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Determination of puerarin in human plasma by high performance liquid chromatography

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Abstract

Puerarin, an isoflavone *C*-glycoside, has been identified as the major active component isolated from *Pueraria lobata* (Kudzu) responsible for suppression of alcohol drinking. In order to conduct clinical studies of Kudzu's efficacy, a method for measuring its bioavailability and pharmacokinetic profile is needed. We have developed a gradient reversed-phase HPLC system for pharmacokinetic study of puerarin in human plasma. Solid-phase extraction was performed on an abselut Nexus cartridge (60 mg/3 ml) possessing adsorbent function with a recovery of >97% and 4-hydroxybenzoic acid was used as an internal standard. The HPLC assay was performed on a YMC ODS-A column (150 mm × 4.6 mm i.d., 5 μm particle size). The HPLC mobile phase consisted of methanol/0.5% acetic acid with 20–35% methanol gradient at a flow-rate of 0.8 ml/min. The UV wavelength was set at 254 nm. Calibration of the overall analytical procedure gave a linear signal ($r > 0.999$) over a puerarin concentration range of 5–500 ng/ml in human plasma. The lower limit of quantification was ca. at 8 ng/ml of puerarin in plasma. The detection limit (defined as signal-to-noise ratio of about 3) was approximately 3 ng/ml. The preliminary pharmacokinetic study after oral administration of the Kudzu capsules containing 400 mg of puerarin to a healthy volunteer confirmed that the present method was suitable for determining puerarin in human plasma.

Keywords: Puerarin; Kudzu; Human plasma; Reversed-phase HPLC

1. Introduction

Alcohol abuse and alcoholism are serious public health and social problems worldwide. The development of a safe and effective pharmacotherapeutical agent for the treatment of alcohol abuse using traditional Chinese medicine has been our primary objective.

Pueraria lobata (Will.) Ohwi has been used traditionally in China for centuries to reduce alcohol "intoxication" [1]. It is the main ingredient of a Chinese herbal remedy called Xing-Jou-Ling (NPI-028). The crude extract of NPI-028 showed significant reduction of alcohol drinking in alcohol-preferring rats and monkeys [2]. Puerarain (Fig. 1) and several other isoflavones were isolated and identified as the responsible components in the kudzu extract [2–5]. We have demonstrated that the mechanism of anxiolytic effects of alcohol withdrawal for puerarin is as antagonist at the benzodiazepine receptors [5]. Because puerarin is an isoflavone *C*-glycoside, it is more resistant to metabolic deactivation and enzymatic hydrolysis than *O*-glycosides such as daidzin. Recently, Prasain et al. proved that a large amount of puerarin is absorbed without hydrolysis and only a limited amount of puerarin is conversed to its oxidative metabolites in rats [6]. The same authors also demonstrated that puerarin can cross the blood–brain barrier, as evidenced by its presence in the brain tissures of puerarin-treated rats [6]. The above findings provided strong support that puerarin works on the central nervous system.

In order to investigate the pharmacokinetic properties of puerarin in human, we need a highly sensitive and reliable analytical method for quantification of puerarin in human

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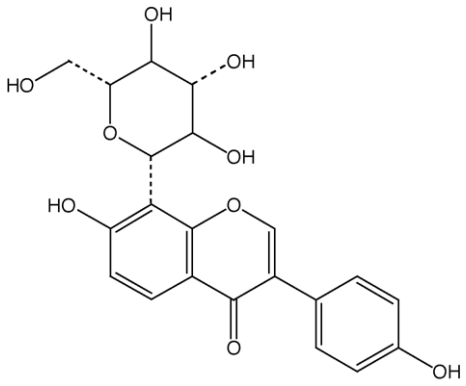

Fig. 1. Structure of puerarin.

plasma. Reversed-phase high performance liquid chromatography (HPLC) is typically the first choice because it has been widely used for the purification and separation of naturally occurring flavonoids [7–9]. HPLC analysis of puerarin in animal plasma by HPLC based on liquid–liquid extraction has been reported [10–13], but unfortunately, these methods could not provide satisfactory sensitivity in human study.

