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Determination of puerarin in rat plasma by rapid resolution liquid chromatography tandem mass spectrometry in positive ionization mode

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ABSTRACT

A highly sensitive and specific method of rapid resolution liquid chromatography tandem mass spectrometry (RRLC–MS/MS) in positive ionization mode has been developed and validated for pharmacokinetic study of puerarin in rat plasma. Chromatography was carried out on a Zorbax XDB C18 reversed-phase column using a mobile phase comprising a mixture of methanol and 0.05% acetic acid in water (35:65, v/v) with a flow rate of 0.3 mL/min from 0 min to 5.4 min and then 0.6 mL/min from 5.41 min to 12 min. The mass spectrometer operated in ESI positive ionization mode. Multiple reaction monitoring (MRM) was used to measure puerarin and tectoridin (internal standard). The method was sensitive with a detection limit of 0.33 ng/mL. A good linear response was observed over a range of 10–2000 ng/mL in rat plasma. The inter- and intra-day precision ranged from 2.97% to 7.52% and accuracy from 93.70% to 101.60%. This validated method was applied successfully to a pharmacokinetic study in rat plasma after intravenous administration of puerarin. The main pharmacokinetic parameters were as follows: $AUC_{0\to t}$ 45.37 ± 13.19 (mg h/L), $AUC_{0\to\infty}$ 47.03 ± 14.78 (mg h/L), MRT 1.03 ± 0.46 (h), $T_{1/2}$ 1.31 ± 0.31 (h), V_{ss} 0.09 ± 0.02 (L), V_z 0.17 ± 0.04 (L), Cl 0.10 ± 0.04 (L/h).

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1. Introduction

Puerarin (chemical name 7,4′-dihydroxyisoflavone-8β-D-glucopyranoside, Fig. 1) is a major isoflavone *C*-glycoside isolated from the traditional Chinese medicine *Pueraria radix* (the root of the *Pueraria lobota* (Willd) Ohwi) and has been widely prescribed in China for patients with cardiovascular diseases. It has been reported that puerarin exhibits beneficial effects on hypertension [1], arteriosclerosis [2], diabetes mellitus [3] and metabolic syndrome [4]. The molecular mechanism underlying these clinical benefits is believed to involve the ability of puerarin to act as a scavenger of reactive oxygen species and inhibit low density lipoprotein oxidation [5,6]. Owing to the short elimination half-life of puerarin in human beings, frequent and high dose injections are often required. Although puerarin has protective effects on cardiovascular diseases, the clinical efficacy of puerarin is limited by severe and acute side effects which develop after several weeks of therapy. In particular, intravenous hemolysis is a key factor limiting the clinical use of puerarin. The mechanism for development of intravenous hemolysis remains uncertain, but may be related partly to higher concentrations of puerarin [7].

To investigate the mechanism for development of intravenous hemolysis due to puerarin in rats, an assay that allows specific, sensitive and accurate measurement of puerarin in biological samples is critical. The pharmacokinetics of puerarin have been investigated in a number of studies, but the pharmacokinetic parameters determined are not consistent among the studies. For example, the mean elimination half-life ($T_{1/2}$) of puerarin in healthy rats was found to be 60.92 min in one study [8] and 3.703 h in another [9]. In these studies, puerarin was determined by high performance liquid chromatography (HPLC), and the limit of detection for puerarin was 195 ng/mL and 83 ng/mL, respectively. An assay with greater specificity and sensitivity for the measurement of puerarin in biomatrices is essential. In addition to HPLC [8–13], puerarin in biological matrices could be quantified by capillary electrophoresis with the limit of detection of 10^{-8} M [14] and liquid chromatography tandem mass spectrometry (LC–MS/MS) with the limit of detection of 0.39–10 ng/mL [15–17]. LC–MS/MS is a well-known technology with high specificity and sensitivity in analysis. In previous studies of analyzing puerarin in biological samples using LC–MS/MS, a negative ionization mode was employed. We used ion trap mass spectrometry operated in negative ionization mode and found that although the ratio of signal-to-noise was high when

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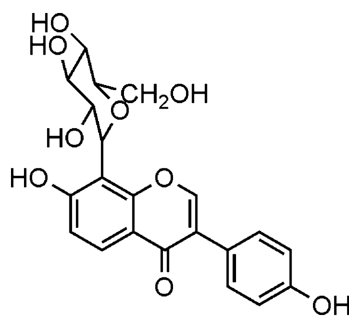

Fig. 1. Chemical structure of puerarin.

