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Determination of Puerarin in Rat Plasma by HPLC with Fluorescence Detection and its Application to Pharmacokinetic Studies

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***SUMMARY*. A simple, rapid, sensitive and selective method of high-performance liquid chromatography with fluorescence detection was developed for determination of puerarin in rat plasma. Chromatography was carried out on a reversed-phase Hypersil ODS column at 30 °C using a mobile phase consisting of methanol - 50 mM NH_4OAc in water (23:77, v/v) with a flow rate of 1 mL/min. The excitation wavelength and emission wavelength were 250nm and 480nm, respectively, PMT-gain was set at 11. The method was demonstrated to be selective and sensitive, and a good linear response was observed over a range of 0.16–120.00 μg/mL in rat plasma. The validated method was successfully applied to the characterization of the pharmacokinetics of puerarin in rat plasma after intravenous administration to rats. The main pharmacokinetic parameters were as follows: $AUC_{0\rightarrow t}$ 41.94 ± 12.90 (mg/L•h), $AUC_{0\rightarrow\infty}$ 44.37 ± 28.90 (mg/L•h), MRT 0.97 ± 0.37 (h), $T_{1/2}$ 1.06 ± 0.39 (h), Vss 0.09 ± 0.02 (L), Vz 0.14 ± 0.03 (L), Cl 0.10 ± 0.05 (L/h).**

INTRODUCTION

Puerarin (chemical name 7,4'-dihydroxy-isoflavone-8β-glucopyranoside, Fig. 1) is a major active ingredient extracted from the traditional Chinese medicine Pueraria Radix (the root of the *Pueraria lobota* (Willd) Ohwi. Puerarin has been widely prescribed for patients with cardiovascular diseases in China. 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 puerarin's ability to act as a scavenger of reactive oxygen species and inhibit LDL oxidation [5,6]. Due to the short elimination half-life of puerarin in human bing [7], frequent and high dose injections are often required. Although puerarin have 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. Especially, intravenous hemolysis is a key factor limiting the clinical use of puerarin. The mechanisms of intravenous hemolysis remain uncertain. Intravenous hemolysis is related to higher concentrations of puerarin [8].

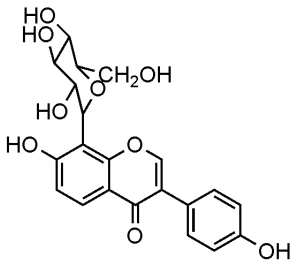

Figure 1. Chemical structure of puerarin.

To investigate the mechanisms of intravenous hemolysis due to puerarin in rats, an assay that allows specific, sensitive and accurate measurement of puerarin in biological samples is critical. Previous methods of analyzing puerarin in biological samples are based on high-performance liquid chromatography separation and ultraviolet detection (HPLC-UV) [9-13] or liquid chromatography-tandem mass spectrometry

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[14,15]. Although the liquid chromatography-tandem mass spectrometry method is specific and sensitive, it should be noted that mass detection is expensive and not easily available in most laboratories. Compared to the poorly selective UV detection method, fluorescence detection provides superior selectivity and sensitivity of analysis. We found that puerarin could emit fluorescence when excited by the ultraviolet light under certain conditions, so it is possible that puerarin can be detected by HPLC with fluorescence detection. Fluorescent methods have been reported [16-18], however, there are some disadvantages of these methods, for example, in two methods, daidzein was used as the internal standard [17,18], but daidzein is one of the urinary or biliary metabolites of puerarin in human and rats [15,19,20], and the other one needed post-column modification [16]. However, the present method is simple as no expensive instrumentation or complicated derivatization procedures or post-column modifications are required. Furthermore, a number of studies on the pharmacokinetics of puerarin have been investigated, but the pharmacokinetic parameters are not consistent in all studies. For example, the mean elimination half-life of puerarin in healthy rats was 60.92 min [21] or 3.703 h [11]. In these studies, HPLC with UV detection or liquid chromatography-tandem mass spectrometry were employed to determine puerarin. The different sensitivity of methods may be responsible for the variance of results. It is therefore necessary to develop a relatively sensitive method to quantify puerarin in biological samples. In this report, we have developed a sensitive, selective analytical method of HPLC with fluorescence detection to study the pharmacokinetics of puerarin in rat plasma.

