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Identification and quantification of baicalein, wogonin, oroxylin A and their major glucuronide conjugated metabolites in rat plasma after oral administration of *Radix scutellariae* product

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ABSTRACT

The current study aims to identify and quantify three flavones (baicalein, wogonin and oroxylin A) and their major metabolites (baicalin, wogonoside and oroxylin A-7-*O*-glucuronide) in rat plasma after oral administration of *Radix scutellariae* product. A simple HPLC/UV method has been developed to simultaneously determine the three flavones and their major metabolites in rat plasma. The chromatographic separation of the six analytes was achieved by a Thermo C_{18} column with linear gradient elution of a mobile phase containing acetonitrile and 20 mM sodium dihydrogen phosphate buffer (pH 4.6). All the tested analytes were detected by PDA detector at a wavelength of 320 nm. The intra-day and inter-day precision for the current assay of the six analytes was within the range of −2.23% to 15.13% and −10.83% to 6.42%, respectively. All the studied analytes could be efficiently extracted from the rat plasma using HLB cartridge with extraction recoveries above 70% and were stable under different storage conditions. The developed assay method was successfully applied to the pharmacokinetic study of baicalin, wogonoside after oral administration of a commercially available *Radix scutellariae* containing capsule at a dose of 3.2 g/kg to Sprague–Dawley rats. In addition to wogonoside, a new metabolite of wogonin has been identified using LC/MS/MS for the first time.

1. Introduction

Radix scutellariae (RS) is an herbal medicine with a variety of pharmacological activities to treat inflammation, cardiovascular diseases, respiratory and gastrointestinal infections. Traditionally, RS functions as a major herb in various herbal decoctions including Xiexin decoction, Shuang-Huang-Lian and Huangqin-Tang. Nowadays, it has been extensively employed in various commercial available proprietary traditional Chinese medicine products such as Huang Qin tablet, Shuang-Huang-Lian capsule, Shuang-Huang-Lian Soft Capsule, Shuang-Huang-Lian Injection, Yin Huang Capsule, and Yin Huang Dripping Pill. The most abundant phytochemical components of RS are flavonoids with over 30 of them being identified and quantified [1]. It has been confirmed that six flavones, namely baicalein (B), baicalin (baicalein-7-glucuronide, BG), wogonin (W), wogonoside (wogonin-7-glucuronide, WG), oroxylin A (OA) and oroxylin A-7-glucuronide (OAG) were the major bioactive components in RS [2,3, Fig. 1]. The six major flavones in RS demonstrated a variety of pharmacological effects including anti-inflammation effect [4–6], anti-cancer effect [7,8], anti-viral effect [9,10], and neuroprotective effect [11].

With the extensive applications of proprietary traditional Chinese medicine (PTCM) products, it is essential to understand their biotransformation *in vivo* including their pharmacokinetic profiles to ensure their safety and efficacy. There have been a few reports on the pharmacokinetic studies of the pure compounds of B, BG and W after administrations to rats. It was found that B could be readily absorbed with a fast and extensive first-pass extraction, while BG was believed to be firstly hydrolyzed to B prior to its absorption [12–15]. There are only two reports on the pharmacokinetics of B and BG in human and the results showed that the glucuronic acid conjugate was the predominant form in plasma and urine [16]. For the pharmacokinetics study of W, it was found that W-7β-D-glucuronide was the dominant form in plasma with only a trace amount of W after its oral administration to Wistar rat [17]. The subchronic toxicity and plasma pharmacokinetic studies of W were also carried out in Beagle dogs. The total area under the plasma concentration–time curve from zero to infinity ($AUC_{0\to\infty}$) was 2137.9 ± 231.4 ng h/ml and $t_{1/2}$ was 1.51 ± 0.43 h after intravenous administration of W [18]. There has been no report about the pharmacokinetic study of pure OA yet.

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In addition to the pure compounds, the pharmacokinetic characteristics of the bioactive components in the herb extract and decoction or of RS, such as Huang-Lian-Jie-Du-Tang and Xiexin decoction were also investigated in rats [14,19]. Besides, Ye et al. monitored the concentrations of BG after administration of Shuanghuang-lian solution via oral, intravenous and intratracheal routes and found the bioavailability of BG was 19.1 ± 9.2% and 36.5 ± 10.2% after intravenous and intratracheal administration, respectively [25]. Since it is usually more than one herb in the decoction, the effects of other co-existing ingredients on the pharmacokinetics of the major components of RS were also investigated in Huang-Lian-Jie-Du-Tang and Huangqin-Tang [14,20]. *Rhizoma coptidis* and *Cortex phellodendri* in Huang-Lian-Jie-Du-Tang could inhibit the activity of β-glycosidase and thus decreased the systemic exposure of BG and WG [14]. Co-existing ingredients in Huangqin Tang could delay the absorption and prolong the circulation time as well as increase the absorption of BG and WG in rat, however the details of the involved ingredients and mechanism are not revealed [20].

Because of the complexity of ingredients in herbal medicines, it is extremely important to simultaneously monitor the bioactive components in biological samples as much as possible for the purpose of pharmacokinetic study. So far, most of the assay methods in plasma focused on the determinations of B, W, BG and WG after the administration of the products containing RS [21–23]. Till now, there were just two articles that have been found to report the *in vivo* pharmacokinetics profiles of OA, but none of them has quantified the plasma concentration of OAG [15,20]. Sharing the similar chemical structure as B and W, it is expected that OA might also undergo extensive Phase II metabolism. In addition, the content of OA also exist in relatively high amount in the reference herb and proprietary traditional Chinese medicine products of *Radix scutellariae* [3]. As a result, it is necessary to develop an assay method to simultaneously determine all the six bioactive flavones, namely B, BG, W, WG, OA and OAG, in plasma.

In the present study, a simple and specific HPLC method has been developed for the simultaneous determination of three bioactive flavones as well as their major glucuronides metabolites in rat plasma. Subsequently, this method has been successfully applied to determine the plasma concentration of the six flavones after oral administration of Shuang-Huang-Lian Capsule (SHL-Cap, a commercial available capsule product of *Radix scutellariae*) to Sprague–Dawley rats.

