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HPLC method for the determination and pharmacokinetic studies on puerarin in cerebral ischemia reperfusion rat plasma after intravenous administration of puerariae radix isoflavone

Bin Yan[a], Dongming Xing[b], Yi Ding[b], Jialin Tao[b], Li-jun Du[b,*]

[a] *Institute of Medicinal Plant, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China*
[b] *Laboratory of Pharmaceutical sciences, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China*

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Abstract

A new HPLC method for the determination of puerarin in cerebral ischemia reperfusion rat plasma is introduced. Puerarin, the principal bioactive component of puerariae radix isoflavone, was extracted from plasma by methanol. The HPLC separation was then performed on a reversed-phase C18 column using water–acetonitrile (89:11, v/v) as eluting solvent system, and UV detection at 252 nm to measure the analyte with a limit of quantitation about 9.44 ng ml^{-1}. The calibration curve for puerarin was linear ($r = 0.9998$) in the concentration range of 9.44–1208.00 ng ml^{-1}, both intra- and inter-day precision of the puerarin were determined and their coefficient of variation did not exceed 10%. The validated method has been successfully applied for pharmacokinetic studies of puerarin from rat plasma after intravenous administration of puerariae radix isoflavone. Another novel finding of this study was that the elimination rate of puerarin was significantly slower in the cerebral ischemia reperfusion rat than in the normal rat, judging by the pharmacokinetic parameters obtained. Since puerariae radix isoflavone was mainly administrated to the patients suffering from cerebralvascular diseases, the pharmacokinetic studies performed on the pathological animal models were suitable references for clinical application.

Keywords: Puerarin; Puerariae radix isoflavone; Pharmacokinetics; Cerebral ischemia reperfusion; HPLC

1. Introduction

Pueraria lobata (*Willd.*) *Ohwi* is one of the earliest medicinal plants to be used in China. Puerariae radix (PR; root of the Pueraria plant) was first described in the Chinese materia medica, Shen Nong Ben Cao Jing (Anonymous, ca. 200 B.C.) and was used as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent-a general antimicrobial agent in today's parlance. In the past decades, medications based on PR have been found useful to treat coronary heart diseases and cerebral vascular diseases in China and Japan, and have produced favorable effects [1,2]. Four major isoflavones were identified in the PR extract and quantified-namely, puerarin, daidzin, genistin and daidzein [3]. Puerarin (Fig. 1), the most abundant component in the puerariae radix isoflavone mixture [4,5] has been reported to significantly dilate coronary arteries, to protect the neurons from damage by glutamine and NMDA and to improve microcirculation both in animals and patients suffering from cardiovascular and cerebralvascular diseases [6–12]. Thus, puerarin has been used as one of the marker compounds to characterize the puerariae radix isoflavone. But the administration of purified puerarin reported in the literature [13] could not be used as suitable references for clinical application because most medications based on PR are administered intravenously as a mixture known as puerariae radix isoflavone. Therefore, it is necessary to perform the pharmacokinetic studies of puerariae radix isoflavone, which have not been previously reported. Our pilot experiments showed that, owing to the complex ingredients of puerariae

* Corresponding author. Tel.: +86 1 62773630; fax: +86 1 62773630.
E-mail address: pharm@mail.tsinghua.edu.cn (L.-j. Du).

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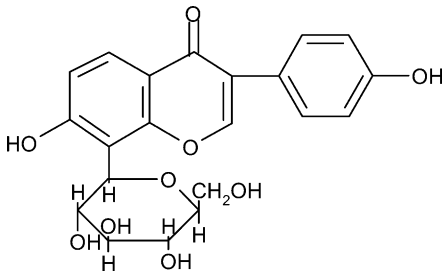

Fig. 1. The chemical structure of puerarin.

radix isoflavone, the regular mobile phase (water–methanol) [14–16] used in HPLC always led to increased retention time or unsatisfactory separation effects. So in the present experiments, HPLC was performed using water–acetonitrile as the mobile phase, which was proved to be satisfactory. Furthermore, puerariae radix isoflavone was mainly administrated to the patients suffering from cerebralvascular diseases and so in this paper, a simple and rapid HPLC method was developed to determine puerarin in cerebral ischemia reperfusion rat plasma after intravenous administration of puerariae radix isoflavone, so as to provide a limited view of its pharmacokinetic profiles.

2. Experimental

2.1. Crude drugs

Puerariae radix was purchased from Beijing Songlan Herbal Pharmaceutical Company, China. The herb was dried under shade and sliced into pieces. The pieces were then refluxed for 1 h in aqueous ethanol (80% v/v) twice and the combined alcoholic extractive was evaporated to dryness. The extract was dissolved in hot water and then filtered. The filtrate was chromatographed on foraminate resin 101 and eluted with water and then aqueous ethanol (70% v/v). The aqueous ethanol fraction was collected and evaporated to dryness to obtain puerariae radix isoflavone (purification 80%).

