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Simultaneous determination of five flavonoids from Scutellaria Barbata extract in rat plasma by LC–MS/MS and its application to pharmacokinetic study

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ABSTRACT

A new liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the determination of five flavonoids including scutellarin, naringenin, apigenin, luteoline and wogonin in rat plasma using sulfamethalazole as internal standard (IS). Plasma samples were pretreated with liquid–liquid extraction procedure and acid hydrolysis method was used for converting conjugated flavonoids to their respective free forms. The chromatographic separation was performed on a C_{18} column with a linear gradient elution using a mobile phase consisted of 0.01% acetic acid and methanol. The detection was accomplished by multiple-reaction monitoring (MRM) scanning with electrospray ionization (ESI) source operating in the negative ionization mode. The optimized mass transition ion-pairs (m/z) monitored for scutellarin, naringenin, apigenin, luteoline, wogonin and IS were 461.1/285.1, 271.0/119.0, 269.0/117.0, 285.0/132.9, 283.0/268.0 and 252.0/155.9, respectively. The method was linear for all analytes over investigated ranges with all correlation coefficients greater than 0.9915. The lower limit of quantitation (LLOQ) of scutellarin was 9.15 ng/mL and other compounds were all less than 2.0 ng/mL. The proposed method showed appropriate accuracy and repeatability and was suitable for pharmacokinetic studies of the five flavonoids after oral administration of Scutellaria Barbata extract.

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1. Introduction

Traditional Chinese Medicine (TCM) is one of the world's most ancient herbal medicines and has been applied by TCM practitioners for thousands of years. Scutellaria Barbata, an important member of Chinese medicinal herbs, is derived from the dried whole plant of *Scutellaria barbata* (Labiatae) and has been listed in the Pharmacopoeia of the People's Republic of China [1]. Modern pharmacological studies have showed that Scutellaria Barbata has the effects of antibacterial, anticancer, as well as antioxidative and so on [2–5]. So it plays an increasingly important role in clinic for the treatment of urinary, ophthalmic, respiratory and digestion system disorders in China [5].

In recent years, some researches have demonstrated that flavonoids are the main and effective components which mostly contribute to the pharmacological efficacy of Scutellaria Barbata [5–7]. Moreover, scutellarin, naringenin, apigenin, luteoline and wogonin are the major bioactive flavonoids as they have high levels in Scutellaria Barbata [4,6,8–10]. They have been reported to show significant activities, such as the inhibition of 3 strains of human immunodeficiency virus (HIV) [11], the selective growth-inhibitory response on hepatoma cells and the anti-proliferation against Hep G2 cell [12], the inhibition on lipopolysaccharide (LPS)-stimulated NF-κB transcriptional activity [13] and the inhibition of malignant glioma and breast carcinoma cells [14], respectively. Zhang and Ma [15] and Wittemer and Veit [16] reported flavonoids always transformed to their conjugated metabolites under the effect of the phase II metabolic enzymes in liver after being administrated to animals or human. In order to better use Scutellaria Barbata, it is necessary to study the pharmacokinetics of their major components, flavonoids and their conjugated metabolites. Accordingly, a simple, convenient and practical method is required to achieve the determination of the free and total flavonoids in biological matrix.

Some assays with HPLC–UV [17–20] and HPLC–MS [21–25] methods have been described for the determination of scutellarin, naringenin, apigenin, luteoline and wogonin in plasma samples. But these assays mainly focused on the quantification of one or two components or their metabolites. Moreover, the HPLC–UV methods always needed a long chromatographic step (16–25 min) to avoid the interference in plasma samples and achieve better separation. Even though rapid and specific HPLC–MS methods have been used to analyze naringenin or apigenin and luteoline in plasma, the methods were not sensitive enough since the LLOQ of naringenin, apigenin and luteoline exceeded the 2.5 ng/mL. In addition, except a HPLC–UV method reported to study the pharmacokinetics of three flavonoids (scutellarin, isoscutellarein-8-*O*-glucuronide

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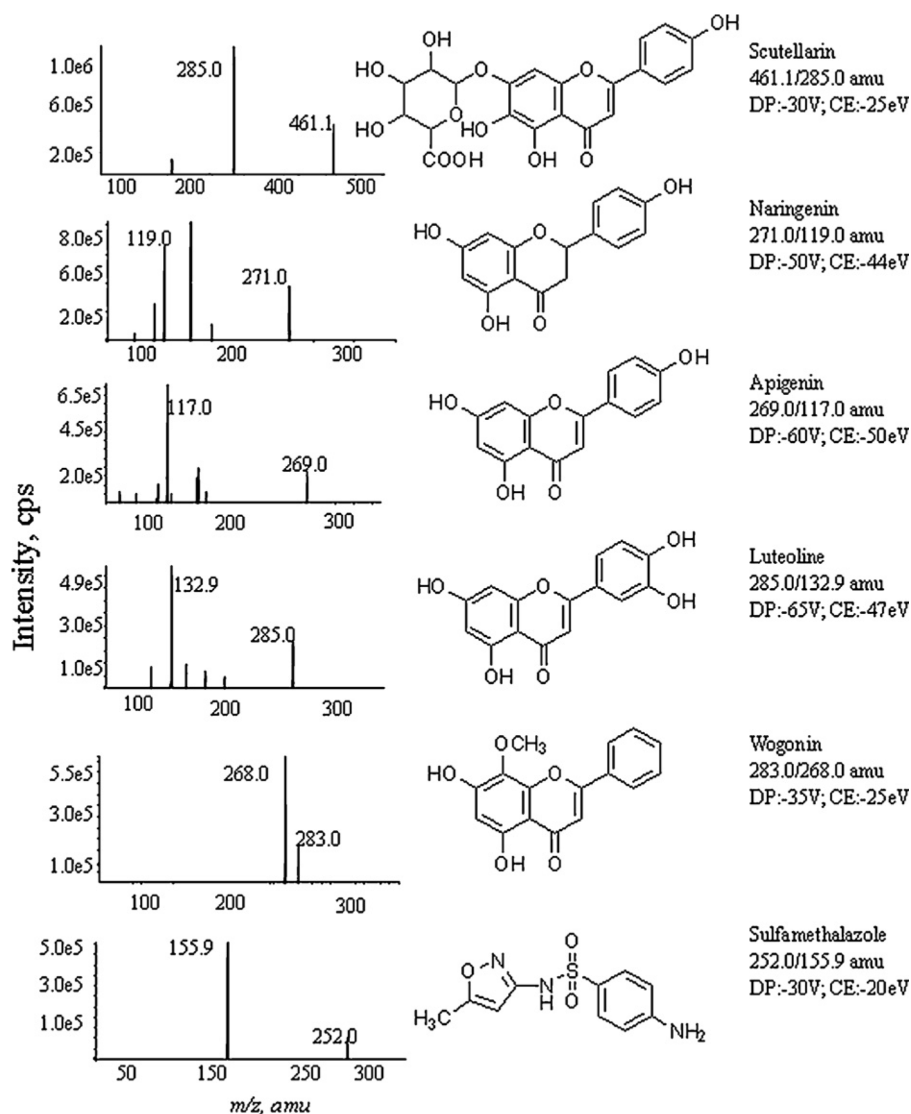