MS^2 spectra was used, the detection of puerarin was inadequate. On the other hand, when MS^3 spectra were applied, a positive ionization mode was successful in quantifying puerarin in rat plasma, although more fragmentation occurred.

Recently, much attention has been paid on the improvement in chromatographic performance achieved by the introduction of rapid resolution LC (RRLC). RRLC takes full advantage of chromatographic principles to perform separations using columns packed with sub-2 μm column particle and high flow rates, resulting in much shorter analysis time and much higher sample throughput, as well as increased peak capacity and sensitivity than ordinary LC [18]. In this paper, we describe a sensitive and specific analytical method of rapid resolution liquid chromatography tandem mass spectrometry (RRLC–MS/MS) in positive ionization mode which can be used to study the pharmacokinetics of puerarin in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Puerarin and puerarin injection were purchased from Guangdong Greatsun Biochemical Pharmaceutical Co., Ltd. (Guangzhou, P.R. China). Methanol and acetonitrile (Merck, Germany) were of HPLC grade without further purification. Acetic acid was purchased from Acros Organics (Geel, Belgium). Tectoridin (internal standard) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Drug-free rat plasma was collected from healthy Sprague-Dawley rats of both sexes and stored at −20 °C. Ultrapure water was always used (Millipore, USA).

2.2. Animals

Six specific-pathogen-free grade Sprague-Dawley healthy rats (180–220 g, there male, there female) were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, P.R. China). The rats were kept in an environmentally controlled breeding room (temperature 25 ± 2 °C, humidity 60 ± 5%, 12 h dark/light cycle) for one week before being used for the experiments. They were fed a soy-free custom diet (Guangdong Medical Laboratory Animal Center, Guangzhou, P.R. China) and water *ad libitum*. All rats were fasted overnight before the experiments. All animal treatments were carried out according to the National Research Council's guidelines.

2.3. Standard solutions

Puerarin stock solution was prepared in methanol at a concentration of 1 mg/mL. Puerarin standard working solutions (100, 500, 1000, 2000, 5000, 10,000, 20,000 ng/mL) were prepared by appropriate dilution with 35% methanol in water (i.e. mobile phase solution without acetic acid). The tectoridin solution (internal standard, 4.5 μg/mL) was prepared in 35% methanol in water. The stock solution and the standard working solutions were stored at −20 °C.

2.4. Sample preparation

An amount of 10 μL of one of the puerarin standard working solutions and 10 μL tectoridin solution were added to 100 μL of rat plasma, respectively. Then 200 μL of a mixture of methanol and acetonitrile (90:10, v/v) was added to precipitate protein, vortex mixed for 5 min, and centrifuged (13,000 rpm) for 10 min. The separated supernatant was centrifuged (13,000 rpm) for 5 min. Samples were injected immediately after preparation.

2.5. RRLC–MS/MS analysis

RRLC–MS/MS analyses of rat plasma samples were performed by using an Agilent 1200 series RRLC and 6330 Ion Trap system consisting of a vacuum degasser, a binary pump, an autosampler, a column thermostat and a 6330 Ion Trap XCT Ultra mass spectrometer (Agilent Technologies, USA). Chromatography was carried out on a Zorbax XDB C18 reversed-phase column (4.6 mm × 100 mm, particle size 1.8 μm) (Agilent Technologies, USA), preceded by a guard column filled with C18 (Zorbax XDB, particle size 1.8 μm). The injection volume was 2 μL. The column temperature was set at 30 °C. The mobile phase consisted of a mixture of methanol and 0.05% acetic acid in water (35:65, v/v). The flow rate was 0.3 mL/min from 0 min to 5.4 min followed by 0.6 mL/min from 5.41 min to 12 min.

The column effluent was introduced into the mass spectrometer operated in ESI positive ionization mode, Nitrogen with a pressure of 40 psi and a flow rate of 10 L/min was used as nebulizing and drying gas at 350 °C. The capillary voltage was set at −4500 V. Multiple reaction monitoring (MRM) was used to measure puerarin and tectoridin. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from mass-to-charge (m/z) 417/399 (puerarin) or 463/301 (tectoridin) at two different segments. Smart parameter setting was used for the ion trap parameters, and the number of ions stored in the ion trap was controlled, with a target number of 50,000 for m/z 417/399 (puerarin) and of 500,000 for m/z 463/301 (tectoridin), and a maximum accumulation time of 200 ms. The scanning m/z range was from 250 to 470 with a scanning speed of 26,000 m/z/s. The RRLC and MS system were controlled by Chemstation version B.01.03 SR2 and Ion Trap software 6.1, respectively.