MATERIALS AND METHODS

Chemicals and reagents

Puerarin and puerarin injection was purchased from Guangdong Greatsun Biochemical pharmaceutical Co., Ltd, (Guangzhou, PR China). Methanol and acetonitrile (Merck, Germany) were of HPLC grade without further purification. Purified water was always used (Molecular, PR China).

Animals

Specific-pathogen-free grade Sprague-Dawley healthy rats (180-220 g, half male, half female) were obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, PR 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, PR China) and water *ad libitum*. All rats were fasted overnight before the experiments. All animal treatments were according to the recommendations of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Standard solution

Puerarin stock solution was prepared in methanol at a concentration of 1.6 mg/mL. Puerarin standard working solutions (1.6, 3.1, 6.2, 12.5, 25.0, 50.0, 100.0, 200.0, 400.0, 800.0, 1200.0 μg/mL) were prepared by appropriate dilution with 77% methanol in water (i.e. mobile phase solution without ammonium acetate). The stock solution and the standard working solutions were stored at –20°C.

Sample preparation

An amount of 10 μL of one of the puerarin standard working solutions was added to 100 μL of rat plasma. Then 200 μL of a mixture of methanol and acetonitrile (90:10, v/v) was added to precipitate protein, vortex mixed for 3 min, and centrifuged (13 000 rpm) for 10 min. The separated supernatant was centrifuged (13 000 rpm) for 5 min. A 10 μL aliquot of the final supernatant was injected into the HPLC system for analysis. Prepared samples were analyzed immediately.

Apparatus and chromatographic conditions

Analysis of rat plasma samples was performed by HPLC using an Agilent 1100 series HPLC system. The system consisted of a vacuum degasser, a quaternary pump, an autosampler, a column thermostat and a fluorescence detector. Separation was achieved using a Hypersil ODS C18 reversed-phase column (250 mm x 4.6 mm i.d., 5 μm particle size), preceded by a guard column filled with C18 (ODS, 5 μm particle size) (Agilent Technologies, USA). The column temperature was set at 30 °C. The mobile phase consisted of a mixture of methanol and 50 mM ammonium acetate in water (23:77, v/v) with a flow rate of 1 mL/min. The mobile phase was degassed by sonication and filtered through 0.45 μm PTFE membranes (Jinteng, PR China) prior

to use. The fluorescence detector was set at excitation wavelength of 250nm, emission wavelength of 480nm and PMT-gain of 11. The injection volume was 10 µL. The HPLC system was controlled by Chemstation version A.10.02.

Method validation

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

Selectivity was established by the lack of interference peaks at the retention time for puerarin.

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 concentration spiked standard solution in the extracted blank plasma. Three different concentrations of puerarin (0.31, 5.0, 80.0 µg/mL) were measured.

Linearity was tested at 11 concentration levels covering the range 0.16–120.0 µg/mL (0.16, 0.31, 0.62, 1.25, 2.50, 5.0, 10.0, 20.0, 40.0, 80.0, 120.0 µg/mL). The calibration curves were established by plotting peak area vs concentration of puerarin. The regression parameters of the slope, intercept and correlation coefficient were calculated by linear weighted least-square regression.

The accuracy and precision (presented as relative standard deviation (RSD)) of this analytical method were determined in six replicates of 0.31, 5.00 and 80.00 µg/mL of puerarin in spiked plasma. Accuracy was determined by comparing the calculated concentration with the known concentration using calibration curves. The mean value of accuracy should be within 15% of the actual value except at lower limit of quantification (LLOQ), where it should not deviate by more than 20%; The RSD determined at each concentration level 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, 30 °C and at –20 °C. The storage stability at –20°C for 2 months was also evaluated. Triplicate samples of 0.31, 80.0 µg/mL of puerarin were determined, respectively.

