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Short communication

Determination of scutellarin in rat plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

A validated high-performance liquid chromatography method is described for the determination of scutellarin in rat plasma using a liquid–liquid extraction and ultraviolet (UV) absorbance detection. The separation used a Diamonsil ODS column (250 mm × 4.6 mm i.d., 5 μm particle size) with an isocratic mobile phase consisting of methanol–acetonitrile–50 mM dihydrogen ammonium phosphate buffer (22:15:63 (v/v/v), adjusted to pH 2.5 with 1 M phosphoric acid). The ultraviolet detector operated at 335 nm. Plasma samples were extracted with ethyl acetate after acidification. The extraction recovery of scutellarin ranged from 68.1 to 80.5%. High selectivity and a low quantitation limit (0.050 μg/ml) were achieved. The linear range was 0.050–12.5 μg/ml, correlation coefficient $r = 0.9981$. The method has a good reproducibility, R.S.D. values were below 7.9% for within-day and between-day precision. The method is simple, rapid, and applicable to preliminary pharmacokinetic studies of scutellarin in rats.

Keyword: Scutellarin

1. Introduction

Flavonoids are widely occurring in the plant kingdom and contained in the common human diet, and comprise of flavones, flavonols, flavanones, flavanonols, isoflavone, flavanol, anthocyanidins. Many of these compounds exist as sugar conjugates [1]. Scutellarin, a flavone glucuronide, extracted from a Chinese herb Erigero breviscapus (Vant.) Hand.-Mazz [2], is not only an important component of a Chinese herb, but also a major constituent of Skullcap, a popular western herb. It has been used in the treatment of occlusive cerebral vascular diseases. It is efficacious in the treatment of cerebral infarction, coronary heart disease, angina pectoris [3–5]. For a better understanding of its pharmacokinetics, it is essential to use a sensitive and precise analytical method to determine the concentration of scutellarin in biological fluids.

There has been active interest in recent years in developing and optimizing analytical methods for detection of flavonoids in foods [6–8] and biological matrix. A number of methods have been described for flavonoids in biological fluids [9–20], including the determination of baicalin and baicalein in rat plasma by HPLC with electrochemical detection [9], and the quantitation of wogonin and its major metabolite wogonin-7β-D-glucuronide in rat plasma by LC–MS/MS [10]. Tsuchiya analyzed polyhydroxyflavones in human plasma using HPLC with

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diode array detection and solid-phase borate-complex extraction [11]. Several recent reports described the analysis of quercetin in human plasma using HPLC with electrochemical detection [12] and the identification of quercetin glucuronides in human plasma by LC–MS/MS [13]. Nielsen et al. determined the apigenin and acacetin in human urine by column-switching HPLC–UV [14]. However, to our knowledge, there is still no method described for the determination of scutellarin in biological fluids.

In the present study, we firstly established an HPLC–UV method that is sensitive, simple and suitable for determining scutellarin in rat plasma.

2. Experimental

2.1. Materials

Scutellarin was from the Delta Information Center for Natural Organic Compounds (99.5%, Hong Kong, China). *Injectio Breviscapini*, which is an injection solution of scutellarin (20 mg/5 ml) was produced by Gejiu Bio-Medicine Industry Ltd. (Yunnan, China). 2-(4-Hydroxy-phenyl)-7-(3-morpholin-4-yl-propoxy)-chromen-4-one (internal standard, IS, see Fig. 1) was a kind of gift from Prof. Chun Hu (Shenyang Pharmaceutical University, Shenyang, China). Methanol and acetonitrile were of HPLC-grade, and other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

The Hewlett-Packard 1100 Series HPLC system (Agilent, USA) consisted of a G1311 quaternary pump, a G1313A autosampler, a G1314A UV-Vis detector, a G1316A column oven, a vacuum degasser unit. The HPLC system was controlled by a computer employing the HP ChemStation software. Turbo Vap® LV evaporator (Zymark Company, USA) was used for the evaporation of samples.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a 250 mm × 4.6 mm, 5 μm Diamonsil C_{18} column (Dikma, Beijing, China) with a 4 mm × 3.0 mm SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA). The column temperature was maintained at 30 °C. The mobile phase consisted of methanol–acetonitrile–50 mM $NH_4H_2PO_4$ buffer (22:15:63 (v/v/v), adjusted to pH 2.5 with 1 M phosphoric acid) at a flow-rate of 1.0 ml/min. The ultraviolet (UV) detector operated at 335 nm.