2.2. Chemicals and reagents

The reference standard of puerarin was purchased from National Chemicals and Biological Products Institute (Beijing, PR China), with batch number: 0703–9914. Acetonitrile and methanol were of HPLC grade (Fisher, Leics, UK). House triple-distilled water from silica glass equipment was always used.

2.3. Animals

Male wistar rats (200–220 g) were obtained from the Laboratory Animal Institute of Chinese Academy of Medical Sciences (Beijing, PR China). They were kept in an environmentally controlled breeding room for 3 days before starting the experiments. They were fed with standard laboratory food and water ad libitum and fasted overnight before the test.

2.4. HPLC system

The HPLC system consists of a 515E pump, a 2487 UV–vis detector set at 252 nm, a 20 ul injection loop, a Millenium 32 workstation (all from Waters, USA) for data collection and an ODS-3 C18 reversed-phase column (5 mm × 150 mm × 4.6 mm) which was protected by RP18 (5 mm) guard column (both from Agilent, USA). The mobile phase was water–acetonitrile (89:11, v/v) filtered through a 0.45 mm millipore filter and degassed prior to use. The flow rate and the column temperature were set at 1 ml min^{-1} and $25 \pm 1\,^{\circ}C$.

2.5. Content of puerarin in puerariae radix isoflavone

The content of puerarin was calculated first, and then puerariae radix isoflavone was intravenously administered to rats at a dose containing 32 mg kg^{-1} puerarin. To calculate the content of puerarin, the puerariae radix isoflavone was dissolved in mobile phase and diluted to a concentration of 0.5 mg ml^{-1}. The mixture was centrifuged at 2500 rpm for 10 min and the supernatant solution was obtained, and then 20 ml of this solution was injected into the HPLC system for analysis. The content of puerarin in the puerariae radix isoflavone was determined to be 32.0%, from the peak area ratios by using equation for linear regression obtained from the calibration curve.

2.6. Calibration curve

Stock solutions of puerarin were prepared with the mobile phase. Puerarin was prepared at concentrations of 0.0944, 0.1888, 0.3775, 0.7550, 1.5100, 3.0200, 6.0400 and 12.0800 ug ml^{-1}. One hundred unit liters of each solution was added together to blank rat plasma so that the resulting plasma contained 9.44, 18.88, 37.75, 75.50, 151.00, 302.00, 604.00 and 1208.00 ng ml^{-1} puerarin. The plasma was then processed according to the procedure below. The limit of quantification (LOQ) in plasma was defined as the lowest concentration on the calibration curve for which assay precision (coefficient of variation, CV) was lower than 10%.

2.7. Liquid phase extraction procedure

Each collected blood sample was immediately transferred to a heparinized glass tube and centrifuged at 1500 rpm for 10 min. The resulting plasma (1.0 ml) was then mixed with 4 ml methanol by vortex for 1 min. The denatured protein precipitate was separated by centrifugation at 2500 rpm for 10 min at room temperature. The supernatant was evaporated to dryness in a water-bath at 37 °C and then dissolved in

0.2 ml mobile phase. A 10-ul volume of this sample solution was injected onto HPLC for analysis. The same sample handling process was used for recovery and precision determinations in plasma.

2.8. Recovery

Plasma samples were spiked with three different concentrations (18.88, 151.00 and 302.00 ng ml^{-1}) of puerarin. After the samples were processed according to the above-mentioned method, the resulting peak areas were compared with the standard of puerarin carried in mobile phase to provide the recovery values.

2.9. Application of the method for in vivo studies

The cerebral ischemia reperfusion was operated by occupation of bilateral carotids for 30 min and reperfusion [17], prior to puerariae radix isoflavone administration. A weight-matched group of rats was left intact served as the normal control. Aqueous solution of puerariae radix isoflavone were intravenously administrated to rats at a dose of 32 mg kg^{-1} of puerarin, and blood samples were collected at times of 5, 10, 15, 30, 45, 60, 120 and 240 min after dosing. Then the samples were centrifuged and the separated plasma samples were processed according to the above-mentioned method. Data from these samples were used to construct pharmcokinetic profiles by plotting drug concentration versus time. All data were subsequently processed by the computer program 3P87 (Chinese Pharmaceutical Association, 1987). Data are expressed as mean ± S.E.M. Student's *t*-test was used to assess statistical significance and statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Recovery

For the isolation of puerarin from rat plasma, liquid phase extraction was employed in this study. In our experiments, the biosamples were obtained and extracted by precipitating protein with methanol to remove non-polar interfering impurities. Puerarin can be easily dissolved in water and methanol and the recoveries obtained by the method were high enough (Table 1).