In this paper we report the development of a reversed-phase HPLC method in combination with solid phase extraction, which shows sufficient specificity, sensitivity and simplicity for the measurement of puerarin in human plasma.

2. Experimental

2.1. Chemical and reagents

Puerarin was obtained from Natural Pharmaceutical International, Inc. (>99%, Belmont, MA, USA). Kudzu capsules (500 mg/each, 20% puerarin) were provided by Natural Pharmaceutical International, Inc. (Belmont, MA, USA). The internal standard 4-hydroxybenzoic acid was purchased from Sigma (>99%, USA), and trichloroacetic acid was purchased from Aldrich (USA). Stock solutions of puerarin and 4-hydroxybenzoic acid were prepared by dissolving these compounds in methanol. All other chemicals and solvents were of analytical grade or HPLC grade without further purification.

2.2. Sample preparation

To each aliquot of 3.0 ml of plasma in a centrifuge tube was added different amounts of puerarin (15.4–1545 ng) and a fixed amount (312.0 ng) of 4-hydroxybenzoic acid as the internal standard (IS). 1 ml of trichloroacetic acid solution (1 g in 5 ml of water) was added to the above centrifuge tube, and the solution was mixed well and centrifuged at 5000 × *g* for 20 min. The supernatant was passed through an abselut Nexus cartridge (60 mg/3 ml, Varian, USA), which had previously been conditioned by washing with 3 ml of methanol and 3 ml of deionized distilled water. The cartridge was first washed with 1 ml of water, purged with air and then eluted with 5 ml of methanol. The methanol eluate was evaporated at 40 °C in vacuo. The residue was suspended in 1 ml of methanol, and the solution was filtered through a 0.45 μm NYL syringe filter (Whatman, Germany). The filtrate was transferred to a 2 ml vial and dried under nitrogen. The residue was dissolved in 50 μl of methanol, and 5.0 μl of the methanol solution was injected to HPLC for analysis.

2.3. HPLC apparatus, LC–ESI-MS apparatus and their conditions

HPLC analyses were performed on a Waters Breeze liquid chromatograph system equipped with a Waters 1525 binary pump and a Waters 2487 dual λ absorbance detector. An Agilent 1100 Series LC/MSD system (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, diode array and multiple wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI) and software of HP ChemStation, Bruker Daltonics 4.1 and DataAnalysis 4.1 software were used for LC–ESI-MS and LC/MS/MS. The HPLC system consisted of an YMC ODS-A column (4.6 mm × 150 mm i.d., 5 μm particle size) and a security guard cartridge (4.0 mm × 20 mm, i.d.) packed with the same material. A gradient mobile phase consisted of methanol (20–35%)/0.5% acetic acid (80–65%) was employed. The flow-rate was set at 0.8 ml/min and the UV detection wavelength was at 254 nm.

2.4. Recovery

The relative recovery of puerarin from human plasma was assessed at three concentrations, 10.3, 103.0, and 515.0 ng/ml. The plasma samples were filtered through abselut Nexus cartridges as described above. The peak-area ratios (puerarin to 4-hydroxybenzoic acid) of the HPLC chromatograms were compared with those of reference solutions to calculate the relative recoveries of puerarin.

2.5. Calibration

Standard samples were prepared by adding known amounts of puerarin (15.4, 30.9, 92.7, 309.0, and 1545.0 ng) to 3 ml blank plasma. To each standard was then added 312.0 ng of 4-hydroxybenzoic acid as the internal standard. The standard samples were prepared as shown in Section 2.2. Standard curves were established following the extraction and HPLC analyses of the spiked plasma samples. After determining the peak-area ratios (puerarin to 4-hydroxybenzoic acid) of the HPLC chromatograms, the calibration curve was established by least-squares linear fitting of the peak-area ratios of puerarin to the internal standard.

2.6. Accuracy

Accuracy was determined by assaying six preparations of 3.0 ml aliquots of human plasma containing three different

amounts (24.7, 247.2, and 1236.0 ng) of puerarin and a fixed amount (312.0 ng) of 4-hydroxybenzoic acid as the internal standard. After preparing the samples for HPLC as described above, the peak-area ratios were determined.

