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Short communication

Determination of aglycone conjugated metabolites of scutellarin in rat plasma by HPLC

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Abstract

A specific, precise and accurate high-performance liquid chromatography (HPLC) method for the determination of aglycone conjugated metabolites of scutellarin in plasma after enzymolysis to scutellarein (the aglycone of scutellarin) was developed and validated. The chromatographic separation was performed on a Lunar C_{18}(2) reversed-phase column at a column temperature of 40 °C. The mobile phase, delivered at 1.0 ml/min, consisted of acetonitrile–KH_2PO_4 buffer (40 mM, pH 2.5) (33:67, v/v). The detection wavelength was set at 335nm. Scutellarein and I.S. (quercetin) were isolated by a liquid–liquid extraction after incubating the plasma samples with β-glucuronidase/sulfatase. The method was validated using scutellarin spiked plasma as standards. Linearity was confirmed in the concentration range of 0.2165–4.329 nmol/ml, R.S.D.s were within 8.32%, and the recoveries of scutellarein ranged from 101.2 to 108.6%. The method is applicable to the pharmacokinetic study of aglycone conjugated metabolites of scutellarin in rats after oral administration of scutellarin.

Keywords: Scutellarin; Scutellarein; HPLC determination

1. Introduction

Flavonoids, a class of naturally occurring phenolic plant constituents, have drawn much attention because of their various beneficial biological activities and low toxicities. They usually exit as glycosides in plants. After flavonoid glycosides are administrated to animals or humans, they are generally absorbed only after being hydrolyzed to the corresponding aglycones by enterobacterial enzymes, and then the absorbed aglycones form their conjugated metabolites (glucurunic conjugates and sulfate conjugates). Thus, besides the concentrations of parent flavonoid glycosides, the concentrations of the aglycone conjugated metabolites are also useful biomarkers and have been adopted to reflect the pharmacokinetic characteristics of flavonoid glycosides after administration [1–6].

Scutellarin (Fig. 1) is the primary active ingredient in breviscapine, a mixture of flavonoid glycosides extracted from a Chinese herb *Erigeron breviscapus* (Vant.) Hand.-Mazz. Pharmacological studies showed that breviscapine could significantly reduce the blood viscosity, improve the blood flow, decrease the vascular resistance, and inhibit platelet aggregation and thrombosis formation. In China, breviscapine has been widely used for the treatment of occlusive cerebral vascular diseases since 1970s [7–10].

After oral administration of breviscapine to rats, scutellarin is metabolized to scutellarein (the aglycone of scutellarin, Fig. 1) and some scutellarein conjugated metabolites [11]. Scutellarin presented in plasma may also be considered as one of scutellarein glucurunic conjugates. Several HPLC methods for the determination of scutellarin in biological fluids after administration of breviscapine have been reported [12–19]. However, no method for the assay of scutellarein conjugated metabolites has been reported. Therefore, it is necessary to establish a method for the assay of scutellarein conjugated metabolites so that it can be applied to clarify the pharmacokinetics of breviscapine following oral administration.

In most papers reporting the assay of aglycone conjugated metabolites of flavonoid glycosides, the corresponding aglycones were used as the standard to spike the standard plasma samples [1–6]. However, using scutellarein as the standard to

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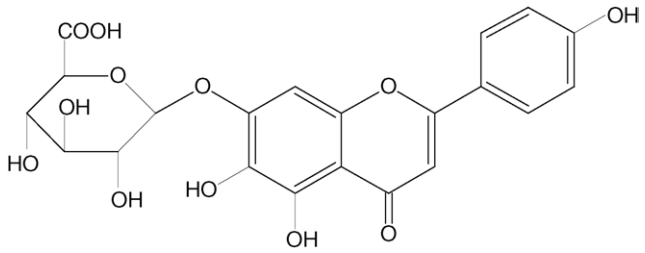

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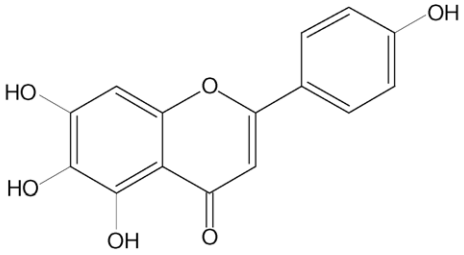