Fig. 1. The product ion scan spectra, chemical structures, monitored transitions, declustering potential (DP) and collision energy (CE) of scutellarin, naringenin, apigenin, luteoline, wogonin and sulfamethalazole.

and luteoline) from total flavonoids of Scutellaria Barbata [26], there were no other studies on the pharmacokinetics of Scutellaria Barbata. The most important was that this method could not offer enough sensitivity due to the LLOQ more than 42 ng/mL. Therefore, in order to more deeply understand the pharmacokinetics of Scutellaria Barbata following oral administration, a more sensitive method for simultaneous determination of multiple components in bio-samples was demanded.

In the present paper, we developed a rather sensitive and selective LC–MS/MS method to simultaneously determine scutellarin, naringenin, apigenin, luteoline and wogonin in rat plasma. The structures are showed in Fig. 1. The method was applied to pharmacokinetics after oral administration of Scutellaria Barbata extract to rats.

2. Materials and methods

2.1. Instruments

The LC–MS/MS system consisted of an Agilent 1200 liquid chromatography system (USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment and a 3200 QTRAP^{TM} system (Applied Biosystems, Foster City, CA, USA) with a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and TurboIonspray interface.

2.2. Chemicals, reagents and materials

Naringenin, apigenin, luteoline, wogonin and sulfamethalazole were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Scutellarin was obtained from Shanghai Tauto Biotech Co., Ltd, China. Methanol and acetic acid (HPLC-grade) were purchased from DIKMA Company (USA). Analytical grade ethyl acetate, hydrochloric acid and dehydrated ethanol (Tianjin Chemical Corporation, China) were used for the preparation of plasma sample and Scutellaia Barbata extract. Deionized water was produced by Heal Force-PWVF Reagent Water System (Shanghai CanRex Analyses Instrument Corporation Limited, China). Scutellaria Barbata was purchased from the Lerentang drugstore in Hebei province in China.

2.3. LC–MS/MS conditions

The chromatographic separation was achieved on an Agilent Zorbax Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm), and the

column temperature was kept at 25 °C. A binary mobile phase system consisted of (A) methanol and (B) 0.01% acetic acid. The run was started at 35% A followed by a linear gradient to 50% A over 1.5 min, turned to 95% A at 8.5 min, held at 95% A for the next 3.5 min, and returned to 35% A over 0.5 min. The liquid flow-rate was set at 0.8 mL/min. In order to reduce the contamination flow into the ion source, the eluent was discharged to waste for the first 2 min.

The ESI interface operated in the negative mode was used. The ion spray voltage was set to −4.5 kV, and the turbo spray temperature was maintained at 600 °C. Nebulizer gas (gas 1) and heater gas (gas 2) was set at 60 and 65 psi, respectively. The curtain gas was kept at 25 psi and interface heater was on. Nitrogen was used in all cases. Quantification was performed using MRM mode. The precursor-to-product ion pairs, declustering potential (DP) and collision energy (CE) for each analyte are listed in Fig. 1. Data acquisition was controlled and synchronized by Analyst software (Versions 1.4.2) from Applied Biosystems/MDS Sciex.

2.4. Preparation of Scutellaria Barbata extract

Two hundred grams of Scutellaria Barbata was soaked in water and extracted three times by decocting with boiling water (1:20, 1:20, and then 1:10, w/v) for 45 min per time. The extracts were combined and concentrated to 750 mL, and then precipitated with one-third volume of dehydrated ethanol, stored at 4 °C for 48 h. After the precipitation was filtered, the ethanol was removed under reduced pressure. The residuary solution was diluted with water to get the Scutellaria Barbata extract with a concentration equivalent to 1.35 g/mL of the Scutellaria Barbata.

The contents of five flavonoids in Scutellaria Barbata extract solution were measured quantitatively by external standard method using the same chromatography conditions as described above. The contents of scutellarin, naringenin, apigenin, luteoline and wogonin in the extract were 7.66, 0.02, 0.18, 1.50 and 0.01 mg/mL, respectively.