Table 1
Recovery of puerarin from rat plasma ($n = 5$)

Spiked concentration (ng ml^{-1})	Recovery (%) mean ± S.E.M.	Average (%)
18.88	96.27 ± 1.46	
151.00	95.73 ± 2.82	96.86
302.00	98.60 ± 0.99	

3.2. Selectivity

The separated peaks of puerarin (Fig. 2) revealed that the retention times of puerarin in puerariae radix isoflavone and puerarin standard were 7.440 and 7.487 min, respectively, and no interfering peaks were detected. This indicated that the selectivity of the elaborated procedure was satisfactory

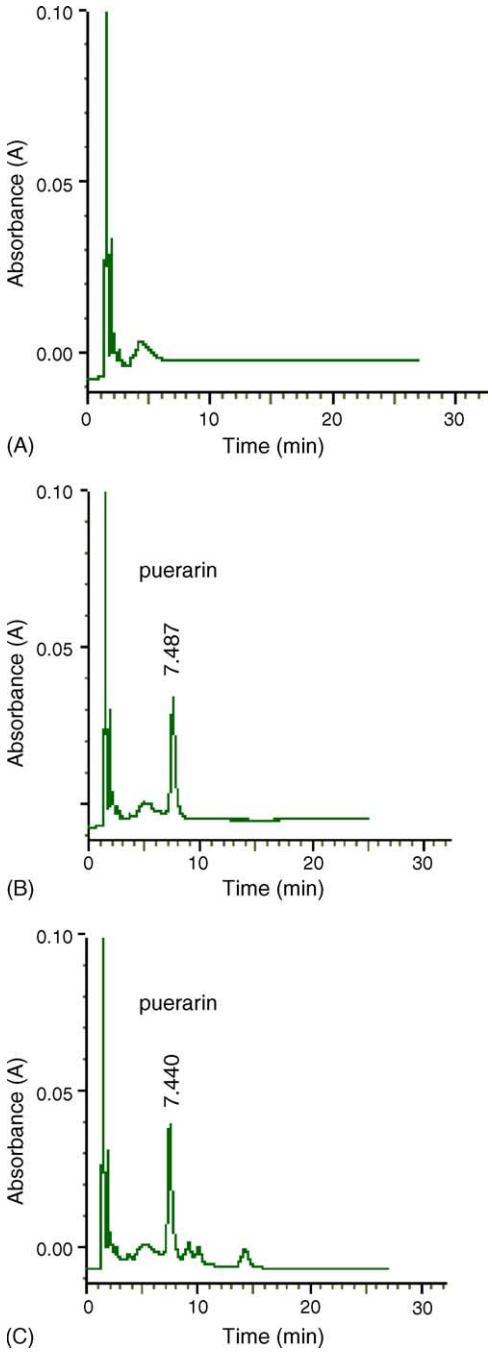

Fig. 2. Typical chromatograms for the determination of puerarin in plasma samples, (A): chromatogram of a blank plasma sample; (B): chromatogram of a plasma sample spiked with puerarin; (C): chromatogram of the plasma sample from a rat after 10 min of intravenous administration of puerariae radix isoflavone.

Table 2
Intra- and inter-day precision and accuracy of puerarin in rat plasma ($n = 5$)

Concentration added (ng ml^{-1})	Concentration measured (ug ml^{-1}) mean ± S.E.M.	Accuracy (%)	CV (%)
Intra-day reproducibility			
9.44	9.22 ± 0.20	97.67	2.17
18.88	18.73 ± 0.70	99.21	3.74
75.50	73.18 ± 1.59	96.93	2.17
302.00	305.61 ± 2.29	101.20	0.75
1208.00	1200.90 ± 12.36	99.41	1.03
Inter-day reproducibility			
9.44	9.29 ± 0.28	98.41	3.01
18.88	17.63 ± 0.57	93.38	3.23
75.50	79.45 ± 3.82	105.23	4.81
302.00	302.66 ± 6.23	100.22	2.06
1208.00	1188.39 ± 18.64	98.38	1.57

and compared with the retention times of puerarin using water–methanol as mobile phase [14–16], the present retention times were shorter.