2. Experimental

2.1. Materials and reagents

Baicalein (B) and baicalin (BG) with purity over 98% were purchased from Sigma–Aldrich Chem. Co. (Milwaukee, WI, USA). Wogonin (W) and wogonoside (WG) with purity over 98% were purchased from AvaChem Scientific LLC (San Antonio, TX, USA). Oroxylin A (OA, purity over 98%) and Oroxylin A-7-O-glucuronide (OAG, purity over 95%) were supplied by Shanghai U-sea Biotech. Co., Ltd. (Shanghai, China). The internal standard (IS), 3,7-dihydroxyflavone with purity of 97%, was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Waters Oasis® hydrophilic–lipophilic-balanced (HLB, 1 cm^3) copolymer extraction cartridges were purchased from Waters. Acetonitrile (Labscan Asia, Thailand) and methanol (TEDIA Company, Inc., USA) were in HPLC grade and used without further purification. Distilled and deionized water was prepared from Millipore water purification system (Millipore, Milford, USA). All other reagents were of at least analytical grade.

2.2. Instrumentation and chromatographic conditions

2.2.1. HPLC/UV

The HPLC system consists of Waters 600 controller (pump), Waters 717 auto sampler and Waters 996 Photodiode Array UV detector. Separation of all the analytes was performed by using a Thermo BDS Hypersil column (250 mm × 4.6 mm; 5 μm particle size) connected to a guard column (Delta-Pak C18 Guard-Pak, Waters). Data were collected by Waters Millennium software (version 3).

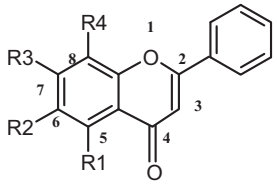
The mobile phase consisting of eluent A (20 mM sodium dihydrogen phosphate buffer, pH 4.6) and B (acetonitrile) was run at 1 ml/min. The linear gradient elution program was set as follows: eluent A decreased from 90% to 70% in the first 10 min, decreased from 70% to 40% in the next 2 min, maintained at 40% for 4 min, then increased from 40% to 90% from 16 to 20 min followed by equilibration for 5 min before the next injection. The detection wavelength was set at 320 nm for all the analytes and the injection volume was 100 μl.

2.2.2. LC–MS/MS

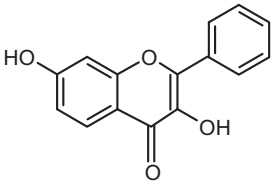
The LC–MS/MS system consisted of ABI 2000 Q-Trap triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI), TWO PerkinElmer PE-200 series micropumps and auto-sampler (PerkinElmer, Norwalk, CT, USA). All the analytes were separated using an Alltima C_{18} column (150 mm × 4.6 mm, i.d., 5 μm particle size, Alltech) protected by a collared frit (4/4.6 mm i.d., 0.5 μm pore size, Thermo Scientific).

The elution gradient for LC analysis consisted of two solvent compositions: acetonitrile (A) and 0.1% acetic acid (B). The gradient began with 35% eluent A for 1 min, changed linearly to 60% A in 6 min and remained for further 3 min before changing back to 10% A in 0.5 min and equilibrating for 2.5 min prior to next injection. Throughout the LC process the flow rate was set at 1 ml/min. The temperatures of auto-sampler and the analytical column were set at 4 °C and ambient, respectively. The sample injection volume was 20 μl and 60% of the LC eluent was split off and only 40% of it was introduced to the ESI source.

The mass spectrometer was set at positive ion mode. Typical instrumental conditions were: ion spray voltage at 5000 V; nitrogen as nebulizer gas, auxiliary gas, curtain gas at 30, 70, 30 and collision gas at 3 psi, respectively; auxiliary gas temperature at 350 °C and interface heater temperature at 100 °C. Other instrumental parameters were analyte specific and were optimized prior to analysis.



Flavones



3, 7-dihydroxyflavone (IS)

Compound	R1	R2	R3	R4
Baicalein (B)	-OH	-OH	-OH	-H
Wogonin (W)	-OH	-H	-OH	-OMe
Oroxylin A (OA)	-OH	-OMe	-OH	-H
Baicalin (BG)	-OH	-OH	$-C_6H_9O_7$	-H
Wogonoside (WG)	-OH	-H	$-C_6H_9O_7$	-OMe
Oroxylin A-7-O-glucuronide (OAG)	-OH	-OMe	$-C_6H_9O_7$	-H
Wogonoside isoform (WG')	$-C_6H_9O_7$	-H	-OH	-OMe

Fig. 1. Chemical structures of major bioactive flavones in *Radix scutellariae* and 3,7-dihydroxyflavone (internal standard).

