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Sensitive liquid chromatography–tandem mass spectrometry method for the determination of scutellarin in human plasma: Application to a pharmacokinetic study

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Abstract

A sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for the determination of scutellarin in human plasma has been developed. Samples were prepared using solid phase extraction and analyzed on a C_{18} column interfaced with a triple quadrupole tandem mass spectrometer. Positive electrospray ionization was employed as the ionization source. The mobile phase consisted of methanol–water (0.1% formic acid), using gradient procedure. The analyte and internal standard baicalin were both detected by use of selected reaction monitoring mode. The method was linear in the concentration range of 0.2–20.0 ng/mL. The lower limit of quantification (LLOQ) was 0.2 ng/mL. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 12.4%. The accuracy determined at three concentrations (1.0, 5.0 and 10.0 ng/mL for scutellarin) was within ±5.0% in terms of relative error. The method herein described was successfully applied for the evaluation of pharmacokinetic profiles of scutellarin guttate pills in 20 healthy volunteers.

Keywords: Scutellarin; LC–MS/MS; Pharmacokinetics

1. Introduction

Scutellarin is a flavone glucuronide extracted from a Chinese herb Erigero breviscapus (Vant.) Hand. -Mazz [1]. It is not only an important component of a Chinese herb, but also a major constituent of skullcap, which is a popular western herb. Scutellarin has been proved to be effective in dilating blood vessels, improving hemodynamics, decreasing the viscosity of blood, reducing the blood platelet count and preventing platelet conglomeration, etc. [2,3]. In clinic, scutellarin is widely used in treating various cardiovascular diseases such as coronary heart disease, angina pectoris, and thrombosis [4]. For a better understanding of its pharmacokinetics and developing new dosage form, it is essential to use a sensitive and precise analytical method to determine the concentration of scutellarin in biological fluids. Some methods have been developed for detection of scutellarin in animal plasma in recent years [5–8]. However, to our knowledge, there is still no method described for the determination of scutellarin in human plasma. It is difficult to detect the scutellarin in human plasma, because it appears to low plasma concentration by administrating a clinic dosage orally. Therefore, some researchers had to investigate the pharmacokinetics through the way of determining the metabolite of scutellarin [9] which is not considered to show the vivo procedure really. In order to investigate the pharmacokinetics exactly, we firstly established a LC–MS/MS method to determine scutellarin in human plasma in this study, and it is sensitive enough to be applied to a pharmacokinetic study under a low clinic dosage (60 mg).

2. Experimental

2.1. Materials

Scutellarin (98.0% purity) and baicalin (internal standard, 98.0% purity) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC-grade) was purchased from Merck Company (America). Formic acid (analytical grade) and phosphoric acid (analytical grade) were from Nanjing Chemical Co.

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Table 1
Gradient procedure of scutellarin

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0–1.0	85	15
1.01–9.0	40	60
9.01–16.0	30	70
16.01–18.0	85	15

(Nanjing, China). Heparinized blank (drug free) human plasma was obtained from Nanjing Blood Donor Service (China). Distilled water, doubly distilled in the laboratory, was used throughout the study.

2.2. Instrumentation

A Thermo Finnigan TSQ quantum ultra tandem mass spectrometer equipped with electrospray ionization (ESI) source (San Jose, CA, USA), a Finnigan surveyor LC pump and autosampler were used for LC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.1 software (Thermo-Finnigan). Peak integration and calibration were carried out using LC Quan software (Thermo-Finnigan). The Alltech Extract-clean SPE columns (500 mg, 4 mL) were purchased from Alltech Company, and the DL-1 solid phase extraction equipment was purchased from Dalian Institute of Chemical Physics of Chinese Academy of Sciences.

2.3. LC–MS/MS conditions

The chromatographic separation was achieved on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Dikma, Beijing, China). Mobile phase A was water with 0.1% formic acid, mobile phase B was methanol. The gradient procedure was shown in Table 1.

The liquid flow-rate was set at 1.0 mL/min, and the column temperature was maintained at 30 °C.

Mass spectrometer was operated in the positive mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z 463.0 → m/z 287.0 for scutellarin and m/z 447.0 → m/z 271.0 for baicalin (internal standard, I.S.), respectively, with a scan time of 0.3 s per transition.

In order to optimize all the MS parameters, a standard solution (1 μg/mL) of the analyte and I.S. was infused into the mass spectrometer. For both scutellarin and baicalin, the following optimized parameters were obtained. The spray voltage was set at 4.0 kV. Nitrogen was used as the sheath gas (40 psi) and auxiliary gas (5 l/min). The heated capillary temperature was set to 350 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of approximately 1.5 mTorr. The optimized collision energy of 12 eV was chosen for both scutellarin and I.S.