2.5. Preparation of standard solution and quality control (QC) samples

The appropriate amounts of scutellarin, naringenin, apigenin, luteoline and wogonin were separately weighed and dissolved in methanol as the stock solutions. Then, the five stock solutions were mixed and diluted with methanol to prepare a final mixed standard solution containing 11,450.00 ng/mL of scutellarin, 972.50 ng/mL of naringenin, 126.50 ng/mL of apigenin, 985.00 ng/mL of luteoline and 632.50 ng/mL of wogonin, respectively. A series of working solutions of these analytes were freshly prepared by diluting mixed standard solution with methanol at appropriate concentrations in the range of 22.88–11,450.00 ng/mL for scutellarin, 1.95–972.50 ng/mL for naringenin, 0.25–126.50 ng/mL for apigenin, 1.52–985.00 ng/mL for luteoline and 1.28–632.50 ng/mL for wogonin, respectively. For the validation of the method, three concentrations of standard solution containing scutellarin (57.25, 1145.00 and 9160.00 ng/mL), naringenin (4.85, 97.25 and 778.00 ng/mL), apigenin (0.625, 12.65 and 101.20 ng/mL), luteoline (4.93, 98.5 and 788.00 ng/mL) and wogonin (3.15, 63.25 and 506.00 ng/mL) were used for preparing the QC plasma samples.

In order to quantify the total concentrations of the four flavonoids naringenin, apigenin, luteoline and wogonin, a series of working solutions of these analytes were prepared as the same processing. The reasonable range of the concentrations was 3.90–1944.00 ng/mL for naringenin, 0.53–253.20 ng/mL for apigenin, 3.93–1968.00 ng/mL for luteoline, 2.53–1264.00 ng/mL for wogonin. The QC samples were also separately prepared at three concentrations of standard solution containing naringenin (9.73, 194.40 and 1555.20 ng/mL), apigenin (1.28, 25.33 and 202.55 ng/mL), luteoline (3.93, 196.80 and 1574.40 ng/mL) and wogonin (2.53, 126.40 and 1011.20 ng/mL).

IS solution of 112.80 ng/mL and 235.20 ng/mL was prepared in methanol for quantify the free and total flavonoids, respectively.

2.6. Preparation of plasma samples

2.6.1. For the determination of free five flavonoids in rat plasma

To a 50 μL aliquot of rat plasma, 20 μL of the IS and 20 μL of methanol (volume of the corresponding working solution for calibration curve and QC samples) and 50 μL of 0.25 mol/L hydrochloric acid were added. This mixture was extracted with 1 mL ethyl acetate by shaking on a vortex-mixer for 2 min. The organic and aqueous phases were separated by centrifugation at 4000 r/min for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. Then the residue was dissolved in 50 μL 50% methanol and mixed on a vortex-mixer. A 20 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.6.2. For the determination of total (free and conjugated) four flavonoids in rat plasma

To a 50 μL aliquot of rat plasma, 50 μL of 4.0 mol/L hydrochloric acid was added. This mixture was vortexed for 30 s and then hydrolyzed in a water bath at 80 °C for 60 min. After cooled to the room temperature, the acid hydrolyzed samples were added with 20 μL of IS, 20 μL of methanol and 1 mL of ethyl acetate. Then the following processing was the same as descried above. A 10 μL aliquot of the pretreated solution was injected into the LC–MS/MS system for analysis. The plasma samples which concentrations exceeded the upper quantification limits were accurately diluted with blank plasma (volumetric ratio was 1:1) and then were processed by the same approach described above.

2.7. Method validation

To evaluate linearity, plasma calibration curves consisted of seven concentration levels and each concentration was prepared and assayed in duplicate on 3 separate days. The calibration plasma samples were prepared by adding 20 μL of working solutions mentioned above and 20 μL of IS solution to 50 μL of blank plasma, and then were extracted and assayed with the method described above. The ratios of peak area of the analytes to that of IS were plotted against nominal concentrations of the analytes and standard curves were in the form of $Y = aX + b$ ($1/X^2$ weighed).

LLOQ was determined at the lowest concentration point of the standard curve, at which the concentration that could be reliably and reproducibly measured on 3 different validation days. The determined processing is the same as the QC samples.

Six replicates of QC samples with three batches were assayed to calculate the precision and accuracy of this method on 3 different validation days. The precision is expressed by relative standard deviation (RSD) between the replicate measurements. Accuracy is defined as relative error (RE) which is calculated using the formula RE % = [(measured value − theoretical value)/theoretical value] × 100.

The extraction recoveries of analytes at LLOQ and three QC levels were evaluated by determining the peak area ratios of the analytes in the post-extraction spiked samples to that acquired from pre-extraction spiked samples. The matrix effects were measured by comparing the peak areas of the analytes dissolved in the pretreated blank plasma with that of pure standard solution containing equivalent amounts of the analytes. LLOQ and three QC levels of

