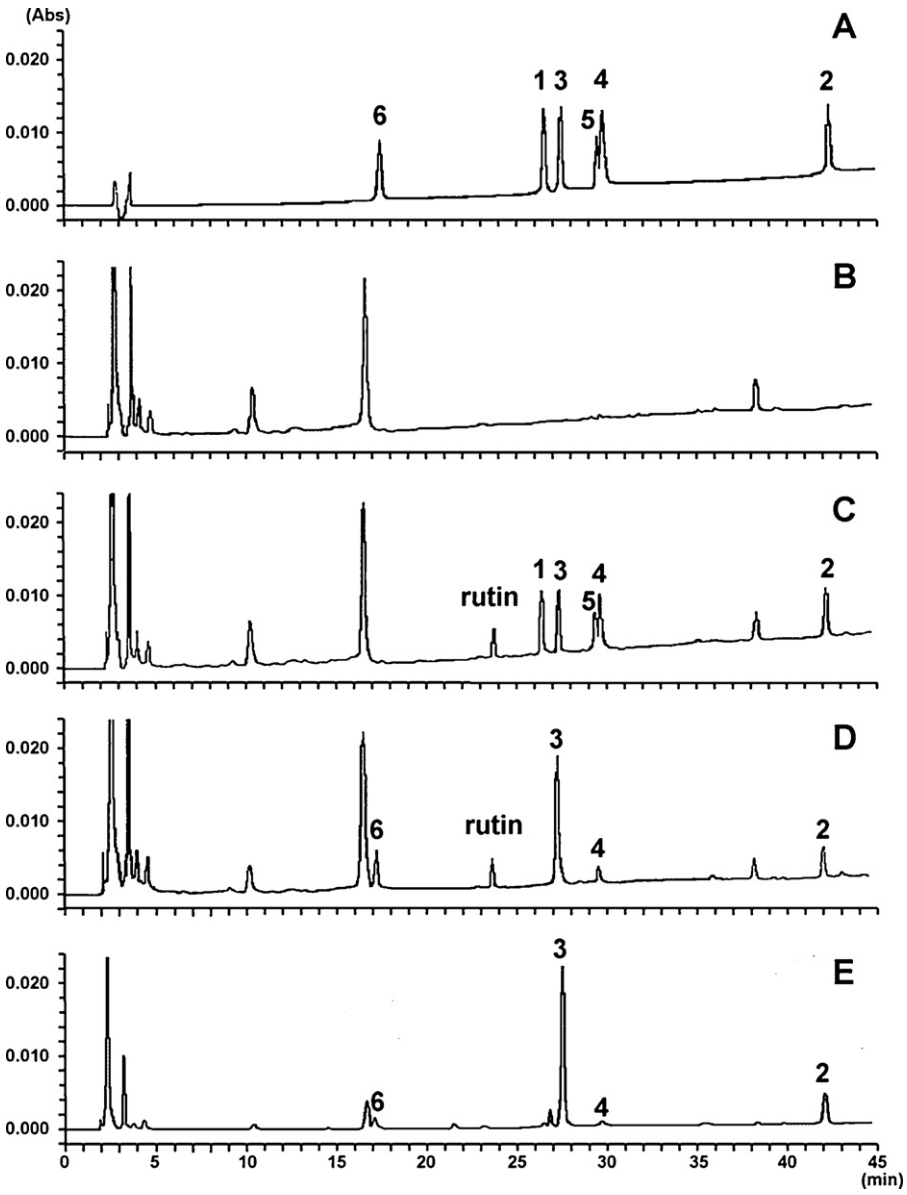

Fig. 3. Representative HPLC chromatograms of a mixed standard solution (A), blank rat plasma sample (B), a blank rat plasma sample spiked with Te-7G-4′S (**6**), tectoridin (**1**), Te-7G (**3**), Te-4′S (5), Te-7S (**4**), tectorigenin (**2**) and rutin (C), plasma sample spiked with rutin collected for 0.5–1.5 h (D), and a hepatic portal venous plasma sample collected for 1–1.5 h (E) after a single oral administration of tectoridin. Peaks were identified by the retention times of authentific compounds, and spectra of UV and TOF-MS shown in Fig. 2 and Supplemental Fig. 1. The detailed chromatographic conditions are described in Section 2.

its fast biotransformation in intestine, rather than its instability in plasma samples.