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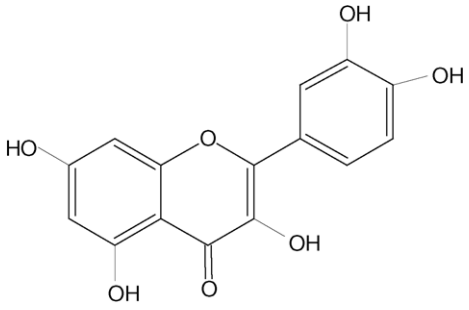

quercetin

Fig. 1. Chemical structures of scutellarin and its corresponding aglycone scutellarein, and quercetin (internal standard).

spike the plasma may not be practical due to its chemical instability. Scutellarein, a polyhydroxyflavonoid possessing 4,5,6-trihydroxy in a benzene ring (A), is very prone to oxidation, which leads to strict conditions for preparation and storage. The objectives of the present study were to establish an HPLC method using scutellarin to spike the standard plasma samples for the assay of its aglycone conjugated metabolites, and to compare the results with the method using aglycone-scutellarein as standard.

2. Experimental

2.1. Materials

Scutellarin (96.8%) and quercetin (>98%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Scutellarein was prepared in our laboratory by the following procedures: scutellarin was dissolved in methanol, mixed with ascorbic acid aqueous solution and further diluted with acetate buffer. The solution was hydrolyzed by β-glucuronidase at 37 °C for 16 h. After acidification with hydrochloric acid, the hydrolysate was extracted with ether and the supernatant was evaporated to dryness. The residue was reconstituted with methanol and purified by preparative reversed-phase HPLC using C_{18} as the stationary phase and a mixture of methanol–0.2% acetic acid solution (13:7, v/v) as the mobile phase. Scutellarein was confirmed by HPLC–MS and ^{1}H NMR and its purity was 98.36% determined by HPLC-DAD.

β-Glucuronidase (Type B-1 from bovine liver) and sulfatase (Type H-1 from *Helix pomatia*) were purchased from Sigma Chemical Co. (St. Louis, Mom, USA). Breviscapine, containing more than 90% scutellarin determined by HPLC method, was supplied by Kunming Pharmaceutical Co. Ltd. Breviscapine suspension was prepared in our laboratory by dispersing 100 mg breviscapine in 20 ml of 0.25% sodium alginate solution. Methanol, acetonitrile and methyl *t*-butyl ether were of HPLC grade (Tedia, Tedia Company INC, USA), and other reagents and chemicals were of analytical grade. Water was purified by a Milli-Q Reagent Grade Water System from Millipore (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The Agilent 1100 Series HPLC system (Agilent, USA) consisted of a G1311A quatpump, a G1322A degasser, a G1316A column oven and a G1315 UV–vis Diode-Array Detector (DAD). The HPLC system was controlled by HP ChemStation.

2.3. Chromatographic condition

The chromatographic separation was performed on a Lunar C_{18}(2) reversed-phase column (5 μm, 4.6 mm × 150 mm) equipped with a SecurityGuard C_{18} guard column (5 μm, 4 mm × 3 mm, Phenomenex, USA) at a column temperature of 40 °C. The mobile phase, delivered at 1.0 ml/min, consisted of acetonitrile–KH_2PO_4 buffer (40 mM, adjusted to pH 2.5 with phosphoric acid) (33:67, v/v). The detection wavelength was set at 335 nm.

2.4. Preparation of standard and quality control samples

Stock solutions of scutellarin, scutellarein and quercetin (all about 2 μmol/ml) were prepared in methanol. The standard working solutions of scutellarin and scutellarein were obtained by further dilution of the stock solutions with water and methanol–water (1:9, v/v), respectively. The samples for calibration or quality control (QC) during validation and pharmacokinetic study were prepared by spiking 100 μl of drug free plasma with 10 μl of standard working solutions of scutellarin or scutellarein.

The I.S. working solution was a 16.54 nmol/ml quercetin solution in methanol–water (1:9, v/v).

The solutions of scutellarein were stored in darkness at −20 °C and the other solutions were stored at 0–4 °C. All solu-

tions were brought to the room temperature and ultrasonicated for 10 min before use.