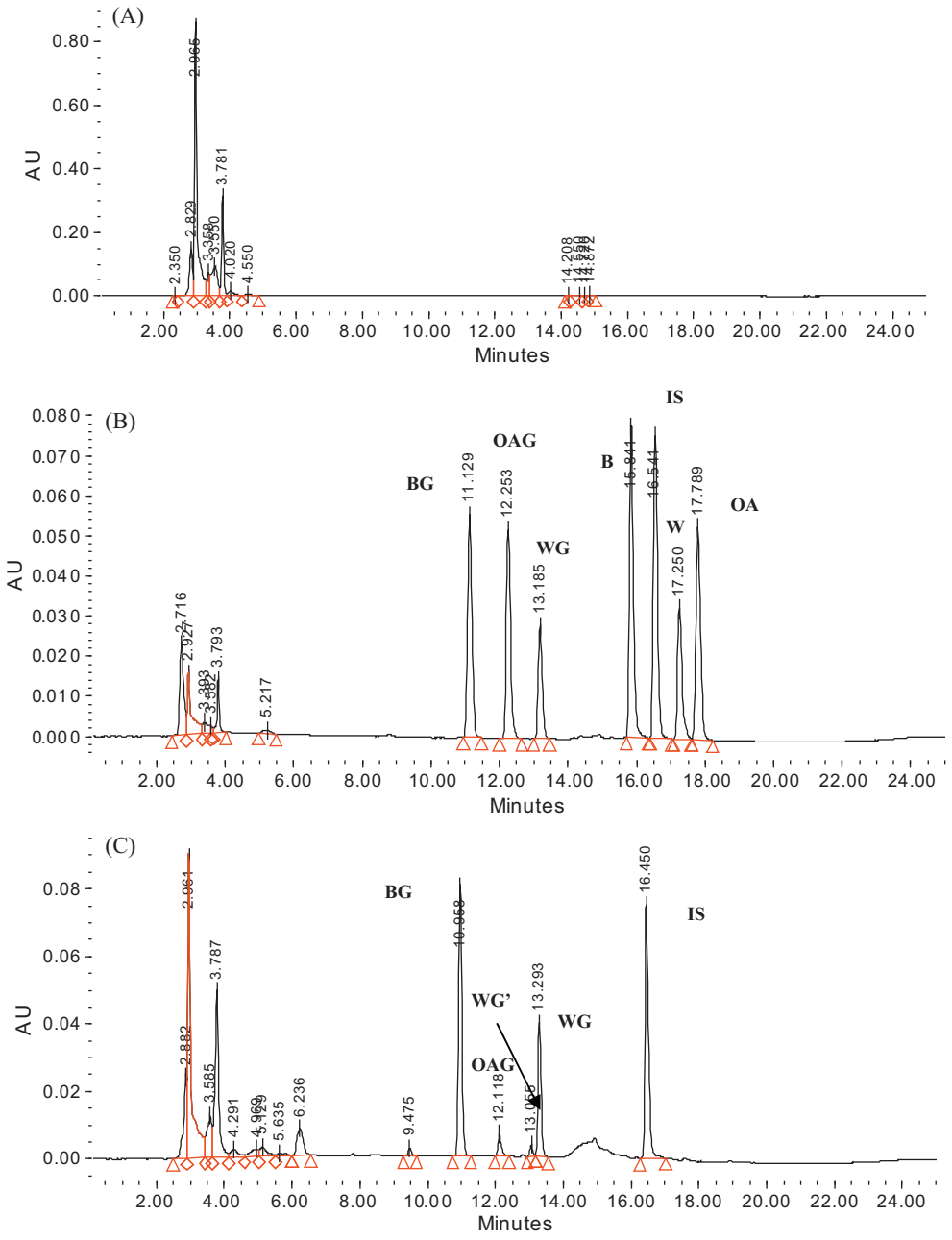


Fig. 2. HPLC/UV chromatograms of (A) blank rat plasma; (B) blank plasma spiked with BG, WG, OAG, B, W and OA at the concentration of 2.0 μg/ml; (C) rat plasma sample obtained at 480 min after oral administration of SHL-Cap (3.2 g/kg).

Data acquisition was conducted at multiple reaction monitoring (MRM), with *m/z* 271 → *m/z* 122.9 for B, *m/z* 285 → *m/z* 270 for W and OA, *m/z* 447 → *m/z* 270.9 for BG, *m/z* 461 → *m/z* 285 for WG and OAG.

2.3. Preparation of standard solutions

The stock solutions of B, BG, W, WG, OA and OAG (1 mg/ml) were prepared by dissolving appropriate amount of each authentic compound in DMSO separately. The working solutions of calibration curve were prepared by mixing and diluting the stock solutions of each compound with methanol and phosphate buffer (25 mM, pH 2.5) comprising 1% ascorbic acid (50:50, v/v) to yield concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0 μg/ml, respectively. Standard solution of IS was prepared by dissolving appropriate amount of 3,7-dihydroxyflavone in methanol at a final concentration of 10 μg/ml.

The samples of calibration curve in plasma were prepared by spiking 50 μl of the above working solutions into 100 μl of blank plasma to yield the concentrations at 0.1, 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0 μg/ml for all the six flavones. Low, medium and high concentrations of quality control (QC) samples were chosen to be 0.8, 4.0 and 8.0 μg/ml for each of the six flavones.

2.4. Plasma sample preparation

Oasis® HLB cartridges were preconditioned with 1 ml of methanol, followed by 1 ml of 25 mM sodium dihydrogen phosphate buffer (pH 2.5). In 100 μl of plasma sample, 50 μl of 50% methanol in phosphate buffer (pH 2.5) comprising 1% ascorbic acid

Table 1
Linearity, intra-day and inter-day precision and accuracy as well as recoveries of the six studied flavones in rat plasma QC samples.

Flavones	Linear range (μg/ml)	Regression equation	R^2	Nominal concentration (μg/ml)	Recovery (%, $n = 3$)	Intra-day ($n = 5$)			Intra-day ($n = 5$)		
						Determined conc. (μg/ml)	Precision (%R.S.D.)	Accuracy (%R.E.)	Determined conc. (μg/ml)	Precision (%R.S.D.)	Accuracy (%R.E.)
BG	0.1–20	$y = 0.1249X + 0.0429$	0.9995	0.8	72.04 ± 9.80	0.74 ± 0.05	6.86	7.77	0.79 ± 0.06	7.71	1.11
				4.0	73.30 ± 19.19	3.86 ± 0.30	7.87	3.54	4.15 ± 0.16	3.90	−3.63
				8.0	82.98 ± 13.98	8.18 ± 0.19	2.27	−2.23	7.92 ± 0.54	6.83	1.02
WG	0.1–20	$y = 0.0819X + 0.0088$	0.9998	0.8	70.17 ± 9.24	0.69 ± 0.04	6.00	13.93	0.75 ± 0.04	4.79	6.00
				4.0	72.43 ± 16.94	3.87 ± 0.29	7.61	3.34	4.18 ± 0.16	3.74	−4.48
				8.0	84.65 ± 11.13	8.08 ± 0.21	2.58	−0.94	8.08 ± 0.58	7.12	−1.00
OAG	0.1–20	$y = 0.1523X + 0.0268$	0.9989	0.8	78.01 ± 11.00	0.68 ± 0.06	8.48	15.13	0.78 ± 0.03	4.38	2.50
				4.0	75.28 ± 17.25	3.81 ± 0.29	7.53	4.64	4.23 ± 0.19	4.59	−5.86
				8.0	87.28 ± 11.78	7.98 ± 0.21	2.68	0.26	8.16 ± 0.59	7.25	−1.94
B	0.1–20	$y = 0.2233X - 0.0762$	0.9987	0.8	80.18 ± 11.38	0.78 ± 0.05	5.90	2.41	0.89 ± 0.09	10.01	−10.83
				4.0	79.41 ± 18.42	3.88 ± 0.13	3.42	2.99	4.01 ± 0.24	6.01	−0.21
				8.0	94.79 ± 17.41	7.72 ± 0.28	3.62	3.50	7.98 ± 0.72	9.03	0.24
W	0.1–20	$y = 0.1258X + 0.0100$	0.9996	0.8	85.40 ± 12.69	0.75 ± 0.03	3.62	6.07	0.75 ± 0.04	5.21	6.42
				4.0	81.60 ± 9.39	4.04 ± 0.13	3.20	−0.99	4.05 ± 0.20	4.89	−1.30
				8.0	97.71 ± 17.70	7.81 ± 0.33	4.27	2.43	8.04 ± 0.68	8.50	−0.55
OA	0.1–20	$y = 0.2581X + 0.0137$	0.9999	0.8	78.46 ± 13.05	0.80 ± 0.03	4.28	0.21	0.79 ± 0.03	4.04	1.51
				4.0	76.15 ± 10.64	3.81 ± 0.12	3.15	4.82	4.02 ± 0.18	4.57	−0.39
				8.0	91.48 ± 16.75	7.58 ± 0.24	3.21	5.22	7.93 ± 0.66	8.28	0.93