3.4. Plasma pharmacokinetics of tectoridin and its metabolites in rats

The validated HPLC/UV method was used to quantitatively determine plasma concentrations of four metabolites after an oral administration of 200 mg/kg tectoridin to rats. Their mean plasma concentrations *versus* time profiles are shown in Fig. 4, and the results of two-compartmental analysis are summarized in Table 6. Tectoridin was not detected during 72 h after treatment. In contrast, C_{max} values for Te-7G-4′S, Te-7G, Te-7S and tectorigenin were 21.4, 20.5, 14.3, and 8.67 μmol, and the corresponding T_{max} values were 3.50, 3.17, 5.58, and 4.92 h after treatment, respectively. Furthermore, the mean area under the curve AUC_{0-t} values for Te-7G-4′S, Te-7G, and Te-7S comprised 30.3%, 33.9%, and 22.6% of the four tectoridin metabolites, respectively. It is evident that

Table 2
Regression equations, correlation coefficients (γ), linearity ranges, and lower limit of quantitation (LLOQ) of Te-7G, Te-7S and tectorigenin in rat plasma.

Marker compounds	Calibrations	γ	Linear range (μg/mL)	LLOQ (ng/mL)
Te-7G (**3**)	$Y = 0.5975\,X + 0.0357$	0.9935	0.125–12.5	125
Te-7S (**4**)	$Y = 0.2551\,X + 0.0404$	0.9981	0.20–16.0	200
Tectorigenin (**2**)	$Y = 1.0381\,X + 0.0261$	0.9966	0.025–2.5	25
Tectoridin (**1**)	$Y = 0.1634\,X + 0.1170$	0.9960	0.15–15.0	150

Table 3
Precision and accuracy of HPLC–UV method in rat plasma ($n = 5$).

Marker compounds	Concentration (μg/mL)		RSD (%)		RE (%)
	Added	Found	Intra-day	Inter-day	
Te-7G (**3**)	0.25	0.25	12.4	9.4	−0.4
	1.25	1.24	6.1	10.6	−1.1
	10.00	11.2	8.5	9.2	12.1
Te-7S (**4**)	0.40	0.38	18.2	13.3	−4.2
	2.00	1.84	9.1	8.4	−8.2
	12.80	11.64	14.7	11.1	−9.0
Tectorigenin (**2**)	0.05	0.045	11.8	15.3	−10.5
	0.25	0.29	12.0	11.0	14.5
	2.00	2.10	6.8	11.1	5.0

Table 4
Recovery of Te-7G, Te-7S and tectorigenin in rat plasma by HPLC/UV method ($n = 5$).

Marker compounds	Concentration (μg/mL)	Recovery (%)	RSD (%)
Te-7G (**3**)	0.25	80.7	11.5
	1.25	80.6	10.1
	10.00	78.8	7.8
Te-7S (**4**)	0.40	70.3	13.1
	2.00	88.2	13.7
	12.80	77.8	12.1
Tectorigenin (**2**)	0.05	94.6	5.0
	0.25	87.0	13.1
	2.00	88.4	14.9
Rutin	5.00	105.8	8.0

plasma Te-7G-4′S, Te-7S, and tectorigenin concentrations showed two successive maximum concentrations, which occurred individually at 1.5 and 8 h after treatment. In contrast, a plateau in the plasma concentration–time profile of Te-7G appeared during the same time period.

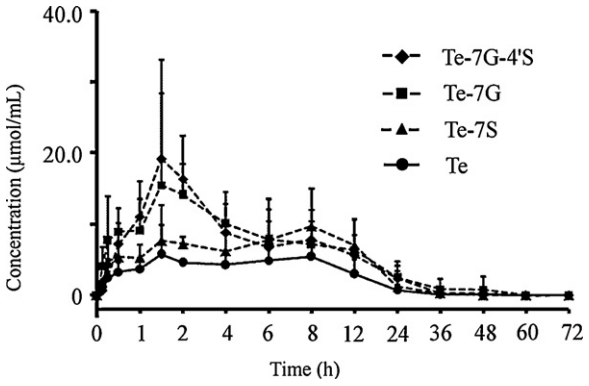

Fig. 4. Plasma concentration–time curves of Te-7G-4′S (**6**), Te-7G (**3**), Te-7S (**4**), and tectorigenin (**2**) after oral administration of tectoridin (200 mg/kg body weight) to rats. Data are expressed as mean ± SD ($n = 6$).