2.5. Sample preparation

The plasma samples were first hydrolyzed with β-glucuronidase/sulfatase and extracted with methyl *t*-butyl ether. For enzymolysis, 100 μl of plasma was mixed with 20 μl of ascorbic acid aqueous solution (200 mg/ml), 20 μl of I.S. working solution, and 50 μl of β-glucuronidase/sulfatase (186 units/ml of β-glucuronidase and 1.84 units/ml of sulfatase, both in pH 5 acetate buffer) and incubated at 37 °C for 16 h in darkness. After enzymolysis, the plasma samples were acidified with 50 μl of 0.1 mol/L HCl and extracted with 1.0 ml of methyl *t*-butyl ether by vortex for 0.5 min. The organic and aqueous phases were separated by centrifugation at 2000 × *g* for 5 min at 10 °C. The upper organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted with 100 μl of methanol–0.1% phosphoric acid (5:5, v/v) and then 20 μl was subjected to the HPLC system.

2.6. Method validation

2.6.1. Specificity

The chromatograms of the samples prepared from six rat blank plasma were inspected visually to assess the potential interferences from endogenous substances. The apparent response at the retention time of scutellarein and I.S. was compared to the response at the lower limit of quantitation (LLOQ) for scutellarein and to the response at the working concentration for I.S.

2.6.2. Extraction recovery

The extraction recovery for the compounds of interest was determined by comparing the peak areas obtained from QC samples (QCs) with the un-extracted standard working solutions at the same concentration in the same solvent.

2.6.3. Calibration and sample quantification

The calibration standards at five levels were extracted and assayed by the above-mentioned method. The calibration curves ($y = ax + b$) were constructed by the plots of the peak-area ratios (y) of the analyte to I.S. versus the concentrations (x) of the calibration standards. A linear least-square regression analysis was performed for the analyte and the calibration curve was repeated if the correlation coefficient was below 0.990. The concentrations of the analyte in unknown samples were determined by interpolation from the calibration curve.

2.6.4. Accuracy and precision

The accuracy and the precision of the assays for intra-day and inter-day determinations were evaluated by the analysis of QCs at the levels of low, middle and high concentrations ($n = 5$ for each level) on the same day and on five different days, respectively. These levels were chosen to demonstrate the performance of the method and to determine the LLOQ of the method. The recovery (%) represents the accuracy, while the relative standard deviation (R.S.D.) indicates the precision.

2.6.5. Stability

The stability of the analyte in reconstituted plasma samples after preparation was evaluated at 0 and 8 h at room temperature (about 20 °C). To assess the stability of the analyte in spiked plasma undergoing three freeze (−20 °C)-thaw (ambient) cycles during frozen storage at −20 °C, the QCs at levels of low, middle and high concentrations and the mixed rat plasma samples after scutellarin intake were analyzed on day 1 and day 20. The stability of the analyte in plasma samples in ambient was evaluated by determining the QCs and the mixed rat plasma samples after storage at room temperature (about 20 °C) for a period of time.

2.6.6. Comparison between the standard plasma samples spiked with scutellarin and the standard plasma samples spiked with scutellarein

The standard plasma samples spiked with scutellarin were compared with the standard plasma samples spiked with scutellarein in the method validation including specificity, linearity, accuracy, precision and stability.

2.7. Drug administration and blood collection

Male Sprague-Dawley rats (220–250 g) were fasted overnight (16–18 h) before dosing and continued for another 4 h after dosing. Water was freely available. Breviscapine suspension was administered at a dose of 86.58 μmol/kg (calculated with reference to scutellarin). Blood samples were withdrawn from tail vein before dosing and at 0.067, 0.15, 0.25, 0.5, 1, 2, 4, 6, 9, 12, 14 and 24 h post-dosing. All blood samples were taken into heparinized tubes and then centrifuged at 6000 × *g* at 10 °C for 10 min to separate the plasma. The plasma samples were stored at −20 °C for later analysis.

3. Results and discussion

3.1. Optimization of sample preparation

The first step of sample preparation was to convert scutellarein conjugates to scutellarein with β-glucuronidase/sulfatase. Extensive effort was invested to optimize the enzymolysis conditions prior to the liquid–liquid extraction. The amounts of β-glucuronidase, sulfatase and ascorbic acid, and the time course of enzymolysis were investigated using the standard plasma samples spiked with scutellarin at 4.329 nmol/ml.

The results showed that a complete enzymolysis of scutellarin could be achieved after 24 h with 62 units/ml β-glucuronidase or with 0.46 units/ml sulfatase. In addition, a certain amount of scutellarein (0.59–1.34 nmol/ml) was detected in the standard plasma samples incubated with no adscititious enzyme. The amount of the released scutellarein was different using different drug-free plasma. No scutellarein could be detected in standard water solution incubated with no adscititious enzyme. The results suggested that endogenous β-glucuronidase/sulfatase

existed in rat plasmas, and the level was different among the individuals.