2.7. Stability in human plasma

Stability of puerarin in human plasma was examined at room temperature and at −20 °C. Puerarin (10.3, 103.0, and 515.0 ng/ml) was added to the blank plasma samples, and they were then left at room temperature for 7 days. Storage stability at −20 °C for 60 days was also determined.

2.8. Application of the assay and sample collection

After a single oral administration of four Kudzu capsules (containing 400 mg of puerarin) to a healthy volunteer, plasma samples were collected just before administration (0 h) and at 0.5, 1, 2, 3, 4, 6, 7, 8, and 11 h time points.

3. Results and discussion

The analytical method for pharmacokinetic study of flavonoids glycosides in human plasma and urine using a reversed phase HPLC with an ODS column has been reported in the literature [14–19]. The plasma clean-up procedure is a prerequisite for a successful HPLC-UV analysis, especially in the case of human plasma, which contains highly polar components. In previous studies, a Sep-Pak C18 cartridge and an anion exchanger cartridge were used for the clean up [14–19]. However, a Sep-Pak C18 cartridge, an abselut Nexus cartridge or a polyamide cartridge was less effective for analysis of puerarin in human plasma, especially at lower concentrations of puerarin, when the plasma samples were passed directly through these cartridges. Instead, after deproteinization of human plasma using 20% trichloroacetic acid solution (w/v), the supernatant was loaded to the cartridges. We found that the abselut Nexus cartridge (60 mg/3mL), rather than the Sep-Pak C18 cartridge or polyamide cartridge, could effectively eliminate the interfering materials in plasma and provide a quantitative extraction of puerarin in human plasma. The abselut Nexus cartridge was designed to extract a wide range of organic compounds from different materials using a non-conditioned solid phase extraction (NC-SPE) technique, as stated by the manufacturer. It is a highly cross-linked, spherical polymeric sorbent with a unique combination of hydrophilic and lipophilic moieties.

An appropriate internal standard is needed for accurate quantitative analysis of analytes in biological fluids. In this study, we chose 4-hydroxybenzoic acid as the internal standard. 4-Hydroxybenzoic acid is a stable phenolic compound, and it displayed a similar solubility to solvent extraction and adsorbent function to cartridge, compared with those of puerarin. Both compounds contain a *p*-hydroxybenzene ring system, and show maximum absorption at the wavelength of around 254 nm. Based on these observations, 4-hydroxybenzoic acid was employed as the internal standard for quantitative analysis of puerarin in human plasma.

Methanol was found to be the best solvent to elute puerarin from the abselut Nexus cartridge. For calculating the relative recoveries of puerarin from human plasma, the peak area ratios (puerarin to 4-hydroxybenzoic acid) of the HPLC chromatograms were compared with those of reference solutions. The recoveries of puerarin were 102.5% (10.3 ng/ml, $n = 3$), 97.3% (103.0 ng/ml, $n = 3$), and 99.3% (515.0 ng/ml, $n = 3$), respectively.