Pharmacokinetic analysis

In a single-dose pharmacokinetic study, puerarin was intravenously administered (into the jugular vein) to 6 rats (half male, half female) respectively at 20 mg/kg body weight and serial blood samples (about 0.3ml) were obtained from the other jugular vein at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 360 min post-dosing under pentobarbital sodium anesthesia (30mg/kg body weight). From the point of 1h of post- dose, 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 compartmental methods (Kinetica 4.4.1, Thermo Fisher Scientific Inc., MA, USA). The elimination rate constant *(Lz)* was obtained from the least-square fitted terminal log-linear portion of the serum concentration–time profile. The elimination half-life $(t_{1/2\beta})$ was calculated by 0.693/ *Lz*. The area under the plasma concentration–time curve of puerarin from time zero to time t ($AUC_{0\rightarrow t}$) was determined by the trapezoidal rule to the last measurable concentration (*Ct*), the area under the plasma concentration–time curve of puerarin from time zero to infinity ($AUC_{0\rightarrow\infty}$) was determined by $AUC_{0\rightarrow t}$ plus the additional area from time t to infinity, calculated as *Ct*/*Lz*. The ratio between partial area under the moment curve(AUMC) and AUC yielded mean residence time (MRT). The apparent volume of distribution during the terminal phase (Vz) = dose/(AUC·*Lz*), the apparent volume of distribution at steady-state (Vss) = (dose ·MRT)/AUC, total clearance(Cl) = dose/ AUC.

RESULTS AND DISCUSSION

Optimization of analytical condition

Puerarin was widely determined by a reversed-phase HPLC-UV method. Acidic solution (acetic acid or phosphoric acid) in the mobile phase was important for good peak shape, probably by preventing the ionization of puerarin [10]. However, we found that the fluorescence intensity of puerarin was very low when acetic acid was in the mobile phase. In contrast, the fluorescence intensity was high enough to be determined when ammonium acetate (50 mM) was added to the mobile phase. In this case, the hydroxyl groups of the isoflavones dis-

sociates, transforming the isoflavones into negative ions, and the fluorescence intensity is increased; therefore, the higher fluorescence intensity could be achieved only when puerarin was ionized. Under the alkaline condition (for example, dipotassium hydrogen phosphate, 10-100 mM, pH 7.9-8.5), the retention time was too short, however, an appropriate retention time, a satisfactory resolution and a good peak shape were obtained when 50 mM ammonium acetate (pH 6.4) was added to the water of mobile phase. We set the optimal exciting wavelength and emission wavelength according to the isoabsorbance plot of puerarin. To obtain higher sensitivity, we set the PMT-gain of the fluorescence detector at 11.

The column was also important. We tried different columns, including Eclipse C8, Venusil XBP-C18 and Hypersil ODS C18, when Eclipse C8 or Venusil XBP-C18 column was used, the retention time of puerarin was not suitable enough, too short or too long, but the peak shape would not be good enough when the proportion of the mobile phase was changed, at last, Hypersil ODS C18 column was employed. A mobile phase consisted of methanol-50 mM ammonium acetate in water (23:77, v/v) with a flow rate of 1 mL/min was the best condition to get satisfying resolution.

Methanol is usually used for protein precipitation [11,20]. In the present study, a mixture of methanol and acetonitrile (90:10, v/v) was used for protein precipitation, as it provided the advantages of being rapid, simple and yielding a higher recovery compared with liquid–liquid extraction using ethyl acetate. The mixture was also more effective for protein precipitation than methanol alone, but the peak shape was not good enough when the ratio of acetonitrile was higher.

To sum up, an appropriate mobile phase, suitable parameters of fluorescence detection, a suitable column and an effective method for protein precipitation provided us with good sensitivity, peak shape for puerarin and adequate separation from interfering peaks.

Since puerarin could emit fluorescence under certain conditions, the method does not require expensive instrumentation or complicated derivatization procedures, and direct protein precipitation was adopted in the process of sample preparation, it consumed about 20 min for preparing one sample, it was simple and rapid.