2.4. Preparation of standard and quality control samples

Stock solutions of scutellarin (500 μg/ml) and internal standard (200 μg/ml) were prepared in methanol. A series of standard working solutions with concentrations in the range of 0.20–50.0 μg/ml for scutellarin were obtained by further dilution of the stock solution with mobile phase. A 40 μg/ml internal standard working solution was prepared by diluting the stock standard solution with methanol.

The standard working solutions (50 μl) were used to spike blank plasma samples (200 μl) either for calibration curves of scutellarin or for quality control (QC) in prestudy validation and during the pharmacokinetic study.

All the solutions were stored at 4 °C and were brought to room temperature before use.

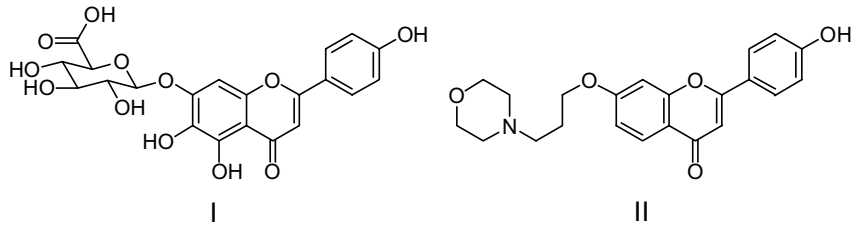

Fig. 1. The structures of: (I) scutellarin and (II) 2-(4-hydroxy-phenyl)-7-(3-morpholin-4-yl-propoxy)-chromen-4-one (internal standard).

2.5. Sample preparation

To a 200 μl aliquot of rat plasma, 50 μl of the internal standard working solution and 100 μl of 1 M phosphoric acid were added. This mixture was extracted with 3 ml of ethyl acetate by shaking for 10 min. The organic and aqueous phases were separated by centrifugation at 2000 × *g* for 10 min. 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 dissolved in 100 μl mobile phase, and vortex mixed. A 50 μl aliquot of the solution was injected into the HPLC system for analysis.

2.6. Method validation

2.6.1. Quantitation of scutellarin in rat plasma

During prestudy validation, the calibration curves were defined in three runs based on triplicate assays of the spiked plasma samples, and quality control samples from three concentrations (see Table 1) were determined in replicates ($n = 6$) on the same day to calculate the accuracy and precision of the method. Calibration curves were constructed using a $1/x^2$ weighted linear regression of the peak-area ratios of the analyte to internal standard versus the plasma concentration of the analyte. During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns.

The matrix effect was investigated by extracting "blank" biological fluids from six different sources, reconstituting the final extract in the mobile phase containing a known amount of analyte, analyzing the reconstituted extracts and then comparing the peak areas of analyte.

Extraction recovery was determined using QC samples at low, mid, and high levels of the calibration range, for each particular assay. Each control level was prepared with *n* of 6. After extraction and analysis, the samples were analyzed and the peak areas were recorded for the analytes. In order to calculate the extraction recovery, the peak areas for plasma extracts were compared to the peak areas of the analyte that was added to extracted blank plasma samples at the same concentration.

The freeze–thaw stability was evaluated by analyzing QC samples at concentrations of 0.125, 1.25 and 12.5 μg/ml after undergoing three freeze (−20 °C)–thaw (ambient) cycles. The stability of scutellarin in rat plasma was assessed by placing QC samples at room temperature for a fixed period of time before being extracted and analyzed. The stability of scutellarin in the reconstitution mobile phase was determined after the QC samples being kept at room temperature for a fixed period of time before analysis.

Table 1
Summary of precision and accuracy from quality control samples of rat plasma extracts ($n = 3$ days, six replicates per day)

Added concentration (μg/ml)	Found concentration (μg/ml)	Accuracy (%)	Precision (%)	
			Intra-run	Inter-run
0.125	0.120	96.0	7.6	7.9
1.25	1.29	103.2	3.9	5.0
12.5	12.3	98.2	4.4	2.4