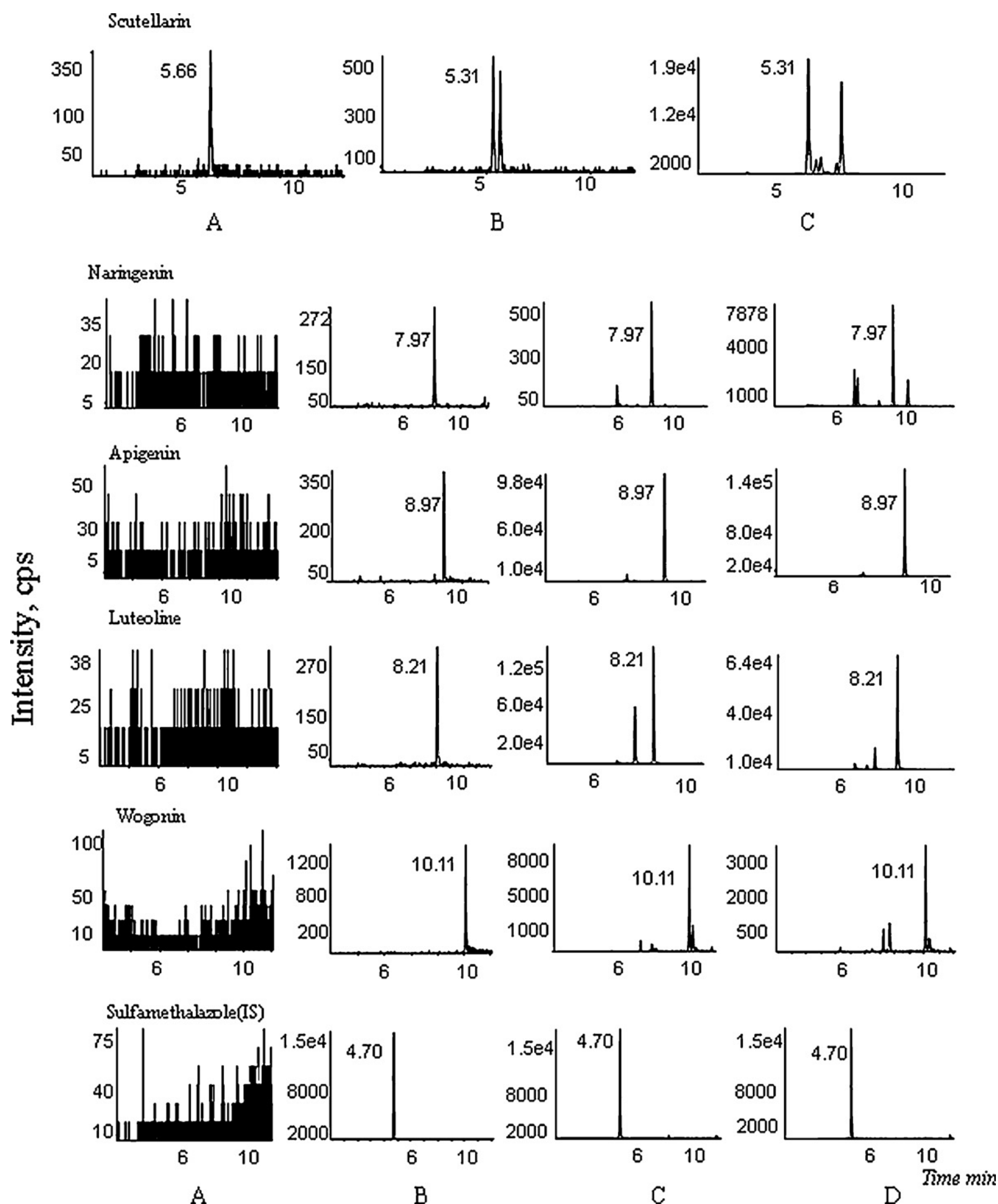

Fig. 2. Representative MRM chromatograms of scutellarin, naringenin, apigenin, luteoline, wogonin and sulfamethalazole in (A) blank plasma, (B) blank plasma spiked with the five analytes and IS at LLOQ, (C) 4 h plasma and (D) 4 h plasma sample with acid hydrolysis after a single oral administration of Scutellaria Barbata extract.

compounds were evaluated by analyzing six samples at each level for both of the extraction recovery and of the matrix effect.

The stability of the analytes in rat plasma was assessed by analyzing QC samples at three concentration levels approached to three different conditions. The short-term stability was determined with untreated QC samples stored for 24 h at room temperature. The long-term stability was assessed after the untreated QC samples had been stored at −20 °C for a week. The freeze–thaw stability was determined after three freeze–thaw cycles (−20 °C to room temperature as one cycle). The post-preparative stability was evaluated with the reconstituted solution of 50% methanol kept for 8 h at room temperature.

2.8. Application of the LC–MS/MS quantitative analysis

Six male Sprague–Dawley rats (weighing 250 ± 20 g, Experimental Animal Research Center, Hebei Medical University, China) were housed in a stainless steel cage with free access to water, and were fed daily with a laboratory diet of rat. Before being administered Scutellaria Barbata extract, the rats were fasted for 12 h but with access to water. Blood samples of approximately 0.3 mL were collected from the vein of the eye ground at 0.08, 0.5, 4, 5, 6, 8, 12, 24, 36, 48 h after a single oral administration of Scutellaria Barbata extract (8 mL/kg). Plasma was obtained by centrifugation and duplicate 50 μL plasma samples were dispensed to heparinized tubes

Table 1
The regression equations, linear ranges and LLOQs for the determination of the free and total analytes.

Compounds	$Y=aX+b$	R	Linear range (ng/mL)	LLOQ (ng/mL)
The free				
Scutellarin	$Y=0.0326X+0.0169$	0.9932	9.15–4580.00	9.15
Naringenin	$Y=0.0295X+0.0157$	0.9970	0.78–389.00	0.78
Apigenin	$Y=0.0571X+0.0292$	0.9975	0.10–50.60	0.10
Luteoline	$Y=0.0455X+0.0181$	0.9953	0.79–394.00	0.79
Wogonin	$Y=0.1180X+0.0496$	0.9915	0.51–253.00	0.51
The total				
Naringenin	$Y=0.0447X+0.0225$	0.9982	1.56–777.60	1.56
Apigenin	$Y=0.1140X+0.1130$	0.9972	0.20–101.28	0.20
Luteoline	$Y=0.0735X+0.0921$	0.9977	1.57–787.20	1.57
Wogonin	$Y=0.1740X+0.1580$	0.9964	1.01–505.60	1.01

and stored at −20 °C until use. Blank plasma was obtained from the rat without oral administration and was used to investigate the assay development and validation.