The HPLC conditions for analysis of puerarin were optimized according to two aspects, i.e., crude Kudzu extract and human plasma. The main constituents of crude Kudzu extract are puerarin, 3′-methoxypuerarin, daidzin and daidzein (Fig. 2A). Though the retention times of puerarin, daidzin and daidzein are significantly different, the retention time of 3′-methoxypuerarin is very close to that of puerarin. Previous reports showed the possibility that the peaks of puerarin and 3′-methoxypuerarin may partially overlap in the HPLC chromatograms [19–23]. It is essential to choose suitable HPLC conditions for the analysis of the Kudzu extract, so that 3′-methoxypuerarin can be completely separated from puerarin. In this study, we optimized the HPLC condition for the separation of puerarin and 3′-methoxypuerarin (Fig. 2B) by using a gradient solvent system consisted of methanol (20–35%)/0.5% acetic acid (80–65%). Furthermore, we found that the solvent system at a flow rate of 0.8 ml/min provided good chromatographic profiles of puerarin and 4-hydroxybenzoic acid (IS) for human plasma. Fig. 3 shows the HPLC chromatograms of extracts prepared from a blank plasma (A), a LOQ standard (B), and a patient plasma who received puerarin (C). Under the conditions described above, the retention times for puerarin and its internal standard were at 17.7 and 11.7 min, respectively. Comparison of the chromatograms shown in Fig. 3A and B, it is evident that under the above HPLC conditions there are no significant interferences to the puerarin peak by other endogenous components. Furthermore, the positive ion ESI mass spectrum (Fig. 4A) of the peak corresponding to puerarin on the HPLC chromatogram (Fig. 3C) showed an abundant ion peak at *m/z* 417, which was the proton adduct of puerarin (416 μm plus 1). The MS–MS analysis of the *m/z* 417 peak as precursor ion gave the major fragments of *m/z* 399, 381, 363, 351, 321, 297, and 267 (Fig. 4). The ion peaks are closely similar to those in the literature [24], and are consistent with those of our standard puerarin. Thus, the puerarin existence in patient plasma was definitively identified.

Calibration curves were established by using 3.0 ml aliquots of blank plasma spiked with different amounts of puerarin ranging from 15 to 1500 ng and 312.0 ng of 4-hydroxybenzoic acid as the internal standard. The peak area ratios of puerarin to the internal standard were calculated. A good correlation was found between the peak-area ratios