Using ascorbic acid as an antioxidant was necessary to prevent the oxidation of scutellarein. The concentrations of scutellarein produced were affected remarkably by the amount of ascorbic acid, and a 40 μl of 50 mg/ml ascorbic acid aqueous solution could efficiently protect scutellarein from oxidation during the enzymolysis. In our experiment, a 20 μl of 200 mg/ml ascorbic acid aqueous solution was chosen to ensure that no oxidation occurred.

A wide range (4–18 h) of enzymolysis durations for the aglycone conjugates of other flavonoids were reported. Thus, the time course of the enzymolysis of scutellarein conjugates was examined to determine its end-point [1–4]. The standard plasma samples were treated with different amounts of enzyme, and drug recovery greater than 90% was set as the criterion for complete enzymolysis. Fig. 2A presents the time-course of the concentration of released scutellarein during enzymolysis with different amounts of the β-glucuronidase. While 16 h was required to reach the complete cleavage of scutellarein glucurunic conjugates with 62 units/ml β-glucuronidase and with 124 units/ml β-glucuronidase, the complete enzymolysis was shorten to 8 h by incubation with 186 units/ml enzyme. Fig. 2B shows that the complete enzymolysis of scutellarein sulfate conjugates was achieved by incubation with 3.68 units/ml sulfatase for 4 h, with 1.84 units/ml sulfatase for 8 h, or with 0.92 units/ml sulfatase for 16 h. Based on these results, we established the optimal enzymolysis conditions as described in Section 2.5.

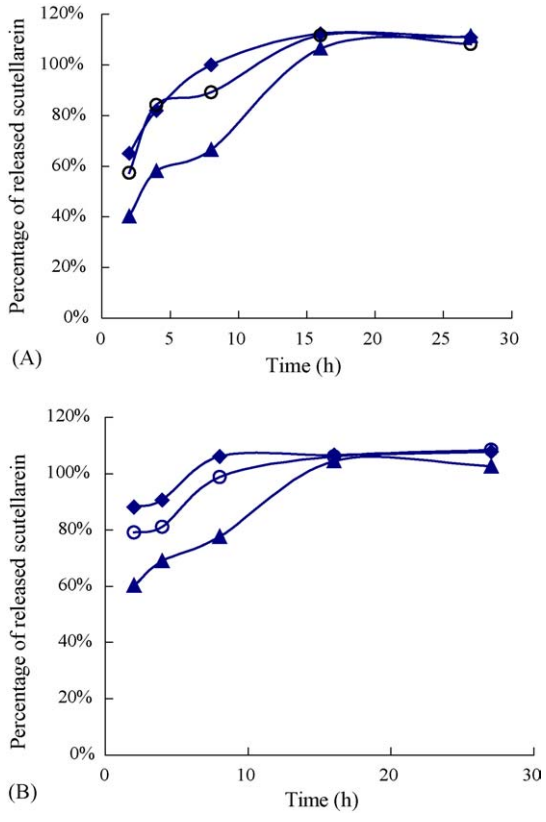

Fig. 2. Time-course for the enzymolysis of scutellarin by (A) β-glucuronidase at the concentrations of 186 units/ml (♦), 124 units/ml (○), and 62 units/ml (▲), and by (B) sulfatase at the concentrations of 3.68 units/ml (♦), 1.84 units/ml (○), and 0.92 units/ml (▲).

3.2. Retention times and specificity

Typical chromatogram of scutellarein standard is showed in Fig. 3A, and the retention time of scutellarein was 3.6 min. Typical chromatograms obtained after enzymolysis of drug-free plasma, drug-free plasma spiked with quercetin, QCs spiked with scutellarin and plasma sample obtained from a SD rat after oral dosing with scutellarin (86.58 μmol/kg) are illustrated in Fig. 3B–E. Scutellarein produced by enzymolysis and I.S. were eluted in 3.6 and 5.0 min, respectively. No interfering peaks due to plasma components, impurities of internal standard or metabolites were eluted at the retention time of the analytes of interest.

3.3. Extraction recovery

The extraction recoveries of scutellarein in plasma spiked with scutellarin were 81.87 ± 5.903, 75.54 ± 4.060 and $77.18 \pm 4.052\%$ at the concentration of 0.4329, 2.165 and 4.329 nmol/ml ($n = 6$), respectively.