2.6. Method validation

The method validation process followed the guidance for industry: bioanalytical method validation (FDA, 2001). The analytical method was validated to demonstrate the specificity, recovery, linearity, accuracy and precision of measurements, and stability of samples.

Specificity was established by the lack of interference peaks at the retention time for puerarin and internal standard.

Recovery of the method was determined by comparing the peak area obtained from the extracted plasma samples with the peak area obtained by the direct injection of the corresponding spiked standard solutions in 35% methanol in water. Three different concentrations of puerarin (50, 200, 1000 ng/mL in plasma) were measured.

Linearity was tested at seven concentrations in the range 10–2000 ng/mL (10, 50, 100, 200, 500, 1000, 2000 ng/mL). The calibration curves were established by plotting the peak area ratio of puerarin to internal standard versus puerarin concentration. The regression parameters of the slope, intercept and correlation coefficient were calculated by linear least-squares regression.

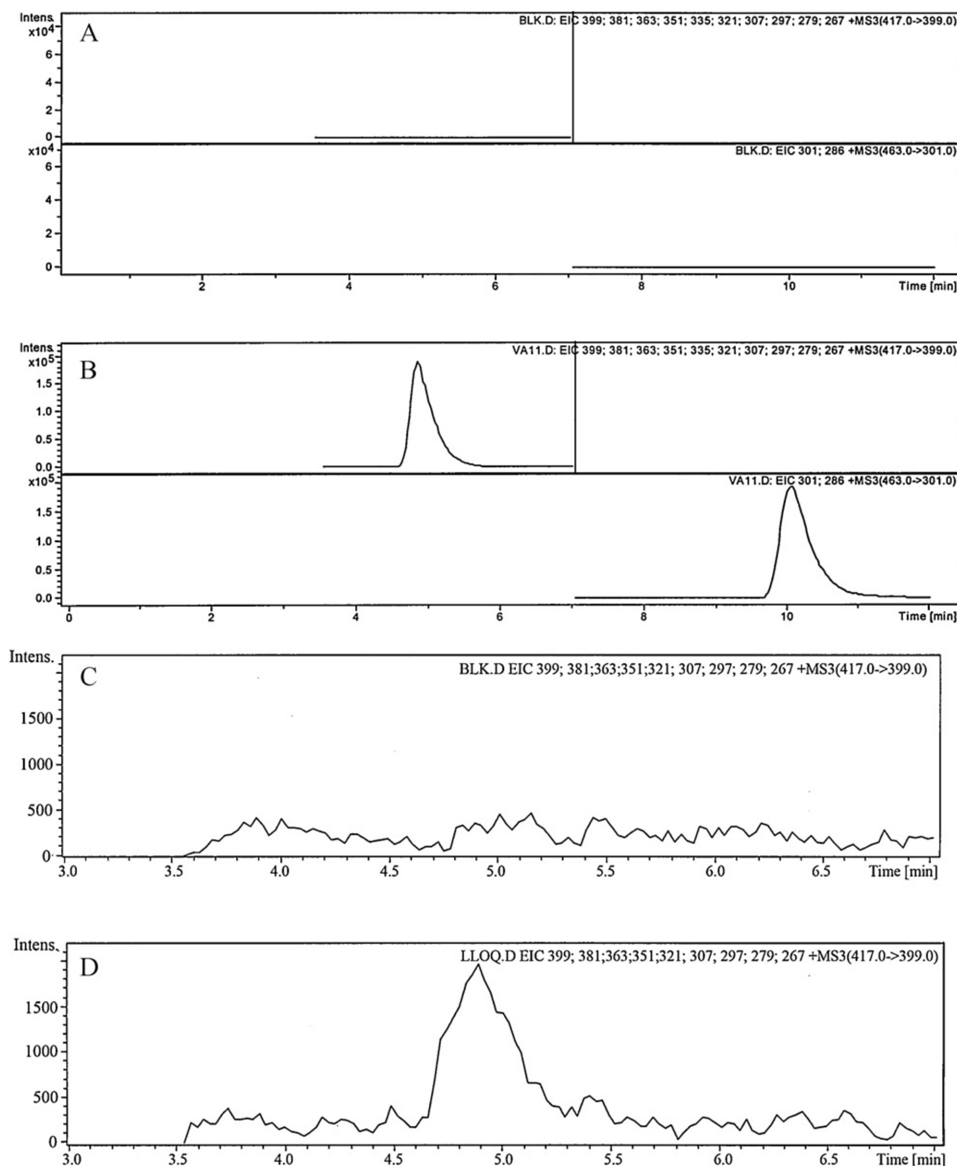

Fig. 2. Representative multiple reaction monitoring chromatograms for puerarin (*m*/*z* 417/399) and the internal standard (tectoridin) (*m*/*z* 463/301). (A and C) Blank rat plasma sample; (B) rat plasma sample 240 min after a single intravenous administration of puerarin (20 mg/kg). Retention time for puerarin = 4.9 min; tectoridin (IS) = 10.0 min; (D) blank rat plasma sample spiked with LLOQ of puerarin.