3. Results and discussions

3.1. Method development

3.1.1. Mass spectrometry

The first step in developing the method was to select precursor ions and product ions of the analytes and IS for MRM mode analysis from the characteristic mass spectra by syringe pump infusion. The standard solution (50 ng/mL) of the analytes and IS were infused into the mass spectrometer separately to obtain detected ions and to optimize mass parameters such as DP and CE. The negative electrospray interface was used for good sensitivity, reproducibility and fragmentation were obtained. In the full scan mass spectra, the deprotonated molecular ions $[M-H]^-$ of scutellarin, naringenin, apigenin, luteoline, wogonin and IS (m/z 461.1, 271.0, 269.0, 285.0, 283.0 and 252.0) were stable and exhibited higher abundance. Under the product ion scan mode, the most intensive ion was $[M-H-C_6H_8O_6]^-$ (m/z 285.1) for scutellarin. As naringenin, apigenin, luteoline are simple substitutional flavonoids, they easily produced $[M-H-C_7H_4O_4]^-$ at m/z 119.0, 117.0 and 132.9, respectively. The most abundant fragments of wogonin and IS were $[M-H-CH_3]^-$ (m/z 268.0) and $[M-H-C_4H_4N_2O]^-$ (m/z 155.9), respectively. Fig. 1 shows the product ions scan spectra of the analytes and IS.

3.1.2. Chromatography

Electrolyte modification of mobile phase can significantly improve the ESI efficiency resulting in enhanced analyte response [27]. At the beginning, we used an electrolyte-free mobile phase water phase/methanol and then tested different electrolyte including acetic acid, formic acid and ammonium acetate in water phase. It was worth noting that the electrolyte concentration had more effect on the intensity of scutellarin than that of any other analyte, which probably attributed that scutellarin is a flavonoid glycoside and its polarity is stronger than that of other compounds. And adding acetic acid in mobile phase could more significantly enhance the sensitivity of scutellarin comparing with adding ammonium acetate, formic acid, and electrolyte-free mobile phase. While the responses of other four compounds were hardly influence. In order to obtain a better peak shape, the concentration of acetic acid in water phase was also optimized. It was found that the peak shapes of analytes were becoming better with the reducing of acetic acid.

Table 2
The intra-day and inter-day accuracies and precisions for the determination of free analytes ($n=3$ days, 6 replicates per day).

Compounds	Intra-day			Inter-day		
Spiked conc. (ng/mL)	Measured conc.[a] (ng/mL)	Accuracy (%)	Precision (%)	Measured conc.[a] (ng/mL)	Accuracy (%)	Precision (%)
Scutellarin						
9.15	9.57 ± 0.29	4.54	2.51	9.81 ± 0.35	5.51	3.62
22.90	22.74 ± 0.74	−0.69	7.52	22.71 ± 1.75	−0.85	9.08
458.00	449.30 ± 13.99	−1.90	3.94	448.35 ± 18.73	−2.11	5.72
3664.00	3587.32 ± 115.24	−2.09	6.31	3545.24 ± 212.95	−3.24	3.24
Naringenin						
0.78	0.78 ± 0.03	0.57	3.42	0.80 ± 0.03	2.40	4.20
1.94	1.99 ± 0.17	2.40	1.19	2.01 ± 0.22	3.61	0.51
38.90	43.22 ± 4.11	11.10	1.20	43.96 ± 4.21	13.01	0.48
311.20	425.02 ± 46.51	4.40	1.45	328.17 ± 47.18	5.45	1.01
Apigenin						
0.10	0.10 ± 0.01	−2.37	14.40	0.09 ± 0.01	1.57	11.80
0.25	0.22 ± 0.02	−12.40	14.80	0.22 ± 0.03	−12.44	5.30
5.06	5.68 ± 0.33	12.20	9.92	5.43 ± 0.55	7.25	11.36
40.48	46.15 ± 2.90	14.00	6.80	44.97 ± 3.30	11.09	10.65
Luteoline						
0.79	0.77 ± 0.04	−2.78	4.60	0.75 ± 0.04	−5.10	5.60
1.97	2.04 ± 0.14	3.50	10.20	2.05 ± 0.20	4.19	7.52
39.40	42.30 ± 0.80	7.40	7.05	44.14 ± 3.18	12.02	8.85
315.20	313.38 ± 6.10	−0.58	7.40	317.76 ± 23.66	0.81	7.51
Wogonin						
0.51	0.50 ± 0.02	−1.52	4.30	0.49 ± 0.02	−3.50	4.90
1.26	1.21 ± 0.11	−3.70	7.90	1.16 ± 0.09	−8.15	10.30
25.30	24.69 ± 1.61	−2.40	12.13	24.70 ± 2.90	−2.37	9.32
202.40	215.33 ± 9.59	6.39	7.30	213.53 ± 18.27	5.50	14.81

[a] Mean ± standard deviation.

Table 3
The intra-day and inter-day accuracies and precisions for the determination of the total analytes ($n = 3$ days, 6 replicates per day).