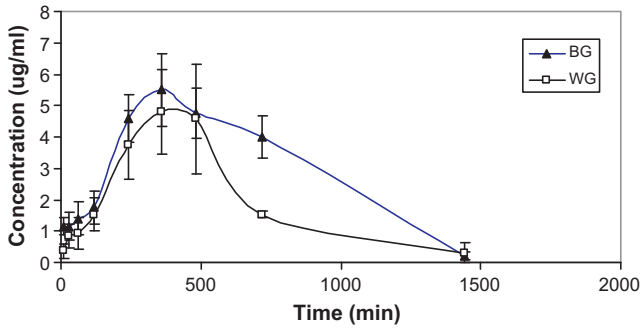


Fig. 3. Plasma concentrations versus time profiles of BG and WG after oral administration of SHL-Cap at 3.2 g/kg to rat ($n = 6$).

and 20 μl of internal standard (3,7-dihydroxyflavone, 10 μg/ml) were added. The mixture was diluted with 1 ml of 35% methanol in 25 mM sodium dihydrogen phosphate buffer (pH 2.5) containing 1% ascorbic acid. After vortexing for 15 s and centrifuging at 16,000 × *g* for 10 min, the supernatant was collected and loaded onto the preconditioned HLB cartridge. The cartridge was then flushed with 1 ml of 25 mM sodium dihydrogen phosphate buffer (pH 2.5) followed by 1 ml of 35% methanol in 25 mM sodium dihydrogen phosphate buffer (pH 2.5) containing 1% ascorbic acid. All the analytes were eluted by 1 ml methanol from the cartridge. The eluent was evaporated to dryness in a Centrivap concentrator, and the residue was reconstituted by 150 μl of 50% methanol in phosphate buffer (pH 2.5) comprising 1% ascorbic acid. After centrifuging at 16,000 × *g* for 10 min, an aliquot of 100 μl supernatant was injected into HPLC system for analysis.

2.5. Validation of the developed assay method

2.5.1. Specificity

The specificity of the method was evaluated by comparing the chromatograms of blank plasma samples from 6 rats with that of blank plasma spiked with standard solutions and rat plasma samples after oral administration of SHL-cap suspension.

2.5.2. Sensitivity

The limit of detection (LOD) was defined as the lowest concentration of the tested compound resulting in a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration of the tested compound resulting in a signal-to-noise ratio of 10:1 with precision below 20% and accuracy within ±20%.

2.5.3. Calibration curve

The calibration curves were plotted using a $1/x^2$ weighted linear regression of the peak–area ratio of each tested compound to internal standard versus the plasma concentrations of each tested compound.

2.5.4. Precision and accuracy

The intra-day precision was determined by analyzing five replicates of QC samples within one day. The inter-day precision was determined by analyzing the QC samples on five separated days. The intra-day and inter-day precision was defined as the relative standard deviation (R.S.D.) and accuracy was defined as relative error (R.E.).

2.5.5. Plasma sample extraction recovery

Plasma sample extraction recovery for each tested compound was calculated by comparing the peak areas of the extracted QC samples to that of the un-extracted standard solutions containing the equivalent amount of each tested compound.

Table 2
Stability of the six flavones under different conditions.

Flavones	Nominal concentration (μg/ml)	Determined concentration (μg/ml)			
		Three freeze–thaw cycle	Room temperature for 2 h	−80 °C for 10 days	Auto-sampler for 24 h
BG	0.8	0.75 ± 0.02	0.67 ± 0.05	0.82 ± 0.02	0.77 ± 0.02
	4.0	3.61 ± 0.17	4.17 ± 0.29	4.03 ± 0.12	4.01 ± 0.06
	8.0	6.88 ± 0.62	8.21 ± 0.21	7.21 ± 0.08	8.00 ± 0.19
WG	0.8	0.73 ± 0.03	0.68 ± 0.04	0.77 ± 0.01	0.75 ± 0.02
	4.0	3.69 ± 0.19	4.15 ± 0.30	4.26 ± 0.08	4.00 ± 0.05
	8.0	7.22 ± 0.80	8.20 ± 0.28	8.12 ± 0.19	7.65 ± 0.21
OAG	0.8	0.74 ± 0.02	0.70 ± 0.04	0.81 ± 0.01	0.76 ± 0.03
	4.0	3.83 ± 0.15	4.21 ± 0.29	4.34 ± 0.08	3.99 ± 0.06
	8.0	7.45 ± 0.78	8.25 ± 0.28	8.13 ± 0.16	7.92 ± 0.21
B	0.8	0.89 ± 0.04	0.91 ± 0.05	0.94 ± 0.01	0.92 ± 0.04
	4.0	3.68 ± 0.07	3.95 ± 0.14	3.94 ± 0.05	3.65 ± 0.09
	8.0	7.42 ± 0.16	8.40 ± 0.64	8.05 ± 0.03	7.73 ± 0.07
W	0.8	0.68 ± 0.00	0.78 ± 0.03	0.77 ± 0.01	0.78 ± 0.02
	4.0	3.70 ± 0.27	4.01 ± 0.14	4.16 ± 0.04	3.94 ± 0.04
	8.0	7.72 ± 0.53	8.12 ± 0.81	7.97 ± 0.18	7.07 ± 0.69
OA	0.8	0.71 ± 0.01	0.76 ± 0.04	0.78 ± 0.01	0.77 ± 0.02
	4.0	3.76 ± 0.23	4.03 ± 0.10	4.05 ± 0.08	3.84 ± 0.07
	8.0	7.46 ± 0.35	8.12 ± 0.71	7.85 ± 0.15	7.84 ± 0.21