The accuracy and precision of this analytical method were determined in six replicates of 50, 200 and 1000 ng/mL of puerarin in blank rat plasma, which was prepared and analyzed on three consecutive days. Accuracy was determined by comparing the calculated concentration with the added concentration, using calibration curves. The intra- and inter-day precision of the assay were assessed by calculating the relative standard deviation (RSD). The mean accuracy should be within 15% of the actual value except at the lower limit of quantification (LLOQ), where it should deviate by no more than 20%. The RSD determined at each concentration should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

The stability of puerarin in rat plasma was examined at room temperature and at −20 °C. The storage stability at −20 °C for 2 months was also evaluated. Triplicate samples of 50 and 1000 ng/mL of puerarin were determined, respectively.

2.7. Pharmacokinetic analysis

In a single-dose pharmacokinetic study, puerarin was administered intravenously to six rats (there male, there female) at 20 mg/kg body weight, and serial blood samples (approximately 0.3 mL) were obtained from the jugular vein at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 360 min after dosing under pentobarbital sodium anesthesia (30 mg/kg body weight). From 1 h after dosing, an equal volume of sterile physiological saline replaced each blood sample. Plasma was isolated by centrifugation of the blood at 13,000 rpm for 3 min. All samples were kept at −20 °C until analysis. The validated analytical method was applied to a pharmacokinetic study of puerarin in rats. Pharmacokinetic parameters of puerarin were determined using non-compartmental methods (WinNonlin Enterprise version 5.2.1, Pharsight Corp., Mountain View, CA, USA).

3. Results

3.1. Specificity, recovery and detection limits

The chromatograms obtained, which included a blank plasma sample and a plasma sample obtained 240 min after intravenously dose of 20 mg/kg of puerarin, were essentially free from endogenous interferences (Fig. 2). The typical retention times for puerarin and internal standard were approximately 4.9 and 10.0 min, respectively, and the peak shapes were acceptable.

The recoveries of puerarin from rat plasma with the present method were 102.45 ± 4.56%, 99.67 ± 2.03% and 98.72 ± 1.95% for the concentrations 50, 200 and 1000 ng/mL, respectively.

The limit of detection (signal-to-noise ratio 3) for puerarin was 0.33 ng/mL, and the limit of quantification (signal-to-noise ratio 10) was 1 ng/mL.

3.2. Linearity

The standard curve was prepared for puerarin in the range of 10–2000 ng/mL described by equations $y = (0.003473 \pm 0.001324)x + (0.00065 \pm 0.00007)$ ($n = 4$), $r = 0.99955 \pm 0.00037$ ($n = 4$), where y is the peak area ratio of puerarin and internal standard, x is the concentration of puerarin in rat plasma, and r is the correlation coefficient.

3.3. Accuracy and precision

The inter- and intra-day accuracies of three different puerarin concentrations (50, 200 and 1000 ng/mL) were estimated. The results showed (Table 1) that inter- and intra-day precision ranged from 2.97% to 7.52% and accuracy ranged from 93.70% to 101.60%.

3.4. Stability

Puerarin stored at −20 °C was stable in methanol and in rat plasma for at least two months. Prepared samples were stable for 24 h at room temperature. Puerarin was found to be stable after three freeze–thaw cycles in rat plasma.

3.5. Pharmacokinetic studies

The developed and validated method was applied to a pharmacokinetic study after a single 20 mg/kg intravenous dose of puerarin to healthy Sprague-Dawley rats. The mean plasma concentration–time curve of puerarin is shown in Fig. 3. Pharmacokinetic parameters of puerarin were determined using non-compartmental analysis. The pharmacokinetic parameters are given in Table 2.