3.4. Linearity

Acceptable linearity was observed over the range of concentration from 0.2165 to 4.329 nmol/ml, using scutellarin as spike standard. The typical calibration curves had a regression equation of $y = 0.4387\ c + 0.0097$ ($r = 0.9996$).

3.5. Accuracy and precision

The data from QCs, using scutellarin as spike standard, were calculated to estimate the intra- and inter-day precision and accuracy of the method. The results are presented in Table 1. The intra- and inter-day % R.S.D. of scutellarein detection ranged from 3.82 to 5.94 and from 7.69 to 8.32, respectively. The percentage of recoveries of the method for scutellarein ranged from 101.2 to 106.3 for intra-day, and from 101.8 to 108.6 for inter-day, respectively. These results were within the acceptable criteria for precision and accuracy.

The LLOQ, defined as the lowest concentration at which the analyte can be quantitated with an accuracy of 80–120% and a precision ≤20%, was 0.2165 nmol/ml.

3.6. Stability

In plasma, the analytes were stable after undergoing three freeze-thaw cycles during frozen storage at −20 °C for 20 days and after being placed at room temperature (about 20 °C) for 2 h. In the reconstitution solution, the analytes were stable at about 20 °C for at least 8 h. Stability data are summarized in Table 2.

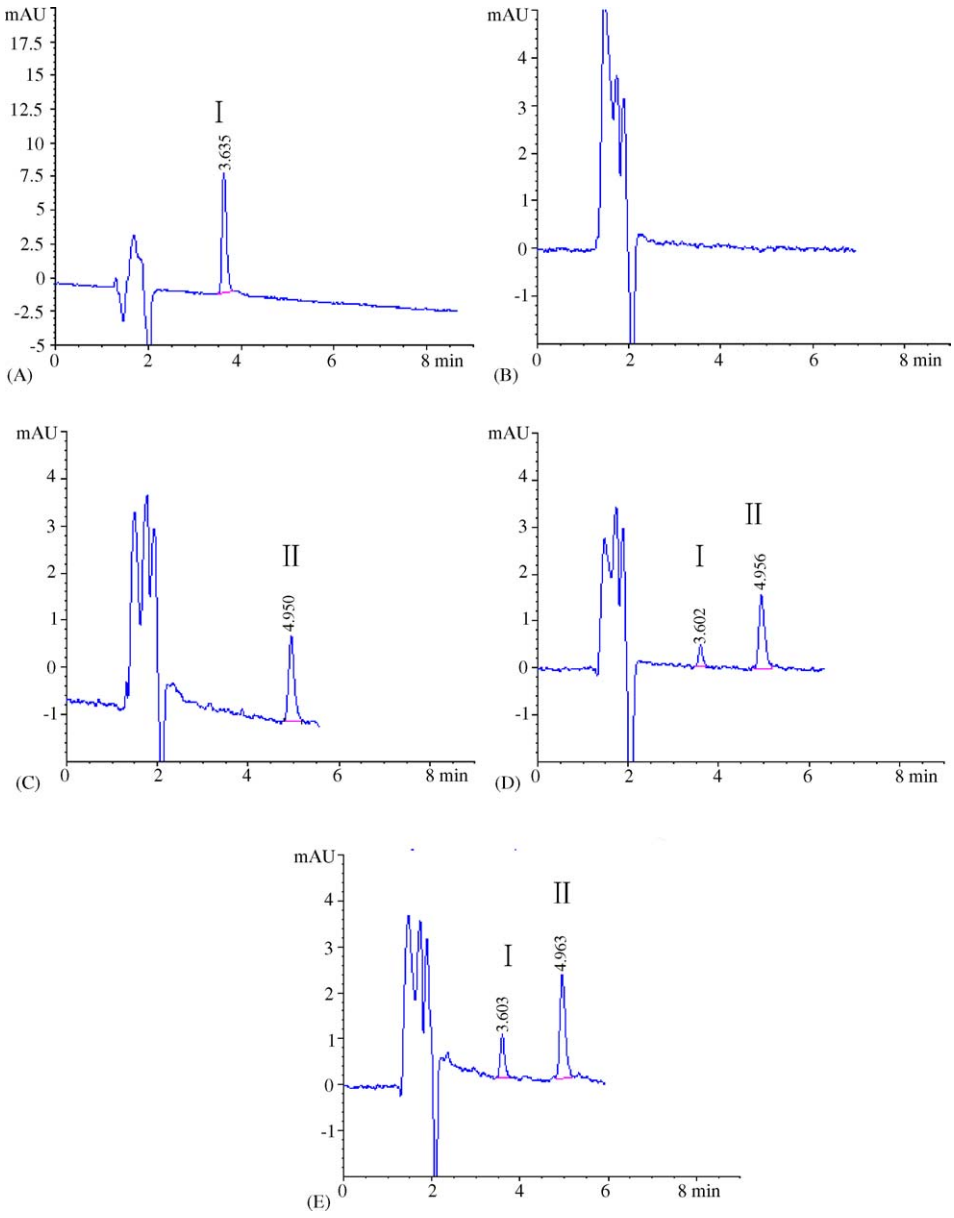

Fig. 3. Typical chromatograms of scutellarein in rat plasma with enzymolysis: (A) scutellarein standard; (B) blank plasma; (C) drug-free plasma spiked with I.S.; (D) quality control sample spiked with scutellarin (0.2165 nmol/ml); (E) a plasma sample from a SD rat 1 h after an oral administration of scutellarin (86.58 μmol/kg). Peak I: scutellarein; peak II: I.S. (quercetin).

3.7. Comparison between the standard plasma samples spiked with scutellarin and the standard plasma samples spiked with scutellarein

No difference was observed between the representative chromatograms of drug-free plasma spiked with scutellarin and those of drug-free plasma spiked with scutellarein. No interfering peaks that could have interfered with the detection of scutellarein and I.S. were observed.

The calibration curves of the standard plasma samples spiked with scutellarein at levels of 0.1748, 0.3497, 0.6993, 1.399, 2.797 and 4.196 nmol/ml were repeated on three different days.

Table 1
Summary of the precision and accuracy of the method ($n = 5$)

Spiked concentration (nmol/ml)	Intra-day		Inter-day	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