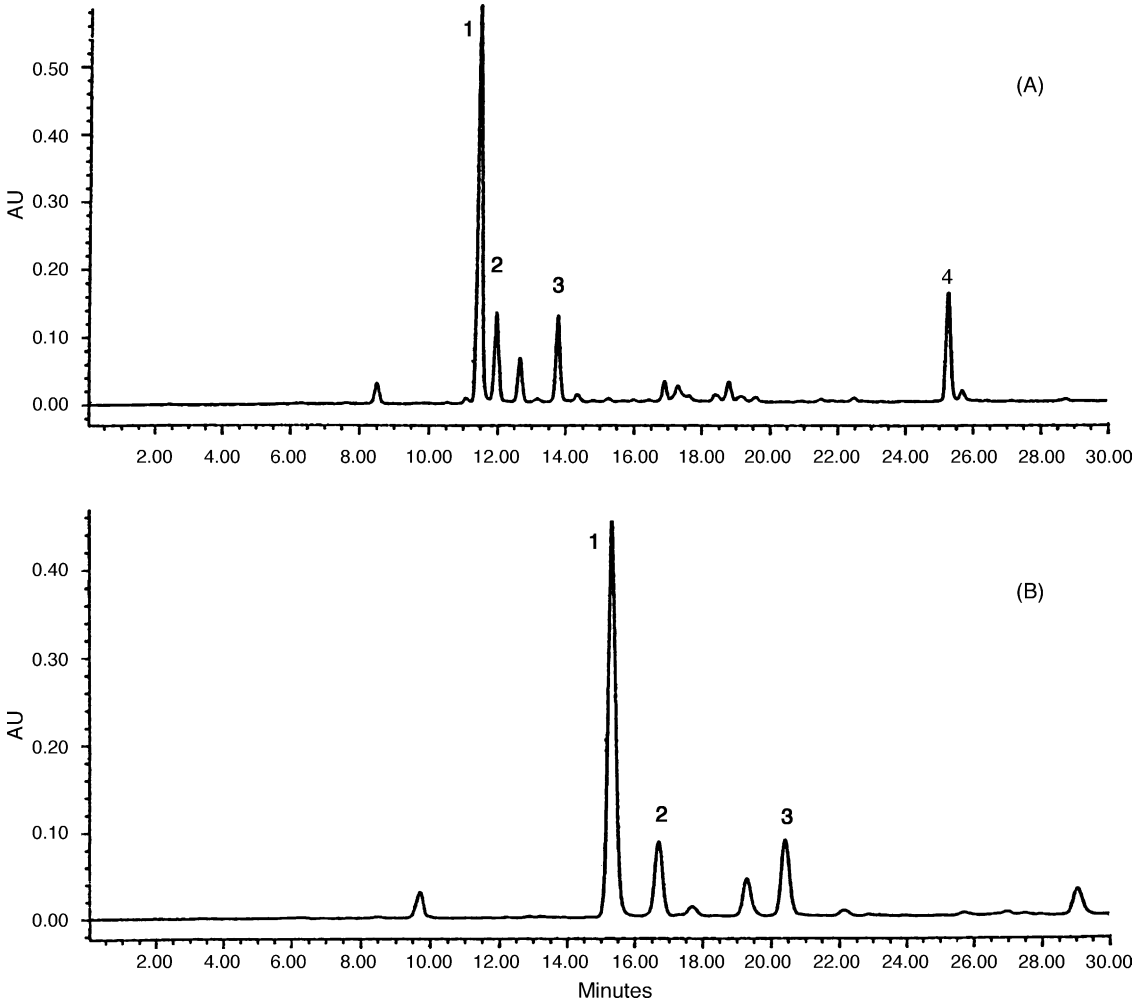

Fig. 2. HPLC chromatograms of Kudzu extract. (A) A gradient mobile phase consisted of methanol (20–65%)/0.5% acetic acid (80–35%), 0.8 ml/min. (B) A gradient mobile phase consisted of methanol (20–35%)/0.5% acetic acid (80–65%), 0.8 ml/min. (**1**) Puerarin, (**2**) 3′-methoxypuerarin, (**3**) daidzin, and (**4**) daidzein.

(y) and the concentrations (x) of puerarin (Table 1). A least-squares regression analysis gave a typical regression line of $y = 0.006147x + 0.01103$ ($r = 0.9999$) for puerarin.

The accuracy of measurement was determined by adding 24.7, 247.2, and 1236.0 ng of puerarin to each 3-ml aliquot of plasma samples with a fixed amount of 4-hydroxybenzoic acid (312.0 ng). Table 2 showed that the amounts for puerarin added for intra-day assay were in good agreement with the amounts of puerarin measured, and the relative errors being less than 7% for 8.24 ng/ml, 5% for 82.4 ng/ml, and 3% for 412.0 ng/ml. The intra-assay relative standard deviations (R.S.D.s) ($n = 6$) were less than 5% for 8.24 ng/ml, 2% for 82.4 ng/ml, and 3% for 412.0 ng/ml. The inter-assay RSDs were less than 5% for 8.24 ng/ml and 3% for 82.4 and 412.0 ng/ml (Table 3). The precision (R.S.D.s) and accuracy (R.E.) at three concentrations, 8.24 ng/ml at the near LOQ, 82.4 ng/ml in the midrange and 412.0 ng/ml at the high end of the range, were acceptable in view of the international recommendations [25]. The sensitivity of the presented HPLC assay (defined as a signal to noise ratio of about 3) was 3.0 ng/ml plasma for puerarin.

Table 1
Statistics on parameters of puerarin standard curves in plasma

Analysis group	Slope	Intercept	Correlation coefficient (r)
1	0.006055	0.01556	0.9999
2	0.006142	0.01217	1
3	0.006294	−0.00495	1
4	0.006171	0.00571	0.9999
5	0.006075	0.02665	0.9995
Mean	0.006147	0.01103	0.9999
S.D.	0.000095	0.01172	0.0002
R.S.D. (%)	1.5	–	–

Puerarin added to the plasma samples was found to be stable after 7 days stored at room temperature and 2 months stored at −20 °C.