3.3. Standard curve

The standard curve was prepared for puerarin in the range of 9.44–1208.00 ng ml^{-1}, which covered the levels following the administration of a single dose of 32 mg kg^{-1} puerarin. The standard curve was described by equations $y = 0.000305x - 2.741111$, $r = 0.9998$, where y is the peak area, x the concentration, and r the correlation coefficient. The LOQ in plasma was defined as the lowest concentration on the standard curve for which the assay precision was reflected by CV ≤ 10%, and it amounted to 9.44 ng ml^{-1}.

3.4. Precision and accuracy

The intra- and inter-day accuracies were estimated and the studied concentrations (9.44, 18.88, 37.75, 302.00 and 1208.00 ng ml^{-1}) were lower than 10%, as indicated by the respective values of CV (Table 2). This showed that the method is quite precise. Moreover, the small difference (≤10%) noted between added levels and the estimated concentrations have documented an appropriate accuracy of the elaborated method.

3.5. In vivo application of the method

In this study, the established method successfully quantified puerarin after intravenous administration of puerariae radix isoflavone, and the pharmacokinetic parameters of puerarin were listed in Table 3. Data showed that there were significant differences in pharmacokinetic parameters in cerebral ischemia reperfusion rats and normal rats, though plasma concentration–time course of puerarin in rats was best fitted to a two-compartment open model. The dynamic equations of puerarin in normal and cerebral ischemia reperfusion rats were $C = 0.1106e - 0.1255t + 0.0593e - 0.0465t$ and $C = 0.1001e - 0.0855t + 0.0501e - 0.0182t$, respectively. The peak plasma concentration of puerarin was reached immediately with the completion of the administration, and then it began to decline and was no longer detected after 4 h. In our study, we found that the plasma concentrations of puerarin in cerebral ischemia reperfusion rats were consistently higher at sampling time points (such as at 45 and 60 min) (Fig. 3) and CL of puerarin in cerebral ischemia reperfusion rats was significantly lower than in normal rats ($P < 0.05$) (Table 3). The reasons why the elimination rate of puerarin slowed down in cerebral ischemia reperfusion rats may be as follows. First, puerarin is partially hydrolyzed to aglycone in the body [18] and in the state of pathophysiology, a low activity of certain enzymes and a low ability of biomembral transfer induced by cerebral ischemia reperfusion damage might lead to the decreased clearance rate of puerarin. Second, puerarin is mainly excreted in the urine [18] and the decreased blood circulation of kidney induced by cerebral ischemia

Table 3
Pharmacokinetic parameters of puerarin in rat plasma after intravenous administration of puerariae radix isoflavone (at a dose containing 32 mg kg^{-1} puerarin)

Parameter	Estimate (mean ± S.E.M.)	
	Normal	Cerebral ischemia reperfusion
V_d (ml kg^{-1})	605.67 ± 104.96	599.91 ± 69.82
$T_{1/2\alpha}$ (min)	6.43 ± 2.46	7.26 ± 2.21
$T_{1/2\beta}$ (min)	18.97 ± 6.80	38.62 ± 5.07**
$AUC_{0-\infty}$ (ng min ml^{-1})	2773.2 ± 572.3	3748.6 ± 566.7**
CL (ml kg min^{-1})	40.18 ± 9.01	26.29 ± 5.06*
MRT (min)	20.62 ± 6.26	35.00 ± 6.65*

V_d, the volume of distribution; $AUC_{0-\infty}$, the area under curve concentration–time; $T_{1/2\alpha}$, distribution half-life time; $T_{1/2\beta}$, elimination half-life time; MRT, mean residence time. Compared with normal rats.

* $P < 0.05$.

** $P < 0.01$ ($n = 5$).

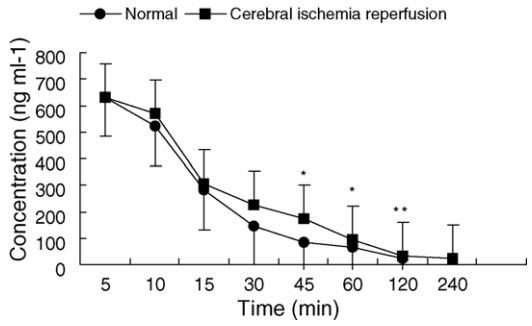

Fig. 3. Plasma concentration–time curve of puerarin after intravenous administration of puerariae radix isoflavone (at a dose containing 32 mg kg^{-1} puerarin). Each point and bar represent the mean ± S.E.M. Compared with normal rats, (*) $P < 0.05$, (**) $P < 0.01$ ($n = 5$).

reperfusion might play an important role in the decreased elimination rate of puerarin.

4. Conclusion

The designed procedure fulfilled the validation requirements and could be applied for in vivo studies. Additionally, the elimination rate of puerarin slowed down in cerebral ischemia reperfusion rats, which offered useful references in drug clinical application.

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