2.5.6. Stability

Freeze (−80 °C)–thaw (room temperature) stability was determined by exposing QC samples to three freeze–thaw cycles before sample preparation. Stability at room temperature and long-term stability were evaluated by keeping QC samples at room temperature for 2 h and at −80 °C for 10 days before sample preparation. The stability of samples in auto-sampler was evaluated by analyzing extracted QC samples after being placed in the auto-sampler at room temperature for 24 h.

2.6. Assay application

Male Sprague–Dawley rats (SD rats) with body weight between 230 and 250 g and age between 6 and 7 weeks were supplied by the laboratory Animal Services Center at the Chinese University of Hong Kong. The experiment was conducted under the approval by Animal Ethics Committee of the Chinese University of Hong Kong. The rats were anesthetized with an intramuscular injection of a cocktail containing 60 mg/kg ketamine and 6 mg/kg xylazine (injection volume, 0.2 ml). Right jugular vein was cannulated with a polyethylene tubing (0.5 mm i.d., 1 mm, Portex Ltd., Hythe, Kent, England) for blood sampling.

The content of SHL-Cap was taken out from the capsule and suspended in water to prepare a suspension of 0.4 g/ml. After oral administration of the suspension to six rats at a dose of 3.2 g/kg, blood samples were taken from the jugular vein catheter at 0, 10, 30, 60, 120, 240, 360, 480, 720 and 1440 min, respectively. Plasma samples were obtained after centrifugation of the collected blood samples at 16,000 × *g* for 3 min and stored at −80 °C until analysis. All the plasma samples were treated within 10 days.

In order to confirm the new metabolite of W, SD rats were orally administrated with pure compound of W, which was prepared in the mixture of PEG 400 and 20% Solutol® HS15 (3:7, v/v). The dose of administration was 5 mg/kg and administrated volume was 1 ml. Blood samples were collected via jugular vein catheter at 10 min after oral administration.

2.7. Data analysis

The plasma concentration versus time profile was analyzed by WinNonlin (Pharsight Corporation, Mountain View, CA, USA, Version 2.1). Non-compartmental model was chosen to calculate the pharmacokinetic parameters including the area under the plasma concentration–time curve from zero to infinity ($AUC_{0\rightarrow\infty}$) and elimination half-life ($t_{1/2\lambda z}$). The peak plasma concentration (C_{max}) and the time reaching C_{max} (T_{max}) were obtained directly from the experimental data. All data was expressed as the mean ± SD (standard deviation).

3. Results and discussion

3.1. Assay validation

The representative chromatograms of blank plasma, blank plasma spiked with standard solution at the concentration of 2.0 μg/ml and plasma sample obtained after oral administration of SHL-cap were shown in Fig. 2. Under the established chromatographic condition, there was no endogenous interference in the plasma and all the six flavones as well as the internal standard could be well separated from each other.

In the range of 0.1–20 μg/ml, the calibration curve of each tested compounds produced good linearity ($r^2 \geq 0.999$) using a $1/x^2$ weighted linear regression. The LOD was 0.05 μg/ml and the LOQ was 0.1 μg/ml for all the six analytes. The intra-day and inter-day precision and accuracy of the current developed assay method were listed in Table 1. The R.S.D. of both intra-day and inter-day precision at low, medium and high concentrations for all the six flavones were below 10.01%. Besides, the intra-day and inter-day accuracy calculated by R.E. were within the range of −10.83% to 15.13%.

As shown in Table 1, the plasma extraction recoveries of six analytes at low, medium and high concentrations as well as internal standard were 72.04–82.98% for BG, 70.17–84.65% for WG, 75.28–87.28% for OAG, 79.41–94.79% for B, 81.60–97.71% for W, 76.15–91.48% for OA. The average recovery for internal standard was 75.28 ± 9.33%.

The results of stability tests under various conditions were listed in Table 2. Stability results indicated that all the six flavones were stable at least for three freeze–thaw cycles or at −80 °C for 10 days. Besides, all the six flavones were stable in the prepared samples for 24 h at room temperature and in unprepared samples for at least 2 h at room temperature.

3.2. Application to in vivo pharmacokinetic study

Although studies on the pharmacokinetic profiles of bioactive flavones in traditional RS decoctions have been reported, none of the studies provided the pharmacokinetic information of bioactive flavones in commercially available PTCM products of RS. The decoc-

tions are the water extract of a single herb or various herbs, while the PTCM products might introduce the impact of other excipients. Due to the extensive application of commercial PTCM products of RS, the current assay is proposed aiming to test the pharmacokinetics of its bioactive flavones in these commercial products. Contents of each studied bioactive flavones are essential for the proposed pharmacokinetics studies. Our previously developed assay has simultaneously quantified the six major bioactive components in the studied SHL-Cap and indicated the contents of six flavones to be 522.24, 7.36, 38.08, 12.48, 3.20 and 0.96 mg/kg for BG, WG, OAG, B, W and OA [3]. The same product was used in the current *in vivo* pharmacokinetic study.