2.4. Sample preparation

The SPE column was activated successively with 2 mL of methanol and 2 mL of water. One millilitre of 0.5% phosphoric acid solution and 1.0 mL of plasma sample were vortex-mixed together for 1min. Then the sample was transferred into the column, and mild suction was applied so that the sample passed through the column at a steady flow rate (about 2 mL/min). After that, 2 mL of 0.5% phosphoric acid solution was used to wash the column. Then the column was eluted with 2.0 mL of methanol. The collected eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 100 μL of mobile phase (methanol:water = 7:3), and 20 μL was injected into the LC–MS/MS system for analysis.

2.5. Preparation of standard and quality control samples

Stock solution of scutellarin was prepared in methanol at the concentration of 100 μg/mL. Stock solution of I.S. was prepared in methanol at the concentration of 100 μg/mL and diluted to 200 ng/mL with methanol. Calibration curves were prepared by spiking the appropriate standard solution to 1.0 mL of blank plasma. Effective concentrations in plasma samples were 0.2, 0.5, 1, 2, 5, 10, 20 ng/mL for scutellarin. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 1.0, 5.0 and 10 ng/mL, respectively. The spiked plasma samples (standards and quality controls) were then treated following the "Sample preparation" procedure on each analytical batch along with the unknown samples.

2.6. Method validation

Plasma samples were quantified using the ratio of the peak area of scutellarin to that of I.S. as the assay parameter. Peak area ratios were plotted against scutellarin concentrations and standard curves were in the form of $y = A + Bx$.

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on separate 5 days. The accuracy and precision were also assessed by determining QC samples at three concentration levels on three different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.%).

Absolute recoveries of scutellarin at three QC levels were determined by assaying the samples as described above and comparing the peak areas of both scutellarin and I.S. with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

The stability of the stock solution of scutellarin was determined by placing the stock solution in the refrigerator (4 °C) for a week. Scutellarin stability in plasma was assessed by analyzing QC samples at concentrations of 1.0, 5.0 and 10 ng/mL, respectively, exposed to different time and temperature conditions. The long-term stability was assessed after storage of the test samples at −20 °C for 5 days. The freeze–thaw stability was determined after five freeze–thaw cycles (−20 to 20 °C) on consecutive days. The extraction storage stability was assessed by placing QC samples being extracted at −20 °C for 5 days and analyzed. The results were compared with those QC samples freshly prepared, and the percentage concentration deviation was calculated.

2.7. Pharmacokinetic study

To demonstrate the reliability of this method for the study of pharmacokinetics, it was applied to determine the plasma concentrations of scutellarin in which 20 healthy Chinese male volunteers (between 18 and 25 years old) received an oral dosage guttate pill (containing 60 mg scutellarin). The pharmacokinetic study approved by the Ethics Committee. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Serial blood samples (4 mL) from a suitable antecubital vein were collected into sodium heparin-containing tubes before and 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15 h after the administration of scutellarin. Plasma was separated by centrifugation at $2000 \times g$ for 10 min and stored frozen at $-20\,^{\circ}C$ until analysis.

Pharmacokinetic parameters were determined from the plasma concentration–time data. To compare the two formulations, an analysis of variance was performed on the appropriate log-transformed and nontransformed *PK* parameters using $BAPP_2$ procedures.

3. Results and discussion

3.1. Mass spectrometry

Because scutellarin and I.S. has numerous hydroxyl functional groups (Fig. 1), the negative ionization mode was initially chosen. But too much interference made the detection difficult to process in negative ionization mode, so we chose positive ionization mode instead. The Q1 full scan spectra of scutellarin and I.S. were dominated by protonated molecules $[M + H]^+$ and no significant solvent adduct ions and fragments ions were observed. In the product spectra of $[M + H]^+$ ions for scutellarin and I.S., along with the raising of the CID energy more fragment ions were observed while the response of $[M + H]^+$ lowered significantly. When the CID energy was set at 12 eV, the main fragment ion at *m*/*z* 287.0 from scutellarin showed a highest MS response. Meanwhile, the most abundant product ion from the I.S. was *m*/*z* 271.0. Additional tuning of the ESI source parameters such as capillary temperature, flow of sheath, auxiliary gas (N_2) and spray voltage onto the transition *m*/*z* 463.0 → *m*/*z* 287.0 (scutellarin) and *m*/*z* 447.0 → *m*/*z* 271.0 (baicalin) further improved the sensitivity.

