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Quantification of puerarin in plasma by on-line solid-phase extraction column switching liquid chromatography–tandem mass spectrometry and its applications to a pharmacokinetic study

Qingqing Wang, Xiaoshuang Li, Shujia Dai, Lun Ou, Xiao Sun, Baozhen Zhu, Fang Chen, Mingmei Shang, Haifeng Song *

Laboratory of Drug Metabolism and Pharmacokinetic, Beijing Institute of Radiation Medicine, Beijing 100850, China

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Abstract

A highly precise, automatic and rapid method for quantification of puerarin in canine and human plasma using an on-line solid-phase extraction (SPE) column switching procedure combined with liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI-MS) was developed. The eluent of SPE column consisted of acetonitrile/methanol/0.1% formic acid (25/25/50) at a flow rate of 0.2 mL min^{-1}. Puerarin was analyzed by a linear ion trap mass spectrometer, LTQ-MS, operating in the negative ion and selective reaction monitoring (SRM) acquisition mode. Method validation results demonstrated that the linear calibration curve covered a wide range of 0.39–400.00 ng mL^{-1}, the correlation coefficients (r^2) were above 0.999. The lower limit of detection (LLOD) with the signal-to-noise (S/N) ratio higher than 12 was 0.39 ng mL^{-1}. The intra- and inter-batch precisions were less than 7.61% and 6.42%, respectively. The accuracy was well within the accept limit. The on-line SPE column switching HPLC–MS system was applied to pharmacokinetic (PK) study of puerarin after a single orally dose in beagles. And the optimum conditions were successfully utilized to quantify puerarin in human plasma, which indicated the feasibility and the reliability of this method for application in preclinical and clinical PK studies of isoflavone drugs.

Keywords: Puerarin; On-line solid-phase extraction column switching; HPLC–MS; Pharmacokinetic study

1. Introduction

Traditional Chinese herbs are a rich natural resource and have been extensively used to prevent and cure many diseases for thousands of years in China. *Pueraria* genus (Leguminosae family) is a rich source of isoflavonoids, in which *Pueraria lobata* (Wild.) Ohwi (Ye-Ge in Chinese) and *Pueraria thomsonii* Benth (Fen-Ge in Chinese), two of the most popular traditional herbs have been used in China for centuries. The major isoflavone in pueraria extracts is puerarin (Fig. 1, chemical name is 7,4-dihydroxyisoflavone-8-glucopyranoside) [1,2]. During the past decade, an increasing number of publications on the health beneficial effects of puerarin have published, such as anti-thrombotic, anti-allergic [3], anti-oxidant activity [4], etc. Others reported that puerarin could also be effective in the treatment of sudden deafness and could improve memory dysfunction [5]. In order to investigate the pharmacokinetic (PK) properties of puerarin, it was urgently needed to establish a simple and effective method for the quantitative analysis of puerarin in biomatrices.

At present, many analytical methods have been used to analyze puerarin, including reversed-phase high-performance liquid chromatography (RP-HPLC) [6–8], capillary electrophoresis (CE) [9], and ion chromatography (IC) [10]. Hyphenated techniques, especially LC–tandem MS [11–13] has been increasingly used to enhance the sensitivity and provide a wealthy information for on-line compositional and structural analysis. However, quantitative determination of purarin in biological samples at a low concentration level remained difficult because of the extremely complex interference in biomatrices.

Sample preparation is a crucial step in the analysis of biological samples. Analysis of puerarin in plasma based on liquid–liquid or solid-phase extraction, which was widely used

* Corresponding author. Tel.: +86 10 6693 0259; fax: +86 10 6821 4653.
E-mail address: songhf@nic.bmi.ac.cn (H. Song).

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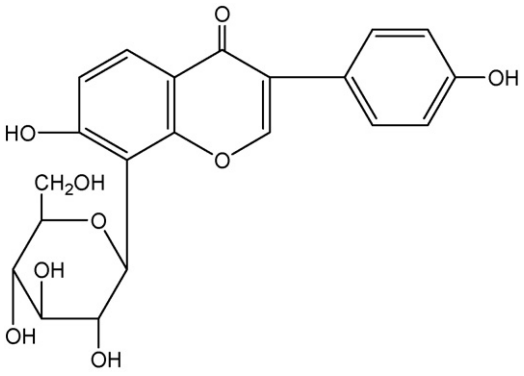