0.4329	102.5	5.94	104.5	7.90
2.165	101.2	3.82	101.8	8.32
4.329	106.3	5.92	108.6	7.69

Table 2
Stability of plasma sample

Plasma sample	Storage stability (−20 °C, 20 days) and freeze-thaw stability R.E. (%)	Short-term stability (about 20 °C, 2 h) R.E. (%)	Stability in the reconstitution solution (8 h) R.E. (%)
QCs 0.4329 nmol/ml ($n = 3$)	<6.01	<3.86	<6.85
QCs 2.165 nmol/ml ($n = 3$)	<4.05	<5.20	<4.08
QCs 4.329 nmol/ml ($n = 3$)	<6.08	<6.38	<3.66
Mixed actual plasma samples ($n = 2$)	<9.47	<4.66	<5.93

R.E. (%): relative error.

There was no significant difference between the calibration curves of standard plasma samples spiked with scutellarein and those of standard plasma samples spiked with scutellarin reported in 3.4. The intra- and inter-day % R.S.D. of scutellarein detection for the standard plasma samples spiked with scutellarein at the concentrations of 0.3497, 1.3997 and 4.196 nmol/ml ranged from 3.59 to 7.87 and from 4.71 to 5.70, respectively. The percent recoveries of scutellarein detection ranged from 97.52 to 100.01 for intra-day, and from 91.63 to 97.71 for inter-day, respectively. These results were also within the acceptable criteria for precision and accuracy.

The stability of scutellarein standard was investigated. The peak area of scutellarein in methanol decreased significantly after 7 days of storage at 0–4 °C. The stability of scutellarein in acid solution such as the reconstitution solution was better than that in methanol solution. In our study, scutellarein standards were stored in hermetically sealed vials and stored at −20 °C. The stock solution and the working solution of scutellarein were also stored at −20 °C and the working solution was stable at −20 °C for at least 3 days (R.S.D. < 4.89%).

The stability of scutellarein in rat plasma was assessed by placing the standard plasma samples spiked with scutellarein at room temperature for 0, 0.5 and 2 h. The amount of remaining scutellarein in the plasma prior to enzymolysis decreased significantly, while the amount of the released scutellarein from those samples after enzymolysis was almost the same (R.S.D. = 12.3%). These results suggested that the decrease of scutellarein in the plasma prior to hydrolysis could not be caused by oxydation. A reasonable explanation could be that the free form of scutellarein rapidly formed its glucuronides/sulfates after it was spiked into the rat plasma. When the spiked plasma samples were hydrolyzed, the same amount of scutellarein would be released.