Selectivity, recovery and detection limits

The typical chromatograms of a blank rat plasma sample, a blank rat plasma sample spiked with puerarin and a rat plasma sample obtained after intravenous administration of puerarin are shown in Figure 2. The typical retention time for puerarin was 8.9 min. No significant interferences of endogenous substances from the blank rat plasma were detected. Furthermore, the mass spectra (performed on a 6330 Ion Trap XCT Ultra mass spectrometer operated in ESI (Agilent Technologies, USA)) of the peak corresponding to puerarin on the HPLC chromatogram showed an abundant ion peak at *m/z* 417 ($M+H^+$) or *m/z* 415 ($M-H^-$) in positive or negative ionization mode (Fig. 3), respectively, the precursor ion and product ions were consistent with those of our standard puerarin, and they are closely similar to those in the literature [22], so, there are no significant mebabolite(s) interfering to the puerarin peak. This indicated that the present HPLC method is selective for the determination of puerarin in rat plasma.

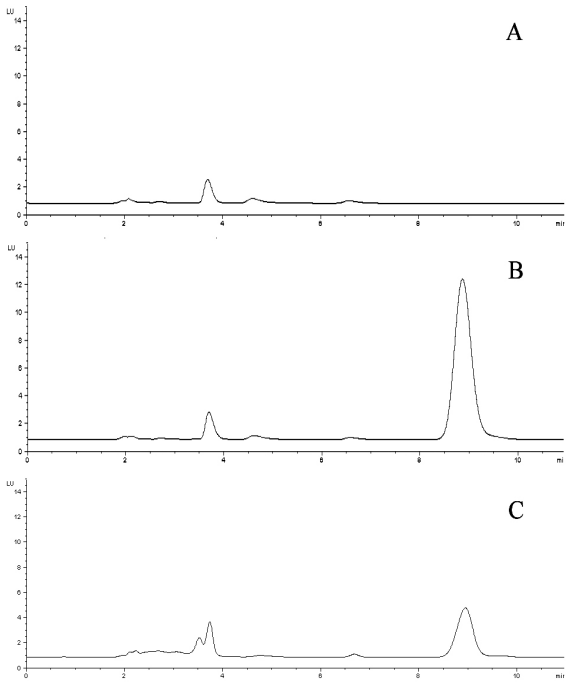

Figure 2. Chromatograms of puerarin in rat plasma samples determined by HPLC with fluorescence detection. **A** Blank rat plasma sample; **B** Blank rat plasma sample spiked with puerarin (20 μg/mL); **C** Rat plasma sample obtained one hour after intravenous administration of puerarin (6.68 μg/mL)