Compounds	Intra-day			Inter-day		
Spiked conc. (ng/mL)	Measured conc.[a] (ng/mL)	Accuracy (%)	Precision (%)	Measured conc.[a] (ng/mL)	Accuracy (%)	Precision (%)
Naringenin						
1.56	1.57 ± 0.05	0.70	2.81	1.60 ± 0.04	2.41	2.48
3.89	4.17 ± 0.50	7.30	7.83	4.12 ± 0.33	5.94	9.74
77.76	72.39 ± 5.62	−6.90	6.21	71.51 ± 4.87	−8.04	10.32
622.08	622.09 ± 11.86	0.00	1.90	622.15 ± 11.67	0.01	1.62
Apigenin						
0.21	0.21 ± 0.01	−2.40	9.70	0.19 ± 0.02	−10.30	7.40
0.51	0.51 ± 0.01	1.50	2.62	0.50 ± 0.01	−0.11	4.11
10.13	9.64 ± 0.13	−4.80	1.30	9.67 ± 0.13	−4.56	1.51
81.02	80.25 ± 2.45	−1.00	3.20	81.14 ± 2.63	0.15	3.20
Luteoline						
1.57	1.55 ± 0.02	−1.30	3.30	1.54 ± 0.02	−2.11	2.23
3.94	3.70 ± 0.07	−6.10	2.97	3.74 ± 0.12	−5.04	4.74
78.72	78.78 ± 0.91	0.07	1.89	78.87 ± 1.59	0.19	3.31
629.76	626.55 ± 7.15	−0.50	1.91	626.71 ± 13.68	−0.48	3.80
Wogonin						
1.01	1.01 ± 0.02	−0.50	3.30	1.02 ± 0.01	0.99	2.06
2.53	2.50 ± 0.08	−1.35	3.30	2.53 ± 0.10	0.20	6.93
50.56	49.45 ± 0.78	−2.19	2.21	49.43 ± 1.19	−2.23	3.80
404.48	389.46 ± 9.91	−3.70	2.10	389.92 ± 8.03	−3.60	1.74

[a] Mean ± standard deviation.

When 0.01% acetic acid was used, the analytes reached to the best symmetrical peak shape and the interference from the biological specimen was also avoided.

3.1.3. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. LLE, protein precipitation and SPE method are commonly used for sample preparation. Protein precipitation method with the advantages of simplicity and time saving was used firstly to analysis. However, this technique resulted in high noise level. Finally, LLE was applicable to use as it offers a pure sample and low matrix effect. Extraction solvent such as ethyl acetate, chloroform and diethyl ether were all considered. Because of the highest extraction efficiency and lowest noise level, ethyl acetate was selected as extraction solvent. However, the stronger polarity makes scutellarin difficult to be directly extracted with ethyl acetate from plasma. So we added appropriate concentration of hydrochloric acid (0.25 mol/L) to acid plasma to improve the extract efficiency and the extraction of other analytes was not influenced obviously.

In the present study, the acid hydrolysis method was used to convert the conjugated metabolites into their aglycone forms so that the total concentrations of the analytes (free and their conjugated metabolites) could be quantified. According to the previous study of Zhao et al. [28], 4 mol/L hydrochloric acid and the incubation temperature at 80 °C were considered to be a good completely hydrolyzed condition with flavonoids conjugated metabolites. And the hydrolysis efficiency had not significant difference between the hydrolysis duration of 30 and 60 min. In order to make sure the conjugated metabolites could be completely hydrolyzed in the present study, the hydrolysis time was investigated at 60 and 70 min with the plasma samples obtained at about 5 h after oral administration

Table 4
The mean extraction recoveries and mean matrix effects for the determination of the free and the total analytes ($n = 6$).

The free				The total			
Compounds	Spiked conc. (ng/mL)	Recovery%[a]	Matrix effect%[a]	Compounds	Spiked conc. (ng/mL)	Recovery%[a]	Matrix effect%[a]
Scutellarin	9.15	53.8 ± 5.6	88.2 ± 4.8	–	–	–	–
	22.90	54.2 ± 2.2	103.0 ± 3.9		–	–	–
	458.00	72.4 ± 4.3	103.2 ± 6.7		–	–	–
	3664.00	81.4 ± 5.3	103.3 ± 5.4		–	–	–
Naringenin	0.78	71.8 ± 9.6	86.3 ± 4.1	Naringenin	1.56	73.6 ± 9.8	95.2 ± 8.2
	1.94	69.6 ± 6.1	89.9 ± 11.3		3.89	85.3 ± 10.0	99.6 ± 3.5
	38.90	83.0 ± 9.1	91.9 ± 7.3		77.76	70.5 ± 4.9	92.2 ± 10.2
	311.20	81.8 ± 2.6	97.6 ± 5.4		622.08	78.2 ± 5.6	96.12 ± 7.9
Apigenin	0.10	78.6 ± 10.2	88.5 ± 9.7	Apigenin	0.20	72.6 ± 6.5	104.6 ± 3.0
	0.25	73.2 ± 7.4	104.0 ± 6.5		0.51	70.5 ± 3.3	85.4 ± 5.6
	5.06	78.0 ± 3.3	97.0 ± 5.6		10.13	78.6 ± 3.6	98.1 ± 7.0
	40.48	82.3 ± 3.4	91.2 ± 7.2		81.02	79.4 ± 7.4	94.6 ± 7.0
Luteoline	0.79	76.8 ± 12.2	102.3 ± 7.2	Luteoline	1.57	84.3 ± 3.9	106.0 ± 6.8
	1.97	75.4 ± 4.6	103.0 ± 7.8		3.94	85.5 ± 4.2	98.6 ± 3.0
	39.40	80.0 ± 5.0	96.4 ± 5.4		78.72	77.6 ± 5.8	93.0 ± 2.6
	315.20	84.2 ± 3.6	99.3 ± 5.2		629.76	83.3 ± 3.7	102.9 ± 11.6
Wogonin	0.51	57.8 ± 8.3	92.5 ± 9.2	Wogonin	1.01	78.7 ± 4.6	94.3 ± 7.7
	1.26	54.3 ± 3.8	86.2 ± 6.4		2.53	82.6 ± 4.6	96.9 ± 3.3
	25.30	72.9 ± 8.9	81.9 ± 2.7		50.56	71.0 ± 3.2	99.6 ± 4.3
	202.40	83.0 ± 2.0	97.9 ± 6.3		404.48	83.5 ± 1.3	98.0 ± 7.4