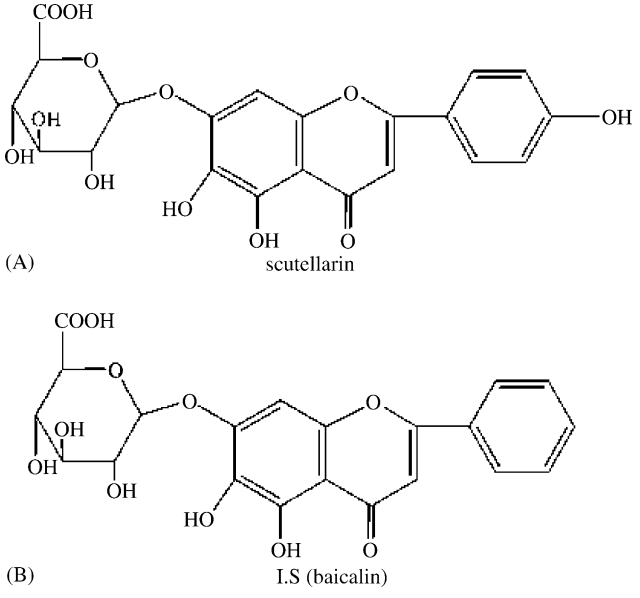

Fig. 1. Structure of scutellarin (A) and I.S. (B).

3.2. Chromatography

There are several hydroxy groups in the structure of scutellarin, so the polarity of scutellarin is so large that it is difficult to retain on the C_{18} column. When we added 0.01 mol/L ammonium acetate into the mobile phase, the retention time of the scutellarin was delayed remarkably, but the response of scutellarin lowered significantly too. So we adjusted the pH of the mobile phase in order to prolong the retention time. When we added 0.1% formic acid in the mobile phase and the gradient procedure was used, the retention time was delayed to 10 min. Under these optimum chromatographic conditions, the symmetry of the peak was good while the analyte and internal standard were free of interference from endogenous substances.

3.3. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). The polar character of scutellarin makes it difficult to extract from plasma by techniques such as conventional liquid–liquid extraction. And protein precipitation is not suitable for scutellarin because it will dilute the plasma concentration, which makes the sensitivity poorer. Thus SPE was used to prepare the plasma samples. The pK_a of the scutellarin is 3.29, therefore eluting the analyte in acid condition will improve the recovery. So 1 mL of 0.5% phosphoric acid solution was vortex-mixed with 1.0 mL of plasma sample before being transferred into the column.

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank, a spiked plasma sample with scutellarin (10 ng/mL) and I.S., and a plasma sample from a healthy volunteer 4 h after an oral administration. There was no significant interference or ion suppression from endogenous substances observed at the retention times of the analytes. Typical retention times for scutellarin and I.S. were about 10.0 min and 15.0 min, respectively.

3.4.2. Matrix effects

To evaluate the absolute matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting matrix compo-