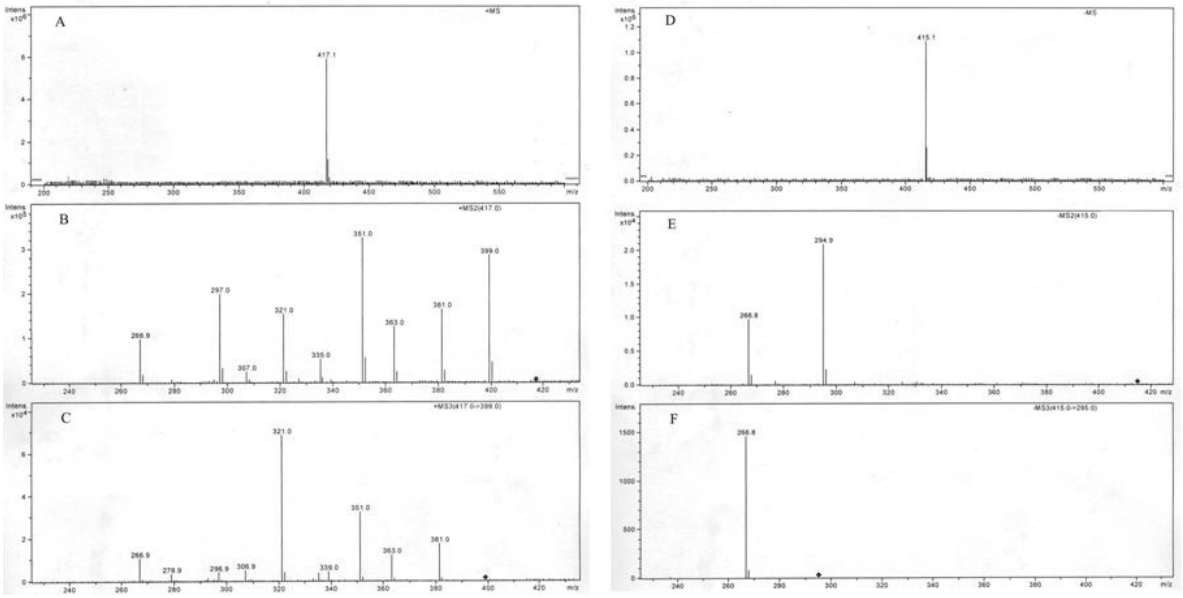

Figure 3. ESI mass spectra of the peak corresponding to puerarin on the HPLC chromatogram. A to C: positive ionization mass spectra; **A**: MS spectrum; **B**: MS2 spectrum (417); **C**: MS3 spectrum (417/399). **D** to **F**: negative ionization mass spectra; **A**: MS spectrum; **B**: MS2 spectrum (415); **C**: MS3 spectrum (415/295).

The recoveries of puerarin from rat plasma with the present method were 101.27 % ± 2.34, 99.40 % ± 1.87 and 98.05 % ± 2.84 (n = 6) for the concentrations 0.31, 5.00 and 80.00 µg/mL, respectively.

The limit of detection (signal-to-noise ratio 3) for puerarin was 10 ng/mL, and the limit of quantification (signal-to-noise ratio 10) was 33 ng/mL. We determined puerarin by the same HPLC system with diode array detector, and the limit of detection was 60 ng/mL. This method was more sensitive than that previously reported detected by the same series HPLC system with diode array detector (detection limit 83 ng/mL) [11]. Certainly, detection limit of 3 ng/mL was reported in the literature [13], but a different HPLC system was used, moreover, solid-phase extraction and condensation process were included.

Linearity

The standard curve was prepared for puerarin in the range of 0.16-120.0 µg/mL, which covered the levels following the intravenous administration of a single dose of 20 mg/kg puerarin. The standard curve was described by equations y = 14.36*x*–0.15, *r* = 0.9999, where y is the peak area, *x* is the concentration of puerarin in rat plasma in µg/mL, and r is the correlation coefficient.

Accuracy and precision

The intra- and inter-day accuracies of there different concentrations (0.31, 5.0 and 80.0 µg/mL) were estimated. The small difference (<5 %) between nominal concentrations and the calculated concentrations documented confirmed appropriate accuracy of the method. Moreover, the lower RSD (<10 %) indicated that the method was relatively precise (Table 1).

Stability

Puerarin was stable in methanol and in rat plasma for at least two months when stored at –20 °C. Prepared samples were stable for 24 h at room temperature and were stored at 30 °C for 2 h. Rat plasmas piked with puerarin were also stable after three freeze and thaw cycles (Table 2).

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 rats. The mean plasma concentration–time curve of puerarin is shown in Figure 4. Pharmacokinetic parameters of puerarin were determined using compartmental analysis. We found the plasma concentration-time course of puerarin was best explained by an open two-compartment model (weighted by 1/Yobs). The pharmacokinetic parameters are given in Table 3. The MRT, $T_{1/2\alpha}$, $T_{1/2\beta}$, Vss and Cl was 0.97h, 0.13h, 1.06h, 0.09L and 0.10L/h, respectively, which indicated that puerarin was

	Concentration added (μg/mL)	Concentration measured (μg/mL) mean ± SD	Accuracy (%)	RSD (%)
Inter-day	0.31	0.32 ± 0.01	103.23	3.67
	5.00	4.97 ± 0.25	99.40	5.09
	80.00	81.55 ± 3.35	101.94	4.11
Intra-day	0.31	0.31 ± 0.03	100.00	8.67
	5.00	5.07 ± 0.24	101.40	4.69
	80.00	81.75 ± 3.22	102.19	3.93

Table 1. Accuracy and precision of method for the determination of puerarin in rat plasma (n=6). Mean: average of six determination, SD: standard deviation. Accuracy (%) = (mean concentration measured/concentration added) x 100. RSD (%) (relative standard deviation) = (SD /mean) x 100.