In the current study, the newly developed and validated plasma assay method has been successfully applied to the pharmacokinetic studies of BG and WG after oral administration of SHL-Cap at a dose of 3.2 g/kg. In all the collected plasma samples, only the glucuronic acid conjugates of B, W and OA rather than their aglycones could be detected. BG and WG were detectable for all the time points

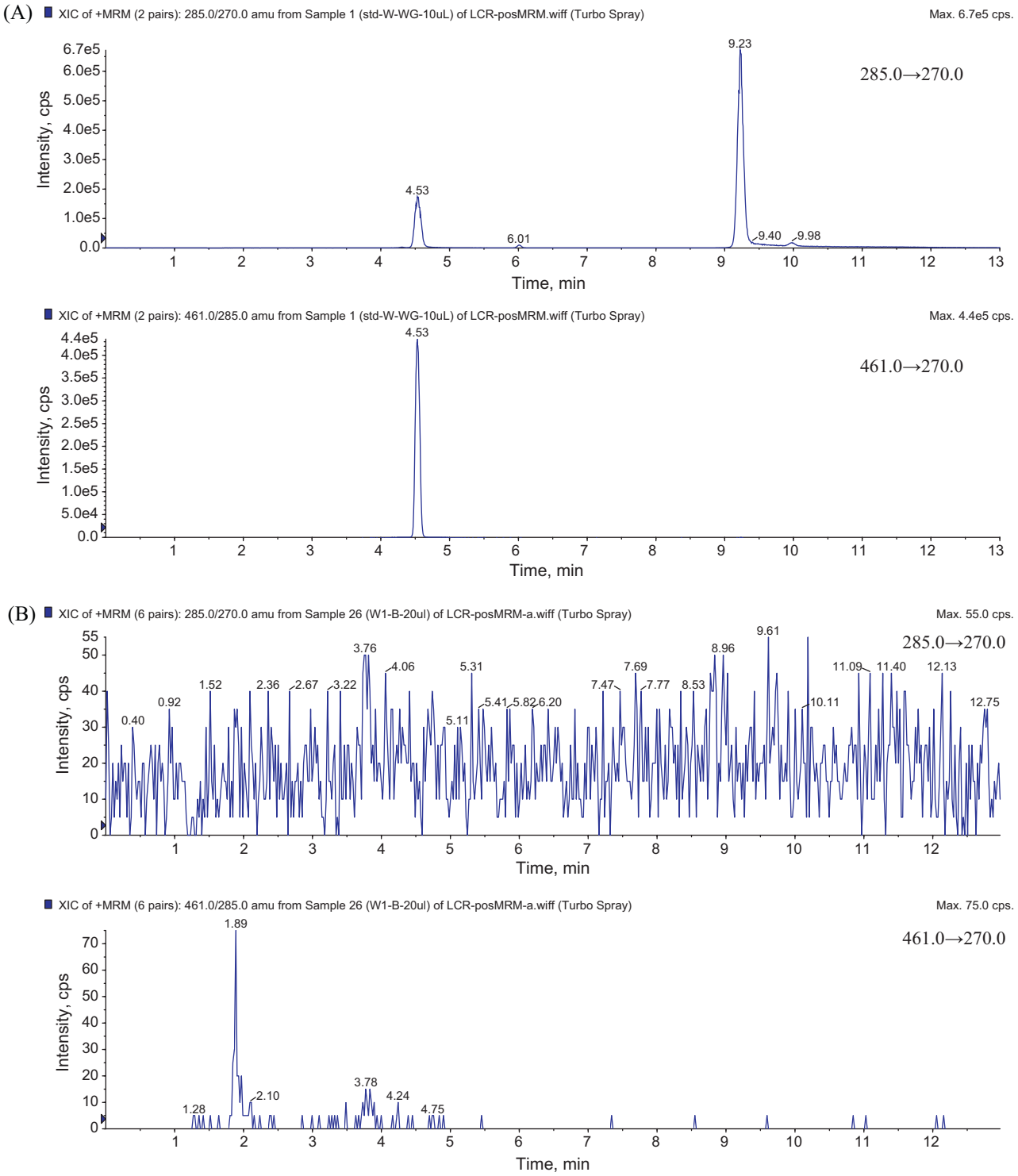


Fig. 4. Positive ion mode MRM chromatograms of (A) standard solution of W and WG mixture at 1 μg/ml; (B) blank rat plasma; rat plasma sample after oral administrations of (C) SHL-Cap at 3.2 g/kg; (D) pure W at 5 mg/kg; (E) pure OA at 5 mg/kg.