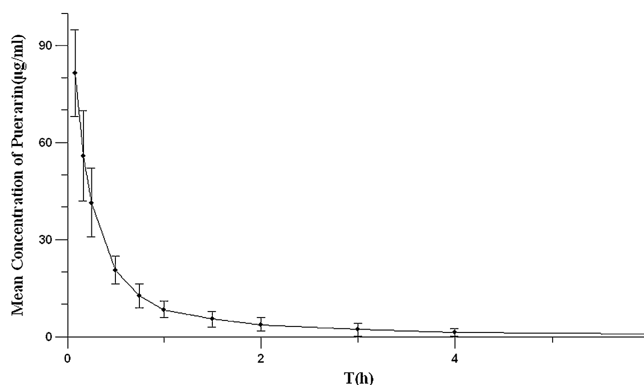

Fig. 3. Mean plasma concentration-time profile of puerarin after intravenous administration of a single 20 mg/kg dose of puerarin to healthy Sprague-Dawley rats.

4. Discussion

Methanol is usually used for protein precipitation [9,19]. But in this study, a mixture of methanol and acetonitrile (90:10, v/v) was used, as it is simple, rapid and has a higher recovery than liquid–liquid extraction using ethyl acetate. The mixture was also produced more protein precipitation than methanol alone; however, the peak shape was unsatisfactory if the proportion of acetonitrile was increased further.

In the positive ionization mode, for ESI–MS analysis, acid solvent must be added in the mobile phase. We found that a mobile phase containing acetic acid resulted in higher sensitivity than that containing formic acid. The highest sensitivity was obtained with a mobile phase comprising methanol and 0.05% acetic acid in water at ratio of 35:65 (v/v), and the peak shape and retention time were satisfactory.

In the case of *C*-glycosides, the sugar is directly linked to the flavonoid moiety via an acid-resistant C–C bond. MS/MS experiments allowed the characterization of *C*-glycosides both in positive and in negative ionization modes [20]. Under certain conditions, the positive ionization mode was suitable for determining puerarin in rat plasma. In this report, the limit of detection (signal-to-noise ratio 3) for puerarin was 0.33 ng/mL, indicating that the method was highly sensitive. The results obtained for accuracy

Table 1
Accuracy and precision of the method used for determination of puerarin in rat plasma ($n = 6$).

	Added concentration (ng/mL)	Concentration found (ng/mL)	Accuracy (%)	Precision (%)
Inter-day	50	49.56 ± 3.73	99.11	7.52
	200	194.57 ± 12.10	97.29	6.22
	1000	1000.50 ± 29.70	100.05	2.97
Intra-day	50	48.23 ± 3.42	96.46	7.08
	200	187.41 ± 9.85	93.70	5.25
	1000	1016.00 ± 55.71	101.60	5.48

Table 2
Pharmacokinetic parameters for puerarin after single dose intravenous administration in rats (20 mg/kg) ($n = 6$).

	$AUC_{0\to t}$ (mg h/L)	$AUC_{0\to\infty}$ (mg h/L)	MRT (h)	$T_{1/2}$ (h)	V_{ss} (L)	V_z (L)	Cl (L/h)
Mean	45.37	47.03	1.03	1.31	0.09	0.17	0.10
SD	13.19	14.78	0.46	0.31	0.02	0.04	0.04

$AUC_{0\to t}$: area under the concentration–time curve from time zero to the last sampling time point; $AUC_{0\to\infty}$: area under the concentration–time curve from time zero to the infinity; MRT: mean resident time; $T_{1/2}$: elimination half-life; V_{ss}: volume of distribution at steady-state; V_z: volume of distribution at terminal state; Cl: total apparent clearance. Mean: average of six rats; SD: standard deviation.

and precision indicated that the method was reliable and reproducible.

This validated method was successfully applied to a pharmacokinetic study in rat plasma after intravenous administration of puerarin. The results showed that puerarin was distributed rapidly and eliminated quickly after intravenous administration of puerarin to healthy rats. $T_{1/2}$ was longer than reported previously (11.4 min) [21], possibly for the following reason: (a) in the literature previously reported only one concentration–time curve was a plot of the mean puerarin concentration at a series of time points of different rats rather than the same rat; (b) daidzein was used as the internal standard in the determination of puerarin, but daidzein was one of the metabolites of puerarin in rats [19,22], and if daidzein was present in the blood, the concentration of puerarin would be underestimated. Accordingly, our results may be closer to the true value.

5. Conclusions

A method of LC–MS/MS operated in positive ionization mode has been developed and validated for quantitative determination of puerarin in rat plasma. The method had good specificity, sensitivity, linearity, accuracy and precision, and was successfully employed in a pharmacokinetic study of puerarin in rats.

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