4. Discussion

The present study provided detailed quantitative concentrations of three glucuronyl and sulfate conjugates of tectorigenin in rat plasma after tectoridin administration, the major isoflavone found in the flowers of *P. thomsonii*. Flavonoids with multiple hydroxyl groups are generally metabolized as conjugates with glucuronic or sulfuric acid during the phase II biotransformation pathway in vertebrates [22,23]. Previously, several pharmacokinetic studies of tectoridin were conducted in rats [12,24] but they did not investigate phase II metabolites.

We first isolated Te-7G-4′S from rat bile and confirmed its structure by MS and NMR analysis because LC–MS alone could not confirm whether the glucuronyl or sulfate groups are attached to the C-7 position. Three conjugate metabolites, Te-7G-4′S, Te-7G, and Te-7S, as well as tectorigenin, were then identified in both the plasma and hepatic portal venous plasma of rats orally

Table 5
Stability of Te-7G, Te-7S and tectorigenin in rat plasma by HPLC–UV method ($n = 3$).

Marker compounds	Concentration (μg/mL)	Stability (RE, %)			
		−20 °C/20 days	Freeze–thaw (3 cycles)	20 °C/24 h	Post-extraction
Te-7G (**3**)	0.25	2.9	8.2	−4.1	−2.1
	1.25	11.0	14.8	13.2	3.5
	10.00	13.9	8.1	10.5	2.6
Te-7S (**4**)	0.40	4.4	−7.1	4.3	−3.1
	2.00	14.6	6.2	6.3	4.3
	12.80	1.2	−14.8	−3.0	0.7
Tectorigenin (**2**)	0.05	−9.0	−10.1	5.1	−2.1
	0.25	5.1	9.5	12.9	4.5
	2.00	3.6	−13.3	3.3	−0.8
Tectoridin (**1**)	0.3	−7.4	9.8	6.7	−7.0
	1.50	9.7	10.5	14.0	6.0
	12.00	−10.1	−2.6	−4.3	13.8

Table 6
Pharmacokinetic parameters for metabolites in rat plasma after oral administration of 200 mg/kg tectoridin.

Parameters	Units	Te-7G-4′S (**6**)	Te-7G (**3**)	Te-7S (**4**)	Tectorigenin (**2**)
T_{max}	h	3.50 ± 1.87	3.17 ± 1.81	5.58 ± 3.07	4.92 ± 2.87
C_{max}	μmol L^{-1}	21.4 ± 13.8	20.5 ± 9.70	14.3 ± 3.30	8.67 ± 3.07
$AUC_{(0-t)}$	μmol h L^{-1}	164 ± 52	184 ± 73	123 ± 63	72.0 ± 22.0
$AUC_{(0-\infty)}$	μmol h L^{-1}	197 ± 79	198 ± 78	199 ± 91	98.0 ± 47.7
$MRT_{(0-t)}$	h	9.79 ± 4.47	10.7 ± 4.30	8.12 ± 3.37	8.54 ± 2.01
$MRT_{(0-\infty)}$	h	20.4 ± 16.7	13.7 ± 6.00	15.9 ± 7.12	12.5 ± 3.90
CL/F	L h^{-1} kg^{-1}	2.13 ± 0.96	2.35 ± 0.76	3.28 ± 1.85	8.13 ± 3.57
V/F	L kg^{-1}	31.3 ± 13.8	31.0 ± 23.1	37.9 ± 20.9	80.7 ± 33.1

Data are the mean ± SD ($n = 6$).

administered tectoridin. On the basis of the metabolite profiles, the possible metabolic pathways of tectoridin in rats were presumed to be as follows (Fig. 1). First, tectoridin is hydrolyzed by bacterial β-glucosidases in the intestine and colon to release aglycone tectorigenin [10]. Then, tectorigenin is absorbed from the intestinal tract by passive diffusion, and undergoes glucuronidation and/or sulfation by UDP-glucuronosyltransferases and sulfotransferases to form Te-7G-4′S, Te-7G, and Te-7S at least in the intestinal epithelium because they were also found in hepatic portal venous plasma. The same pathways were also demonstrated in the metabolism of daizein, genistein, and quercetin glucosides after their oral intake in humans [25–27]. Thus, tectoridin undergoes extensive conjugation to produce its glucuronide, sulfate, and glucuronide-sulfate bis-conjugate in the body after hydrolysis by intestinal flora. It should be noted that Te-4′S was a metabolite of tectoridin isolated from urine of rats orally administered tectoridin [14]. However, Te-4′S was not detected in either the plasma sample or hepatic portal venous plasma in the present study. These results suggest that Te-4′S could be formed during the deglucuronidation of Te-7G-4′S in the renal pathway, because rat kidney has been shown to possess high deconjugation activity due to the presence of β-glucuronidase [28].