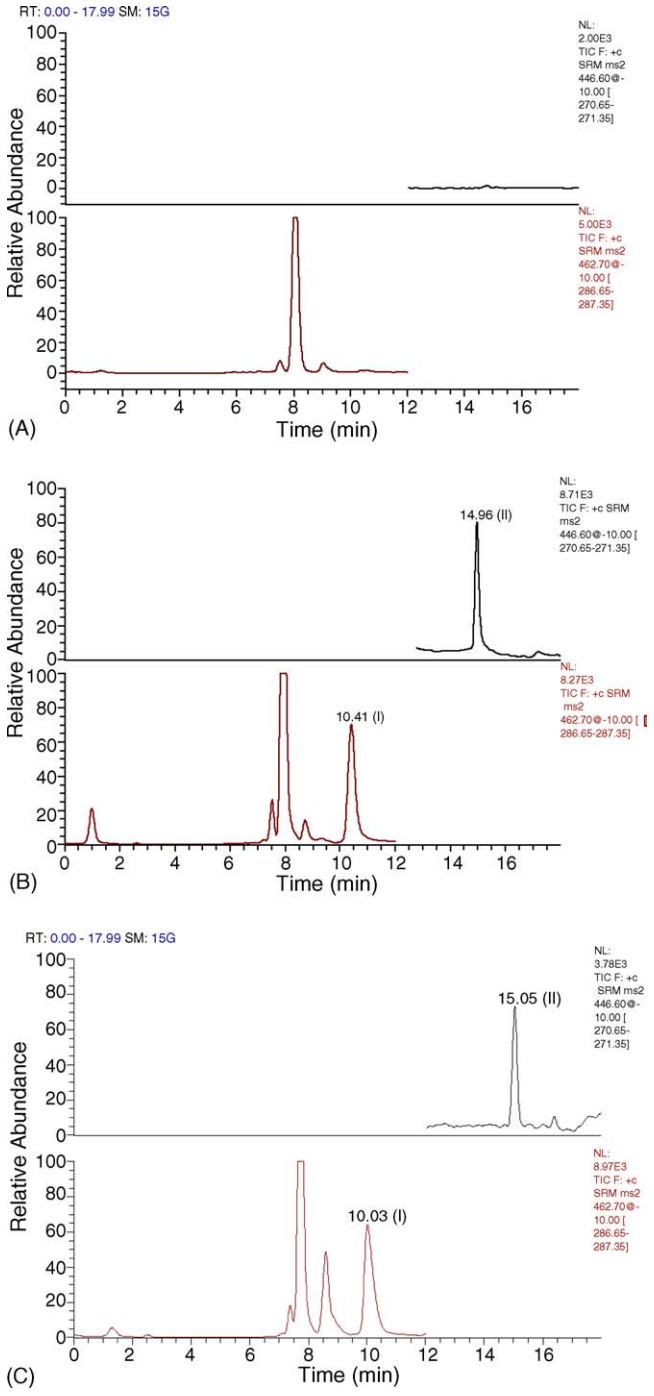

Fig. 2. Representative SRM chromatograms of scutellarin (I) and I.S. (baicalin, II) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with scutellarin at 10 ng/mL; (C) plasma sample from a volunteer 4.0 h after administration of scutellarin.

nents, five different batches of blank plasma were extracted by SPE column and then spiked with the analyte at three QC concentrations. The corresponding peak areas of the analyte in spiked plasma post-extraction (A) were then compared to those of the aqueous standards in mobile phase (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the ME. A ME value of 100% indicates that the responses for scutellarin in the mobile phase and in the plasma extracts were the same and that

Table 2
Matrix effects data for scutellarin at 1.0, 5.0 and 10 ng/mL in five different lots of human plasma ($n = 5$)

Concentration of scutellarin (ng/mL)	Absolute ME (mean ± S.D., %)	Relative ME (%)
1.0	97.85 ± 8.4	8.6
5.0	99.74 ± 6.8	6.8
10	101.99 ± 3.8	3.6

no absolute ME was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots (sources) of plasma. The variability in the values, expressed as R.S.D. (%), is a measure of the relative ME for the target analyte. The ME data at three QC concentrations of scutellarin in five different lots of human plasma are presented in Table 2.

The results showed there was no absolute ME in this study. And the variability was acceptable, with R.S.D. values <8.6% at different concentrations of scutellarin, indicating that the relative ME for the analyte was minimal.

3.4.3. Linearity of calibration curves and lower limits of quantification

Visual inspection of the plotted duplicate calibration curves and correlation coefficients >0.999 confirmed that the calibration curves were linear over the concentration ranges 0.2–20.0 ng/mL for the analyte. Typical standard curve was $f = 0.1269C_i + 0.0053$. Where f represents the ratios of scutellarin peak area to that of I.S. and C_i represents the plasma concentrations of scutellarin.

The lower limit of quantification was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The present LC–MS/MS method offered an LLOQ of 0.2 ng/mL in 1.0 mL plasma sample. Under present LLOQ of 0.2 ng/mL (Fig. 3), the scutellarin concentration can be determined in plasma samples until 15 h after a single oral dose of 60 mg scutellarin, which is sensitive enough to investigate the pharmacokinetic behaviors of scutellarin, to establish the relationship between dose and pharmacological effect in human.

3.4.4. Precision and accuracy

Table 3 summarizes the intra- and inter-day precision and accuracy for scutellarin evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this

Table 3
Accuracy and precision for the analysis of scutellarin in human plasma (in prestudy validation, $n = 3$ days, five replicates per day)

Added C (ng/mL)	Found C (ng/mL)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
1.0	0.21	8.98	12.38	5.0
5.0	5.15	5.83	6.55	3.0
10.0	20.36	2.39	2.92	1.8