Fig. 1. The structure of puerarin.

to remove interfering biomatrix substances, has been reported [14,15]. Grace et al. [16] developed a simple sample preparation procedure required 200 μL of plasma sample and utilized one solid-phase extraction stage. When using isotope dilution LC–MS with SRM mode, limits of detection were in the region of 10 pg mL^{-1} for most analytes. In some cases, protein precipitation has been used in the first step of the extraction process. Prasain et al. [13] analyzed puerarin and its metabolites in plasma and urine sample after protein precipitation with acetonitrile without further extraction by LC–MS/MS method. But all of the published applications were based on off-line procedures, so when the number of biological samples is particularly large, such as in bioavailability and PK studies, manual procedures became relatively tedious, time-consuming, sample-consuming, and expensive due to frequent exchange of the SPE materials. Recently, many researchers [17–19] have paid attention to the on-line SPE procedures combined with LC–MS analysis owing to its higher sensitivity and selectivity. Moreover, instrumentation is now available that will perform conditioning, sample application and clean-up on one SPE cartridge, while chromatographic analysis of a previous sample is running from another SPE cartridge at the same time ("concurrent SPE"), thus significantly saving time.

In this paper, we developed a novel on-line SPE column switching HPLC–MS method for the analysis of puerarin in canine and human plasma. An automated column exchange SPE system coupled to HPLC–MS was optimized. Parameters for SPE column switching system (type of the sorbents and percentage of organic modifier) and MS (ionization method and source parameters and ion optics) were optimized for selectivity and sensitivity. The resulting method was validated for linearity, accuracy, precision, lower limit of detection (LLOD), stability and flexibility. Furthermore, the method was applied to the PK study of puerarin in beagles following oral administration of puerarin–phospholipid complex capsules, in which, the phospholipids were utilized to enhance the absorption of puerarin in the alimentary canal. Finally, this method was also successfully applied to quantify puerarin in human plasma samples. These results showed that the present method provided a sensitive, reliable and high throughput on-line SPE column switching HPLC–MS method for the quantitative determination of prerarin in canine and human plasma and it will become a potent method for preclinical and clinical PK studies.

2. Experimental

2.1. Chemical and reagents

Puerarin–phospholipid complex capsule was produced by PEARL Pharmaceuticals, Inc. (Shenzhen, China). Drug-free canine plasma was collected from six healthy canines of both sexes. Human plasma was prepared from outdated transfusion blood obtained from the North Taiping Rd. Hospital (Beijing, China). Methanol, acetonitrile and other solvents were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid was obtained from Beijing Chemical Co. (Beijing, China). Ultrapure water was produced by a Millipore Simplicity 185 unit (Bedford, MA, USA). All other chemicals and solvents were of analytical grade or HPLC grade without further purification.

2.2. Stock solutions and calibration standard samples preparation

The stock solution of puerarin (0.20 mg mL^{-1}) was prepared in water. The drug-free plasma from the canines was used as the standard dilution. The stock solution was first diluted to the concentration of 20.00 μg mL^{-1} with the mobile phase of analysis column. Working stock solutions were prepared at concentrations of 3.90, 15.60, 62.50, 250.00, 1000.00, 4000.00 ng mL^{-1} by spiking aliquots of the above stock solution with the mobile phase of analysis column. Plasma calibration standard solutions at concentrations of 0.39, 1.56, 6.25, 25.00, 100.00 and 400.00 ng mL^{-1} were got by adding 10 μL of working stock solutions into 90 μL of standard dilution. The preparation procedures of plasma QC samples at concentrations of 0.39, 25.00 and 400.00 ng mL^{-1} were as same as that of plasma calibration standard solutions. All of the standard stock, working stock, working solutions and standard dilution were stored at −20 °C until use. Before the assay, the frozen samples were thawed at ambient temperature, vortex mixed and centrifuged at 10 000 × g for 10 min. For each sample, 100 μL aliquots of the supernatant were transferred to LC vials and 40 μL aliquots of analyte were then introduced onto the SPE column for on-line preparation and LC–MS analysis.