We further validated the simultaneous quantification of the three conjugated metabolites and tectorigenin in rat plasma using a sensitive HPLC–UV method that detected tectorigenin levels and its major metabolites as low as 25–200 ng/mL. A two-compartmental model was applied to the pharmacokinetic evaluation of these metabolites. Tectoridin was rapidly deconjugated by intestinal glucosidases, and was hardly detectable in plasma samples. The concentration peak for Te-7G-4′S, Te-7G, and Te-7S were 2.5–1.6 times higher than that for tectorigenin, and their AUC_{0-t} values were 2.6–1.7 times higher than those of tectorigenin, indicating that phase II metabolism is a predominant pathway for tectorigenin metabolism. In addition, the higher concentration peak and the sharper concentration curve for Te-7G-4′S and Te-7G than those for Te-7S may be partly attributed to the fact that glucuronidation does not readily reach saturation in the body because of adequate supply of UDP-glucuronosyltransferases. In contrast, the lower concentration peak values and a flat concentration curve for Te-7S may be partly related to the saturation of sulfation caused by the limited supply of sulfotransferase. Several studies have demonstrated the physiological and pharmacological effects of conjugated isoflavone metabolites, such as the inhibitory effect of daidzein-4′,7-disulfate on sterol sulfatase in hamster liver microsomes [29], the stimulatory effect of daidzein-7-glucuronide-4′-sulfate on growth of MCF-7 cells [30], and the hypotensive and vasodilator effects of daidzein sulfates in rats [31]. Therefore, these glucuronide and sulfate metabolites could be responsible for the pharmacological and medicinal properties of isoflavone, and should not be ignored. The pharmacological effects of tectorigenin conjugates need further study.

It is well known that isoflavones undergo enterohepatic recirculation after formation of glucuronide conjugates [32–34]. Interestingly, prominent double peaks appeared in plasma concentration profiles of Te-7G-4′S, Te-7S, and tectorigenin, and a flat concentration curve appeared for Te-7G between 1.5 and 8 h after tectoridin administration. The similarities in T_{max} values and variation in plasma concentrations observed between Te-7G-4′S and Te-7G were observed between Te-7S and tectorigenin. Similar pharmacokinetic data have been previously described for plasma concentrations of daidzein, genistein, and their conjugated metabolites in humans after Kinako administration [27]. These results suggest the differential biliary excretion and reabsorption of conjugated tectoridin metabolites and tectorigenin [35], and further study to compare biliary excretion and urinary excretion in bile duct-cannulated rats and control animals is needed to confirm the enterohepatic recirculation of the metabolites.

Our recent study described the plasma pharmacokinetics of kakkalide [36], the 4′-*O*-methyl derivative of tectoridin, which is present in large quantities in flowers of *Pueraria lobata* (Willd.) Ohwi. In spite of their structural similarity, substantial differences were found in plasma dynamics of tectoridin and kakkalide. Following hydrolysis of kakkalide by intestinal flora, the released aglycone irisolidone undergoes direct 7-*O*-glucuronidation, and successive 6- and 4′-*O*-demethylation, while tectoridin undergoes direct glucuronide and/or sulfate conjugation. Thus, the main metabolites of kakkalide in rat plasma were found to be three glucuronide but no sulfate conjugates. Moreover, the glucuronide metabolites of kakkalide showed lower AUC_{0-t} (35.7–133.1 μmol h L^{-1}) and much longer T_{max} (more than 30 h) than tectorigenin conjugates. The lower permeability, more extensive first-pass metabolism and complex enterohepatic recirculation of irisolidone maybe the possible reasons for its poor bioavailability and delayed absorption. Flowers of *P. lobata* and *P. thomsonii* are both used under the same name 'Puerariae flos' in clinical prescriptions of traditional Chinese medicine. Although kakkalide and tectoridin possess similar pharmacological activities, their plasma pharmacokinetic characteristics differ considerably, which may provide scientific evidence to alter the rationale of the use of both traditional flowers.

5. Conclusion

In summary, this was the first study to evaluate plasma pharmacokinetics of the conjugated metabolites in rats orally administered tectoridin. During phase II metabolism, tectorigenin forms glucuronide and sulfate conjugates, and a glucuronide-sulfate bis-conjugate. The conjugated metabolites are found in the plasma at much higher levels than tectorigenin, and also undergo enterohepatic recirculation. These results should provide useful information for pharmacokinetic and pharmacodynamic investigations of isoflavones containing multiple hydroxyl groups.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.017.

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