2.6.2. Application of HPLC quantitative analysis

Wistar rats (female and male, 12 weeks old, 220–260 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University). Before the day of administration, the rats were fasted 24 h but were allowed water ad libitum. The solution of scutellarin was administered to rats (40 mg/kg) by oral gavage or by intravenous administration via the tail vein. Blood samples were drawn from the tail vein at 0.17, 0.5, 1, 2, 3, 5, 8, 12, 24 and 36 h after oral administration, or at 0.08, 0.25, 0.5, 1, 2, 3, 5, 8, 12 and 24 h after intravenous administration, respectively. As soon as possible, the heparinized blood was centrifuged for 10 min at 2000 × *g*, and plasma was obtained and stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of HPLC conditions

In optimizing the chromatographic conditions, the pH of the mobile phase and organic modifier were explored. The mobile phases of pH 6.5 or 4.0 yielded tailing peaks. When the pH value of the mobile phase decreased from 6.5 to 4.0, the peaks became sharper. The peaks were sharp and symmetric when the pH

value of the mobile phase was adjusted to 2.5. The use of an acid modifier is important to suppress ionization of the weak acidic phenolic group and interactions of these groups with residual traces of metals in the stationary phase that are detrimental to peak shape [21]. It was reported that trifluoroacetic acid in mobile phase could enhance the resolution of the catechins and eliminated their peak tailing [22]. Methanol and acetonitrile were used as the organic modifier together. A solvent comprising of a mixture of methanol and acetonitrile and buffer provided a more efficient HPLC separation than a methanol–buffer system. The mobile phase also resulted in a lower pressure in the pump and column as compared with the methanol–buffer system.

3.2. Sample preparation

The clean-up of required components in biological matrix prior to HPLC–UV analysis is a prerequisite for the successful analysis, especially in the case of highly polar components in biological fluids. A simple liquid–liquid extraction was selected for the clean-up of scutellarin in rat plasma. We used an acidic modifier (1 M phosphoric acid) to adjust the pH of plasma samples, in order to improve the recovery of scutellarin by suppressing its ionization. The addition of 1 M phosphoric acid to plasma also denatured and precipitated proteins, which helped to release the bound organic components, including scutellarin, to them. Other extraction solvents were tested, including ethyl

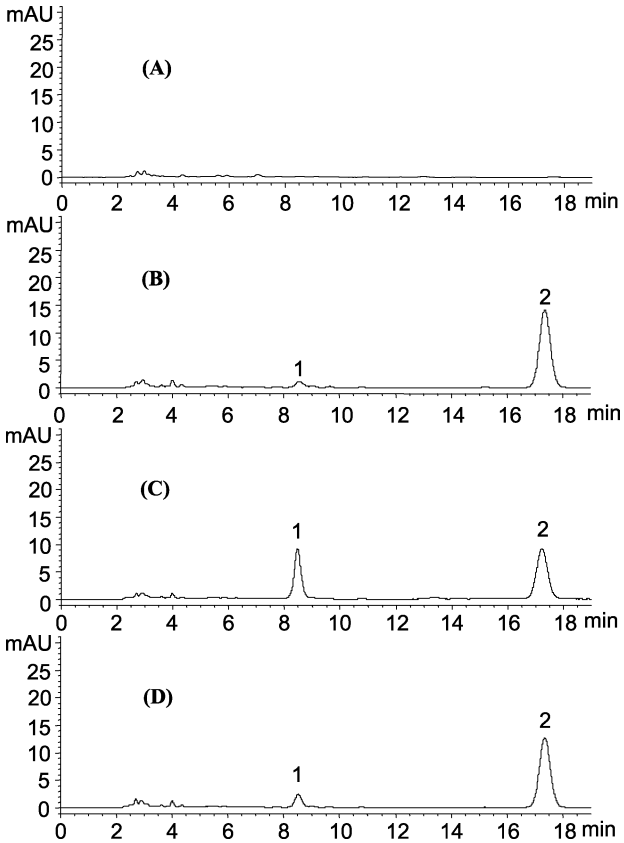

Fig. 2. Representative chromatograms of extracts of: (A) plasma from a rat to which scutellarin had not been administered; (B) from A to which 0.050 μg/ml scutellarin and internal standard (IS, 40 μg/ml) were added; (C) from A to which 1.25 μg/ml scutellarin and internal standard (IS, 40 μg/ml) were added; (D) plasma sample from a Wistar rat 5 h after an oral administration of scutellarin (40 mg/kg). Peak 1: scutellarin; peak 2: IS.

ether, dichloromethane, a mixer of ethyl ether–ethyl acetate (1:1 (v/v)), their extraction recoveries were about 11.6, 0.7 and 56.2% for scutellarin at the concentration of 12.5 μg/ml, respectively. However, the extraction recovery of ethyl acetate was about 72%, which appeared to afford the best recovery.