Although the above results of the method validation using the plasma spiked with scutellarein were within the acceptable criteria, we did not adopt scutellarein to spike plasma but used scutellarin instead. The reasons were as follows: no free form of scutellarein was detected in our previous experiments after oral administration of scutellarin, and the forms of scutellarein in the plasma were its conjugates. Since the spiked plasma standard should imitate the situation of an actual sample, scutellarin, one of scutellarein glucurunic conjugates, would be a better choice than scutellarein to spike the plasma. Moreover, the amount of the enzyme needed to completely hydrolyze the conjugates of scutellarein could be evaluated accurately by spiking scutellarin, not scutellarein. In addition, the very rigorous preparation and storage conditions for scutellarein, caused by its poor stability, made the application of scutellarein very difficult. Therefore, the standard plasma samples spiked with scutellarin were used as QCs for routine analysis in our study.

3.8. Application of analytical method in pharmacokinetic studies

After a single oral administration of 86.58 μmol/kg scutellarin to 12 male SD rats, plasma samples were collected for the determination of scutellarein conjugates and scutellarin. Scutellarein conjugates in the plasma samples was determined by the methods described in this paper, and scutellarin was determined by an HPLC method with solid phase extraction (SPE) developed in our laboratory. The method for the determination of scutellarin will be published in a separated article. Fig. 4 displays the mean plasma concentration–time profiles of scutellarein conjugates and scutellarin ($n = 6$ for each analyte). It showed that the concentrations of the analytes in rat plasma were detectable for at least 24 h after the oral administration of scutellarin. Fluctuations were observed in the concentration–time profiles of both scutellarein conjugates and scutellarin. For scutellarein conjugates, the first peak plasma concentration was 1.36 ± 0.61 nmol/ml obtained at 0.17 ± 0.07 h and the second peak plasma concentration was 1.74 ± 0.92 nmol/ml obtained at 6.00 ± 2.45 h. For scutellarin, the first peak plasma concentration was 0.48 ± 0.20 nmol/ml

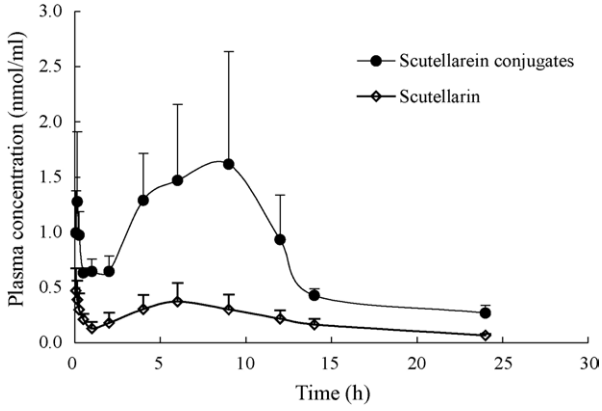

Fig. 4. Mean plasma concentration–time profiles of scutellarein conjugates and scutellarin after oral administration of 86.58 μmol/kg scutellarin to rats ($n = 6$ for each analyte).

obtained at 0.10 ± 0.04 h and the second peak plasma concentration was 0.38 ± 0.16 nmol/ml obtained at 5.67 ± 0.82 h. The area under the plasma concentration–time curve (AUC_{0-24}), calculated using the trapezoidal rule, was 19.40 ± 6.86 nmol h/ml for scutellarein conjugates and 4.85 ± 1.73 nmol h/ml for scutellarin.

4. Conclusion

In this paper, an HPLC method for the determination of scutellarein in plasma samples after enzymolysis with β-glucuronidase/sulfatase was developed and validated. This method is precise, accurate and selective for the determination of aglycone conjugated metabolites of scutellarin in rat plasma samples obtained in the pharmacokinetic studies after oral administration of scutellarin to rats.

There was no significant difference in the results of method validation between QCs using scutellarin or scutellarein to spike the standard plasma. Using scutellarin was more practical due to poor stability of scutellarein, therefore scutellarin spiked plasma samples were selected as QCs for routine analysis in our study. This method could also be a reference to the study of aglycone conjugated metabolites of other polyphenolic flavonoid glycosides, because sometimes the stability of the aglycones of those flavonoids is very poor, which could lead to the limited availability and rigorous storage of the aglycone standards.

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