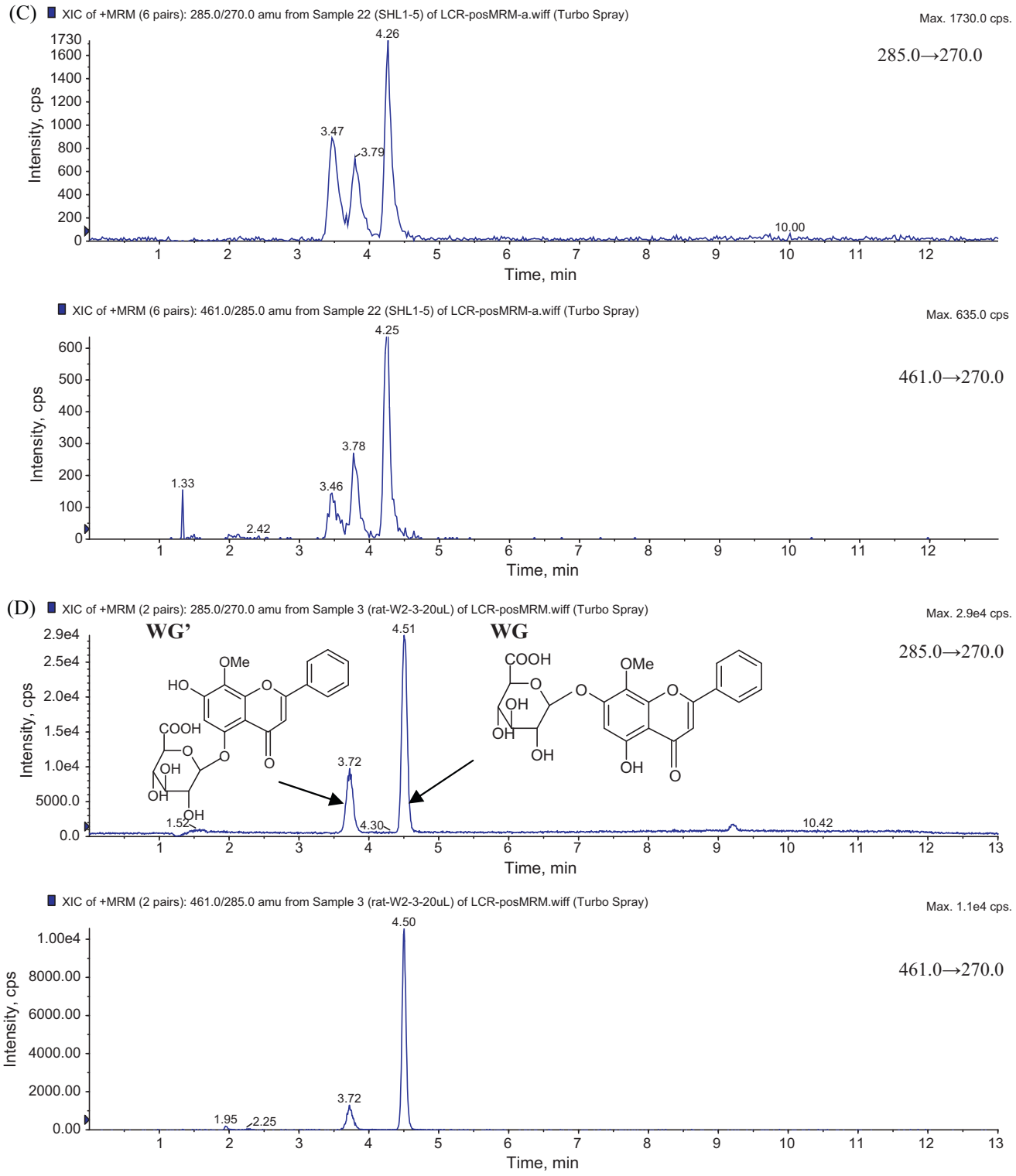


Fig. 4. (*Continued*)

after the oral administration of SHL-Cap, whereas OAG could only be detected in plasma for a few time points around the T_{max} of 360 min, possibly due to the low content of OAG and OA in SHL-Cap. The plot of plasma drug concentration versus time profiles for BG, WG were shown in Fig. 3.

The reason for undetectable aglycones of B, W, and OA could be explained by their potential extensive first pass extraction. Our previous studies of B by *in situ* and *in vitro* models confirmed that B underwent a fast and extensive phase II metabolism during its intestinal absorption to form its glucuronic acid and/or sulfate conjugates. UGT (Urindine 5′-diphospho-glucuronosyltransferase, UDP-glucuronosyltransferase) and SULT (Sulfotransferase) are reported to be involved in such biotransformation [24]. Although the relatively high permeability in Caco-2 monolayer model indicated a good intestinal absorption for B, its extensive intestinal as well as liver first-pass extraction might lead to its low oral bioavail-

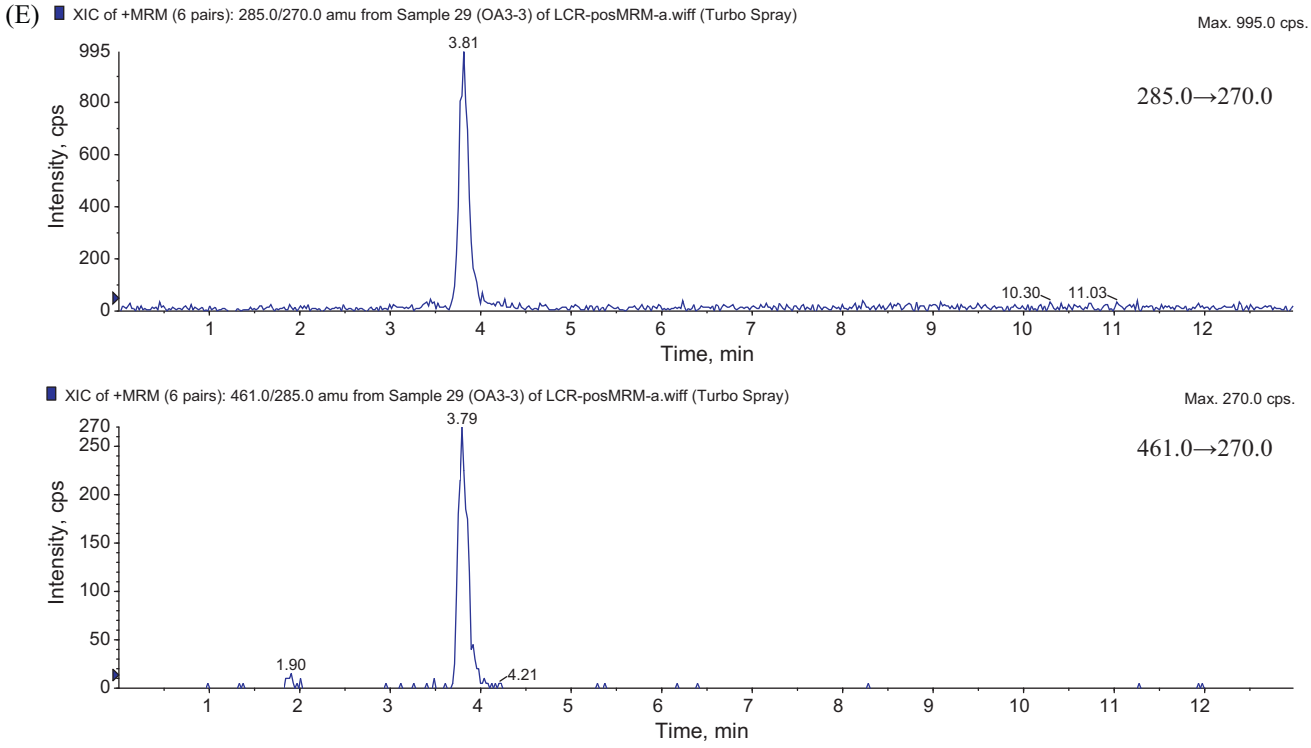


Fig. 4. (*Continued*).

ability. Due to the similarity of chemical structure between B and W and OA, it is expected that W and OA might also undergo extensive Phase II metabolism.