Storage conditions	Concentration added (μg/mL)	Concentration measured (μg/mL) mean ± SD	Accuracy (%)
room temperature / 24 h	0.31	0.32 ± 0.01	103.23
(prepared sample)	80.00	83.44 ± 1.34	104.30
30 °C / 2 h	0.31	0.32 ± 0.01	103.23
(prepared sample)	80.00	83.45 ± 1.34	104.31
room temperature/24 h	0.31	0.34 ± 0.01	109.68
	80.00	79.69 ± 1.01	99.61
–20 °C/ 1 freeze/thaw cycle	0.31	0.33 ± 0.02	106.45
	80.00	82.30 ± 1.44	102.88
–20 °C/ 3 freeze/thaw cycle	0.31	0.29 ± 0.01	93.55
	80.00	81.18 ± 0.46	101.48
–20 °C/ 2 months	0.31	0.30 ± 0.01	96.77
	80.00	81.02 ± 0.38	101.28

Table 2. Stability of puerarin in rat plasma under various storage conditions (n = 3). Mean: average of six determination, SD: standard deviation. Accuracy (%) = (mean concentration measured/concentration added) x 100.

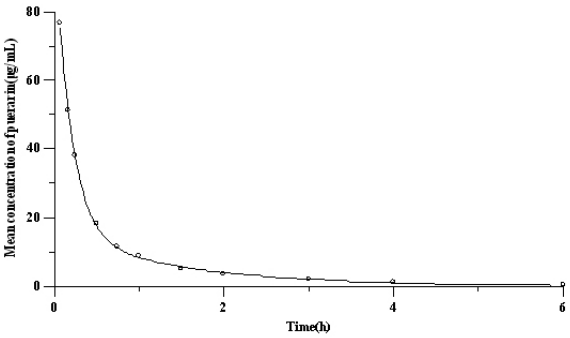

Figure 4. Profile of mean plasma concentration-time of puerarin and pharmacokinetic profile modeled with two-compartment model after a single dose intravenous administration to rats (20 mg/kg, n = 6). The circles denote the mean concentration of puerarin in rat plasma, the line denotes the profile modeled with two-compartment model.

distributed rapidly and widely, and eliminated fast after puerarin was intravenously administered to healthy rats. Both $T_{1/2\alpha}$ and $T_{1/2\beta}$ were longer than that reported before (11-17.82 min) [17,18]. The following reasons may be responsible for it: on the one hand, daidzein was used as the internal standard when puerarin was detected, but daidzein was one of the metabolites of puerarin in rats [19,20], if daidzein existed in the blood, the concentration of puerarin would be underestimated, on the other hand, in one literature [17], the only one concentration–time curve was obtained by the data of different rats, but not a same rat. Accordingly, our results may be closer to practice, notwithstanding absence of an appropriate internal standard in the present method.

	$AUC_{0\rightarrow t}$ (mg·h /L)	$AUC_{0\rightarrow \infty}$ (mg·h /L)	MRT (h)	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	Vss (L)	Vz (L)	Cl (L/h)
Mean	41.94	44.37	0.97	0.13	1.06	0.09	0.14	0.10
SD	12.90	28.90	0.37	0.02	0.39	0.02	0.03	0.05

Table 3. Pharmacokinetic parameters for puerarin after single dose intravenous administration in rats (20 mg/kg) (n=6). $AUC_{0\rightarrow t}$: area under the concentration-time curve from time zero to the last sampling time point; $AUC_{0\rightarrow \infty}$: area under the concentration-time curve from time zero to the infinity; MRT: mean residence time; $T_{1/2\alpha}$: distribution half-life; $T_{1/2\beta}$: elimination half-life; Vss: volume of distribution at steady-state; Vz: volume of distribution at terminal state; Cl: total clearance. Mean: average of six rats; SD: standard deviation

CONCLUSIONS

A simple and rapid method of HPLC with fluorescence detection has been developed and validated for quantitative determination of puerarin in rat plasma. Neither expensive instrumentation nor complicated derivatization procedures is required. Furthermore, the present method offers sensitivity with a lower limit of quantification of 33 ng/ml, selectivity and a good linear response over a range of 0.16–120.00 μg/mL in rat plasma, and it is successfully employed in a pharmacokinetic study of puerarin in rats.

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