As an example of the application of this method, the plasma concentrations of puerarin was analyzed from a healthy volunteer who orally received four Kudzu capsules (containing 400 mg of puerarin). The plasma samples were

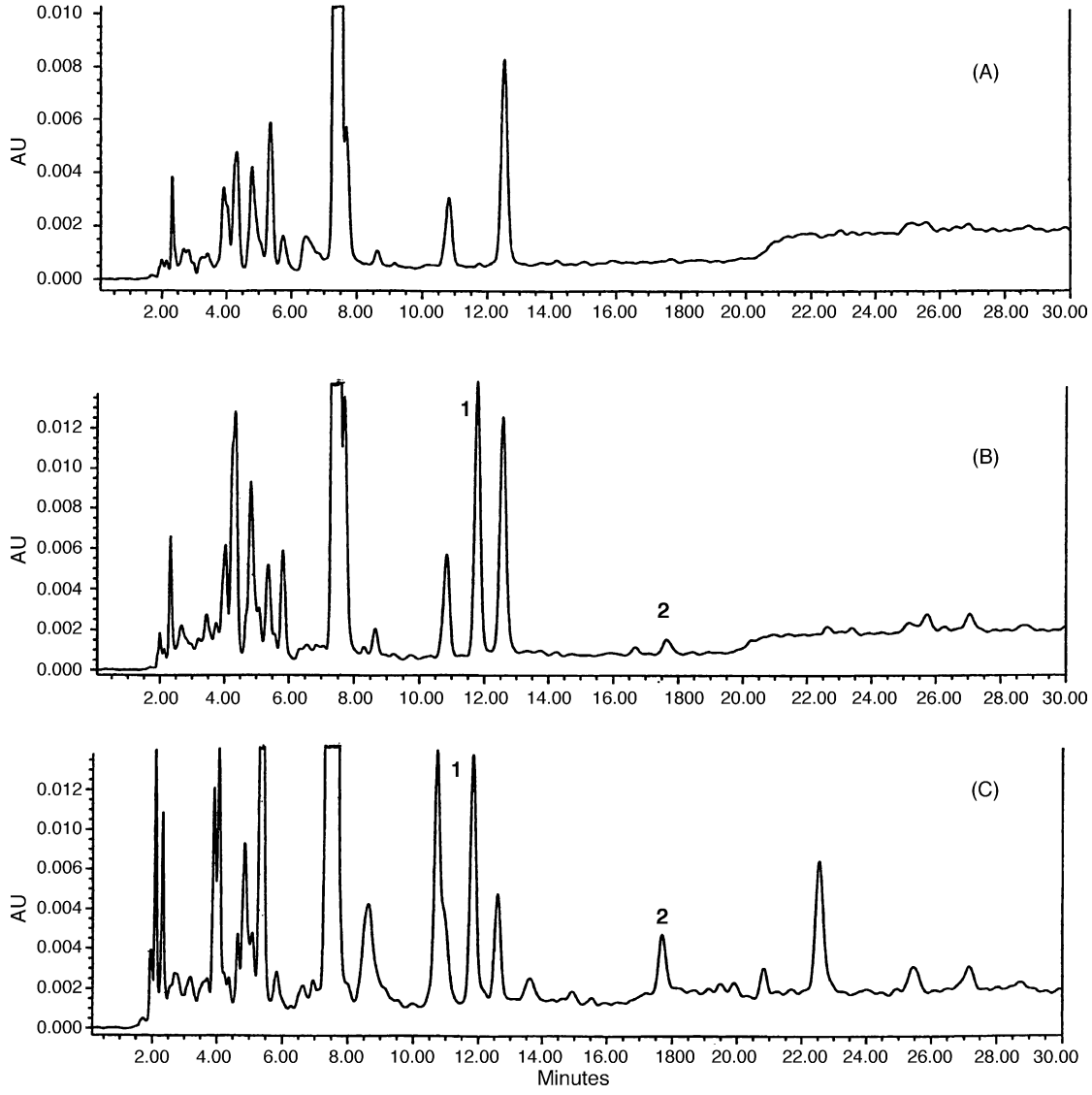

Fig. 3. HPLC chromatograms of extracts of (A) blank plasma (B) plasma spiked with puerarin (8.2 ng/ml) and 4-hydroxybenzoic acid (C) plasma of a patient after the oral administration of Kudzu capsules. (**1**) 4-Hydroxybenzoic acid (11.7 min), (**2**) puerarin (17.7 min).