Because of the first-pass extraction at intestine and liver as well as the relatively low content of B, W and OA, the three aglycones in the PTCM products of RS, their corresponding glucuronides are the predominant form in the rat systematic circulation. Monitoring of the glucuronides including BG, WG and OAG seems to be more important than that of their aglycones, especially after oral administration route. The whole plasma concentration versus time profile could be only obtained for BG and WG. The C_{max} and $AUC_{0\rightarrow\infty}$ were comparable to the results published by Ye et al. after dose adjustment [25]. However, T_{max} and $t_{1/2\lambda z}$ in our study were shorter than their results, which might be resulted from the different rat species and administrated formulation. In previous reports, the typical method for quantification of glucuronides is by enzyme hydrolysis of the samples. Amount of glucuronides is equivalent to the amount of aglycones after hydrolysis treatment subtract that obtained before sample hydrolysis. However, such method may not be specific enough since only the aglycones are quantified and the identities of the glucuronides are usually unknown. Besides, the enzymatic hydrolysis treatments of the samples are quite time consuming and do require large amount of samples from each time point. Thus, the development of the assay method to simultaneously analyze both the aglycones and their glucuronides seems to be more applicable for pharmacokinetics studies.

From the HPLC chromatograms of the plasma sample after oral administration of SHL-Cap in rat, there was a new peak appearing just before the peak of WG. Its UV spectrum resembled that of WG. Subsequently, LC/MS/MS was employed to further identify this new metabolite. Under the mode of positive MRM, there were three peaks under the *m/z* transition from 461 to 285, indicating the loss of a glucuronic acid group. Similarly, there were two peaks found in the plasma sample after oral administration of pure W to rat. The retention time of one peak was identical to that of standard solution of WG. However, there was only one peak appearing under the *m/z* from 461 to 285 after oral administration of pure OA to rat. Additionally, there was just one peak of OAG in the HPLC chromatogram after oral administration of pure OA to rat. The MRM chromatograms were shown in Fig. 4. All the evidences above indicated the existence of an isoform of WG. In addition to 7-OH, there is only one more hydroxyl group in W available for forming glucuronic acid conjugates except for the one. Thus, the isoform of WG might be the glucuronic acid conjugate of W at 5-OH (WG′, wogonin-5-*O*-glucuronide). In the study by Chen et al. [17], there was only one glucuronic acid conjugate at 7-OH found in the plasma samples after oral administration W to Wistar rat at 5 mg/kg. Such discrepancy might be caused by species different between Wistar rat and SD rat. However, Chen et al. also indicated that the existence of wogonin-5β-D-glucuronide in rat plasma could not be excluded since their study was designed mainly to detect the predominant wogonin-7β-D-glucuronide and their method may not be sensitive enough to identify the conjugate of W at 5-OH.

It was expected that OA would have the same metabolic pathways as W due to their structural similarity. However, based on the results from pharmacokinetic study *in vivo*, W could be metabolized into two glucuronic acid conjugates at 5-OH or 7-OH of W, whereas OA could just form the glucuronic acid conjugate at 7-OH. Although both compounds could form the similar intra-molecular H bond between C-4 carbonyl group and C-5 hydroxyl group, the strength of intra-molecular H bond of OA may be different from that of W. The ortho methoxyl group in OA had stronger inductive effect on the hydroxyl group at C-5 than the para methoxyl group in W. As a result, the difference in structure made the electron density at C-5 in OA to be lower compared with that in W, which may lead to a more significant intra-molecular H bond effect in OA so as to prevent a second glucuronide formation at C-5.

The pharmacokinetics parameters of WG and BG after oral administration of SHL-Cap to rats were calculated. Both BG and WG reached their C_{max} (BG: 5.97 ± 0.73 μg/ml; WG: 5.75 ± 1.20 μg/ml) at approximate similar T_{max} (BG: 340 ± 90.33

min; WG: 420 ± 69.28 min). In addition, they eliminated with similar $t_{1/2\lambda z}$ at 131.43 ± 22.63 min and 100.43 ± 20.35 min for BG and WG respectively. In addition, it was also noticed that WG had a comparable exposure to BG ($AUC_{0\rightarrow\infty}$ (μg min/ml) of 3300.00 ± 308.19 and 3175 ± 832.35 for BG and WG, respectively) although the content of W and WG are both in very low content in the capsule (40-folds less than BG, 5-fold less than OAG). For flavonoids, they usually exist in the form of aglycones (B, W and OA) and glycosides (BG, WG and OAG). The glycosides could not be absorbed directly in intestine. They have to be metabolized by intestinal enzymes such as lactase phlorizin hydrolase (LPH) and the hydrolyzed aglycones will be further absorbed [26]. In SHL-Cap, six flavonoids of BG, WG, OAG, B, W and OA all exist in certain amount. For the aglycones of B, W and OA, they could be absorbed directly. The comparable AUC of BG and WG as well as low systemic exposure of OAG could result from the competitions at their relevant content in SHL-Cap in (1) the hydrolysis of glycoside BG, WG and OAG by LPH in GI tract; (2) the permeability across intestinal epithelium of the aglycones; (3) further metabolisms and excretion after absorption. For example, for the hydrolysis of BG, WG and OAG by LPH in GI tract, their competition of binding to LPH determined the net amount of B, W and OA for absorption, which warrant further investigation.

4. Conclusion

A simple and specific HPLC/UV method has been developed and validated for the simultaneous determination of B, W, OA, BG, WG and OAG in rat plasma following a solid phase extraction procedure. The developed assay method demonstrated good sensitivity, accuracy, precision, linearity, stability and recovery. Subsequently, this method was successfully applied to determine the concentration of the flavones in plasma after oral administration of SHL-Cap to male Sprague–Dawley rat. In addition to WG, a new metabolite of W has been found and confirmed using LC/MS/MS for the first time.

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