3.3. Method validation

3.3.1. Assay specificity

The specificity of the method was demonstrated by comparing chromatograms of six independent plasma samples from rats, each as a blank and a spiked sample. Fig. 2 shows that no interferences from endogenous substances with the analyte and internal standard were detected. Typical retention times for scutellarin and the internal standard were 8.5 and 17.3 min, respectively.

3.3.2. Linearity of calibration curves and lower limit of quantitation

Linear calibration curves were obtained over the concentration range of 0.050–12.5 μg/ml for scutellarin in rat plasma. The typical equation of calibration curves was

$$y = 0.0056 + 0.5096x, \qquad r = 0.9981.$$

The lower limit of quantitation (LLOQ) for determination of free scutellarin in plasma, defined as the lowest concentration analyzed with an accuracy ≤15% and a precision ≤15% [23,24], was 0.050 μg/ml.

3.3.3. Assay precision and accuracy

The data from QC samples in validation were examined by a one-way analysis of variance (ANOVA) to estimate the inter- and intra-run precision and accuracy of the method. The intra-run and inter-run mean square of variance was used to calculate %R.S.D. as follows: %R.S.D. = [(men square variance)$^{0.5}$/mean] × 100 [25]. The results are presented in Table 1. The intra-run precisions and inter-run precisions (R.S.D.) were calculated from QC samples, were less than 7.6 and 7.9% for each QC level, respectively. The accuracy as determined from QC samples was within ±4.0% for each QC level.

3.3.4. Extraction recovery

The analyte recoveries under the liquid–liquid extraction conditions were 75.2 ± 5.3, 73.2 ± 4.6 and 72.9 ± 4.8% at concentrations of 0.125, 1.25 and 12.5 μg/ml (QC samples), respectively. The recoveries of the internal standard were 65 ± 3.3% in rat plasma.

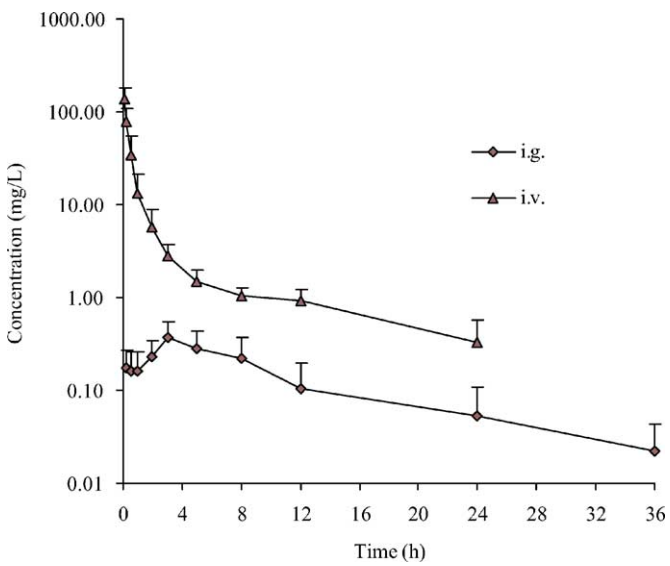

Fig. 3. Mean plasma concentration–time profile of scutellarin after oral or intravenous administration of 40 mg/kg scutellarin to five Wistar rats.

3.3.5. Analyte stability

The analyte was found to be stable after three freeze–thaw cycles in plasma. The accuracy calculated from QC samples ranged from 91 to 105%. The stability of analyte in plasma and mobile phase was investigated. Scutellarin was shown to be stable in rat plasma at room temperature for at least 4 h (relative error, RE $< 8.2\%$) and in the reconstitution mobile phase at room temperature for 24 h (RE $< 7.5\%$).

3.4. Application of analytical method in pharmacokinetic studies

After a single oral or intravenous administration of 40 mg/kg scutellarin to five Wistar rats, plasma concentrations of scutellarin were determined by the HPLC method. Fig. 3 shows mean plasma concentration–time curves ($n = 5$). Plasma concentrations of free scutellarin in rat were detectable for at least 24 h after either oral or intravenous administration.

4. Conclusion

In this study, a specific and sensitive assay for the determination of scutellarin in rat plasma was presented. The method is simple, rapid, and applicable to preliminary pharmacokinetic studies of scutellarin in rats.

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