Table 2
Accurary and precision of puerarin in human plasma during intra-day assay

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values						Mean ± S.D.		
8.24	9.16	8.94	8.67	8.41	9.11	8.32	8.77 ± 0.36	6.4	4.1
82.40	86.42	85.54	87.40	86.16	86.85	84.27	86.11 ± 1.10	4.5	1.3
412.0	403.10	411.96	434.98	424.24	420.55	432.63	421.24 ± 12.19	2.2	2.9

Table 3
Accurary and precision of puerarin in human plasma during inter-day assay

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values						Mean ± S.D.		
8.24	8.32	8.94	8.98	9.47	9.41	9.37	9.08 ± 0.44	10.2	4.8
82.40	84.27	81.11	85.99	82.59	84.65	86.26	84.15 ± 1.99	2.1	2.4
412.0	403.10	416.46	396.54	414.98	404.46	424.45	409.99 ± 10.34	−0.5	2.5

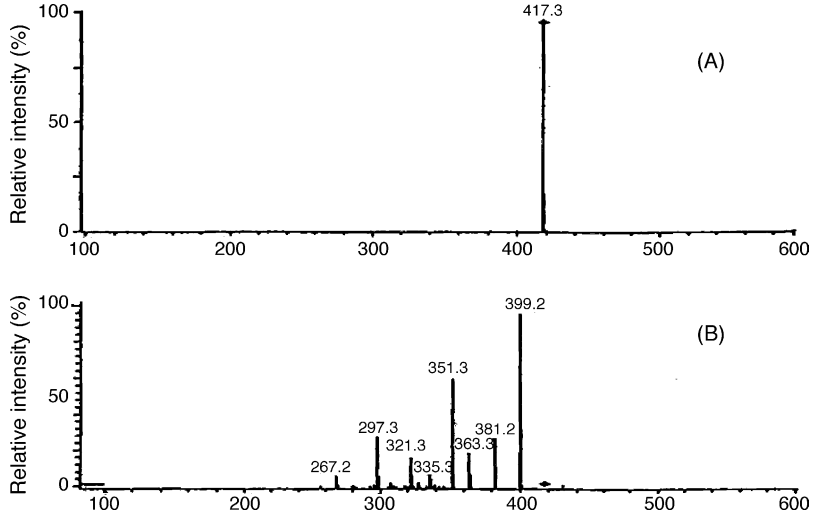

Fig. 4. Positive ion ESI mass spectra ((A) MS spectrum, (B) MS–MS spectrum) of puerarin extracted from a patient who received puerarin.

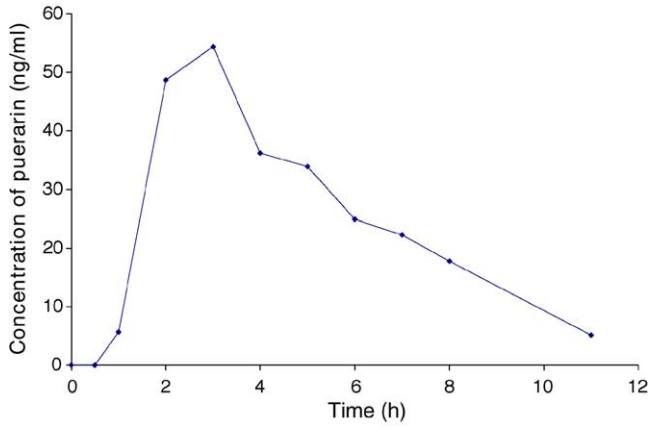

Fig. 5. Plasma concentration–time of puerarin after oral administration of puerarin (400 mg) to a healthy volunteer.

collected at different time points (0, 0.5, 1, 2, 3, 4, 6, 7, 8, and 11 h) after the oral administration. A time course of plasma concentrations of puerarin measured by HPLC is shown in Fig. 5. Puerarin could not be found in the plasma samples collected at 0 and 0.5 h time points. There was a sharp rise of plasma concentration of puerarin between the 1 and 2 h time points that peaked around 3 h. The puerarin concentrations gradually decreased thereafter.

In summary, the present method provides a sensitive and reliable HPLC method for the quantitative determination of puerarin in human plasma and is currently being employed in a pharmacokinetic study of puerarin in human.

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