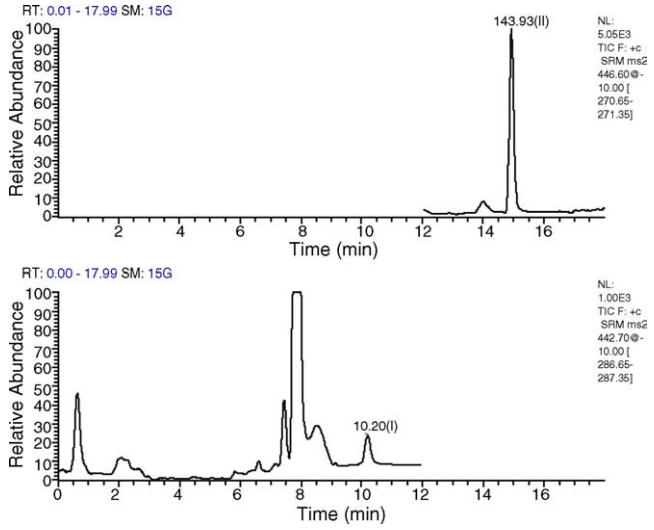

Fig. 3. The LLOQ of the scutellarin in plasma (0.2 ng/mL).

assay, the intra-run precision was 8.98% or less, and the inter-run precision was 12.4% or less for each QC level of scutellarin. The accuracy was within ±5.0%. The results above demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.4.5. Recovery and stability

The recovery of scutellarin, determined at three concentrations (1.0, 5.0, 10.0 ng/mL), were 82.51 ± 10.13%, 85.18 ± 6.62% and 88.14 ± 4.88% ($n = 5$), respectively.

The results of stability experiments showed that no significant degradation occurred at −20 °C for 5 days and after five freeze–thaw cycles for scutellarin plasma samples. The accuracy values of low (1.0 ng/mL), medium (5.0 ng/mL) and high (10 ng/mL) concentrations of scutellarin in human plasma were 108%, 98.4% and 97.5% after five freeze–thaw cycles, and 104%, 103% and 100% at −20 °C for 5 days. The stock solution of scutellarin in methanol was stable at 4 °C for a week. The methanolic solution of I.S. (200 ng/mL) was proved stable at room temperature for more than 8 h and at 4 °C for a week.

3.5. Application of the method to a pharmacokinetic study in healthy volunteers

The method was applied to determine the plasma concentration of scutellarin after an oral administration of reference formulation and test formulation of scutellarin to 20 volunteers. The mean plasma concentration–time curve of scutellarin was shown in Fig. 4. The main pharmacokinetic parameters of scutellarin in 20 volunteers were shown in Table 4.

The relative bioavailability of the test formulation was 104.2 ± 13.0%, and there were no remarkable differences between test formulation and reference formulation.

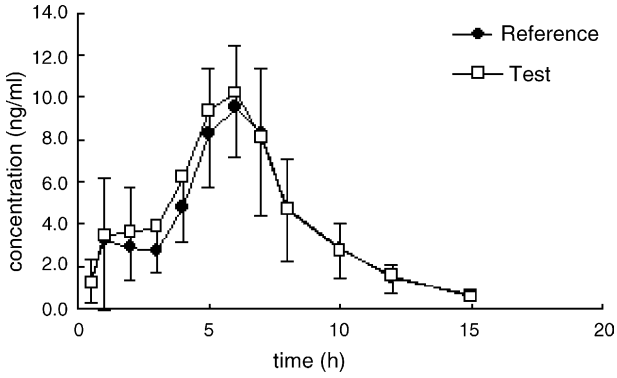

Fig. 4. Mean plasma concentration–time curve of scutellarin after a single oral dose of scutellarin in 20 volunteers.

Table 4
Pharmacokinetic parameters for 20 volunteers after administration of a single dose of scutellarin

Parameter	Test formulation	Reference formulation
$T_{1/2}$ (h)	2.27 ± 0.58	2.25 ± 0.44
C_{max} (ng mL^{-1})	12.02 ± 2.23	11.68 ± 2.67
T_{max} (h)	5.9 ± 0.8	5.6 ± 1.6
$AUC_{0\rightarrow 15h}$ (ng h mL^{-1})	61.7 ± 7.15	58.82 ± 9.50
$AUC_{0\rightarrow\infty}$ (ng h mL^{-1})	63.54 ± 7.54	60.84 ± 9.78

4. Conclusions

In this article, we first developed a LC–MS/MS method to determinate the concentration of scutellarin in human plasma. This method combines the accumulation and purification of SPE with the mass resolution and sensitivity of mass spectrometry, which permits the analysis of scutellarin in plasma. It was then successfully applied for the evaluation of pharmacokinetic profiles of scutellarin in 20 healthy volunteers (the dosage is low as 60 mg). The results proved that the method is rapid, sensitive and highly selective.

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