[a] Mean ± standard deviation.

to rat. It was found that the concentrations of the four flavonoids (naringenin, apigenin, luteoline, wogonin) had no significantly difference when the hydrolysis time of 60 min and 70 min were performed, which indicated that the hydrolysis could be complete in 60 min. Thus, the hydrolysis time of 60 min was selected. However, scutellarin was found to be able to be hydrolyzed for it is a flavonoid glycoside. Thus the total concentration of scutellarin in plasma was not determined.

3.1.4. Selection of IS

Suitable IS is the key of biological sample analysis. It was used to correct the unknown losses during the quantitative processing. Several flavonoids compounds served as IS were investigated, such as resveratrol, rutin and isorhamnetin. But it was found that all of them were unable to be considered as IS. Resveratrol was unstable for it was prone to convert to its cis-trans-isomer in solution due to the influence of "C=C". Rutin was able to be detected in the Scutellaria Barbata extract. Isorhamnetin was one of the metabolites of quercetin [29] which was one of components in Scutellaria Barbata [30]. It was reported that flavonoids are the main chemical constituents in Scutellaria Barbata [30,31], which implied that it would need great effort to find a flavonoid as an ideal IS. Finally, a chemical synthetic drug named sulfamethalazole was chosen as an IS by comprehensive consideration of the interference in biological specimen, stability under this analysis procedure, and consistency with the analytes at retention time and extraction efficiency.

3.2. Method validation

The selectivity of the method towards endogenous plasma matrix was evaluated with plasma of six rats. The typical chromatograms of blank plasma, plasma sample spiked with the analytes and IS, and the plasma sample from a rat after oral administration of Scutellaria Barbata extract are showed in Fig. 2. Blank plasma yielded relative clean chromatograms without interfering peaks both to the analytes and to the IS. The retention times of the scutellarin, naringenin, apigenin, luteoline, wogonin and IS were 5.31, 7.97, 8.97, 8.21, 10.11 and 4.70 min, respectively.

Table 1 lists the typical equation of calibration curves and linearity ranges for the five analytes. All correlation coefficients were higher than 0.9915. The results for LLOQs are also showed in Table 1. The LLOQ of scutellarin was 9.15 ng/mL and other compounds were all less than 2.0 ng/mL. In particular, the LLOQs of naringenin, apigenin and luteoline were 0.78 ng/mL, 0.10 ng/mL and 0.79 ng/mL, respectively. It was indicated that this method was sufficient for pharmacokinetic study. The results of the accuracy and precision of the five analytes in LLOQ and QC samples are summarized in Tables 2 and 3. It could be seen that both the accuracy in term of RE and the precision in term of RSD were less than 15%, which demonstrated that the values were all within the acceptable range.

At four concentration levels of the analytes (including LLOQ and QC samples), the extraction recoveries were all between 53.80% and 85.45%, and no significant matrix effect for scutellarin, naringenin, apigenin, luteoline and wogonin was observed. The data are summarized in Table 4. The recoveries of IS with 112.80 ng/mL and 235.20 ng/mL were 80.3% and 84.3% and the matrix effect of IS were 96.2% and 97.4%, respectively.

The stability of the analytes in rat plasma was investigated under a variety of storage. The analytes were found to be stable with the RE less than 14.0% in rat plasma after three freeze–thaw cycles and for at least 24 h at room temperature. The analytes was also demonstrated to be stable for a week under −20 °C and for post-preparative samples which kept in 8 h at room temperature, with the RE between −11.5% to 10.1% and −9.0% to 3.1%, respectively.

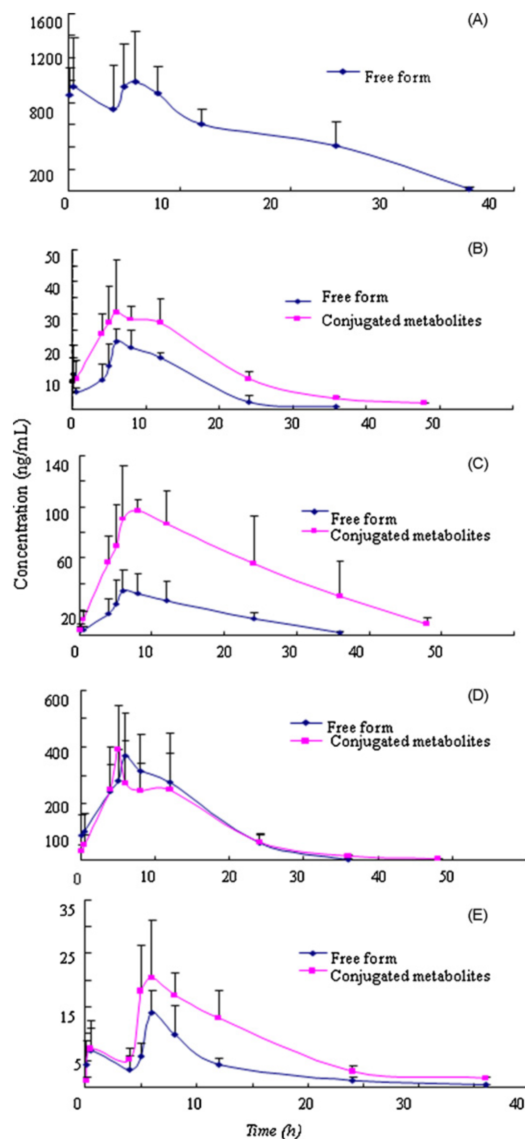

Fig. 3. Mean plasma concentration–time curves of free five flavonoids and their respective conjugated metabolites after a single oral administration of Scutellaria Barbata extract (A) scutellarin, (B) naringenin, (C) apigenin, (D) luteoline, (E) wogonin.

3.3. Application of analytical method in pharmacokinetic studies

The validated LC–MS/MS method was successfully applied to simultaneous determination the five analytes for 36 h or 48 h after oral administration of the Scutellaria Barbata extract. The concentrations of four flavonoids aglycone converted from their respective conjugated metabolites (C_M) were calculated by the formula: $C_M = C_T \times 2 - C_F$. In which, the C_T and C_F represent the concentrations of the total (free and conjugated) four flavonoids and concentrations of the free four flavonoids, respectively. "×2" represents that the volume for analysis of the C_F was twice as much as the volume for analysis of the C_T.

The mean plasma concentration–time profiles of the free analytes and their respective conjugates metabolites are illustrated in Fig. 3. The pharmacokinetic parameters including half-time ($T_{1/2}$), maximum plasma concentration (C_{max}), time to reach the maximum concentrations (T_{max}), elimination rate constants (K), area

Table 5
Mean pharmacokinetic parameters of the free analytes and the conjugated metabolites after oral administration of Scutellaria Barbata extract.

	Parameters	Compounds				
		Scutellarin	Naringenin	Apigenin	Luteoline	Wogonin
The free	$T_{1/2}$ (h)	5.04	5.62	9.56	4.95	9.35
	C_{max} (ng/mL)	1266.33	23.48	36.12	407.65	16.08
	T_{max} (h)	5.75	8.25	6.62	6.53	6.67
	K (1/h)	0.002	0.002	0.001	0.0024	0.0014
	$AUC_{0\to36}$ (ng h/mL)	1,123,868	20,047	40,045	304,934	8419
	$AUC_{0\to\infty}$ (ng h/mL)	1,348,517	20,454	42,572	308,663	8747
The conjugated	$T_{1/2}$ (h)	–	11.43	13.12	6.73	9.31
	C_{max} (ng/mL)	–	36.55	123.28	457.12	32.13
	T_{max} (h)	–	6.25	7.80	6.33	5.67
	K (1/h)	–	0.0012	0.0012	0.0018	0.0013
	$AUC_{0\to36}$ (ng h/mL)	–	–	–	–	15,235
	$AUC_{0\to48}$ (ng h/mL)	–	40,748	157,041	366,087	–
	$AUC_{0\to\infty}$ (ng h/mL)	–	43,848	185,449	369,637	16,975
Total $AUC_{0\to\infty}$ (ng h/mL)		–	64,302	228,021	678,300	25,722
Conjugated form $AUC_{0\to\infty}$/Total $AUC_{0\to\infty}$ (%)		–	68.2	81.3	54.5	66.0

under concentration–time curve ($AUC_{0\to t}$ and $AUC_{0\to\infty}$) calculated by non-compartment model are presented in Table 5. The total $AUC_{0\to\infty}$ which was the sum of the $AUC_{0\to\infty}$ of free and conjugated metabolites is also listed in Table 5.

As seen from Table 5, the free five analytes could achieve the maximum plasma concentration between 5.75 and 8.25 h after oral administration. The values of the K ranged from 0.001 to 0.0024, which indicated that the free five analytes had slow elimination rates. A double-peak phenomenon of scutellarin is presented in Fig. 3. The first peak appeared at about 0.5 h, and the second peak appeared at about 5.75 h which is higher than the first peak. This phenomenon was almost the same with the study of Luan et al. [26], and it was to be relevant to the double-location absorption in stomach and gut. And other free four flavonoids had parallel plasma concentration–time profiles and pharmacokinetic parameters in vivo. It is demonstrated that the flavonoids aglycone had the similar oral administration and absorption from the Scutellaria Barbata extract. Traditionally the Scutellaria Barbata decoction has been taken orally 2 times a day in China. According to this study, the $T_{1/2}$ of the free five analytes was between 4.95 and 9.56 h, so the decoction of the Scutellaria Barbata was suggested to be taken about 3 times a day to achieve the better curative effect.

From Table 5, it also revealed that the $AUC_{0\to\infty}$ of the four flavonoids conjugated metabolites constituted between 54.5% and 81.3% of the total $AUC_{0\to\infty}$. The C_{max} of the conjugates metabolites were also approximately twice more than their respective free form. These parameters clearly demonstrated that the amounts of the four compounds (especially the apigenin) could be extensively converted to its conjugated forms and undergo rapid first-pass effect by the gut and liver after oral administration. This result was in a good agreement with the study of Wittemer and Veit [16] and Spencer et al. [32].

4. Conclusions

In the present paper, a sensitive and specific LC–MS/MS method was developed for simultaneous analysis of five flavonoids in rat plasma for the first time. The analytical procedure was successfully applied to pharmacokinetic study of the free and conjugated forms of the analytes after oral administration of Scutellaria Barbata extract. The results would be helpful to study the action mechanism of major flavonoids in Scutellaria Barbata and provided a useful tool for pharmacokinetic investigation of TCM.

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