2.3. Extraction procedure and chromatography for on-line analysis

A volume of 40 μL of plasma sample was injected for on-line SPE column switching HPLC–MS analysis. In the sample pretreatment step, the valve stayed in position 1 (Fig. 2(A)) for 1.5 min. The analyte was retained on the extraction column with the loading solvent (0.1% formic acid/acetonitrile, 95:5 at 1 mL min^{-1}) delivered by an Agilent 1100 Quatpump (Agilent Technologies, Waldbronn, Germany) through the sample loop onto the 50 mm × 2.1 mm i.d. SPE extraction column (2 μm frits, Alltech Associates, Inc., Shang-

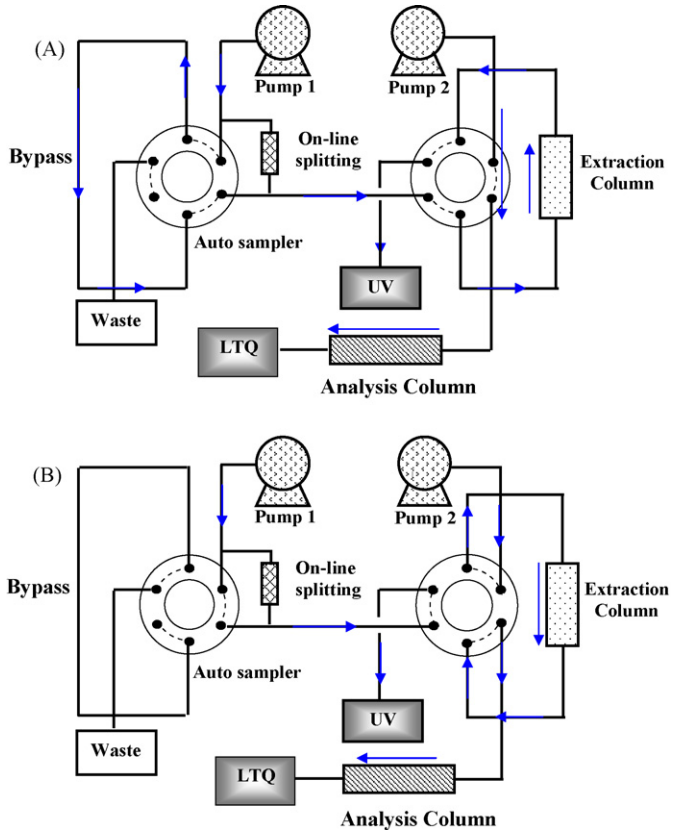

Fig. 2. On-line extraction column-switching scheme. (A) After injection, sample was loaded onto the SPE column. (B) Sample was eluted from the SPE column in back-flush mode and carried onto the analytical column for quantification.

hai, China) packed with reversed phase materials based on octadecyl-bonded (Zorbax C_{18}, spherical, average particle size 50 mm, pore size 80 Å, Agilent Technologies, Waldbronn, Germany). This course was monitoring with UV detection at 254 nm.

After 1.5 min the valve switched to position 2 (Fig. 2(B)), and the puerarin was back-flushed onto the analytical column (Grace Vydac C_8 column, 5 μm, 10 mm × 2.1 mm i.d., Hesperia, CA, USA) for separation and quantification. The mobile phase was acetonitrile/methanol/0.1% formic acid (25/25/50) at a flow rate of 0.2 mL min^{-1} directed by an Agilent 1100 Binpump (Agilent Technologies, Waldbronn, Germany). During sample analysis, the Quatpump delivered the solvent with a linear gradient of 0.1% formic acid from 95% to 2%, and acetonitrile from 5% to 98% at a flow rate of 0.2 mL min^{-1}.

Finally, the switching valve shifted to position 1 (Fig. 2(A)) again at 3.5 min disconnected the extraction column and analytical column. The extraction column was washed with 0.1% formic acid/acetonitrile (2:98) at a flow rate of 1 mL min^{-1} from 3.5 to 5.0 min after sample injection. From 5.0 to 6.5 min, the extraction column was equilibrated again with 0.1% formic acid/acetonitrile (95:5) at a flow rate of 1 mL min^{-1}. The whole time schedule of on-line SPE switching LC–MS system was listed in Table 1.

2.4. Mass spectrometric conditions

The HPLC–MS system comprised the binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, ThermoFinnigan, San Jose, CA, USA). LTQ equipped with an atmospheric pressure ionization interface operating in ESI mode. Data were processed using LCQuan software (version 2.0). Computer was controlled by Xcalibur 1.4 software. The operational parameters of the mass spectrometer were as shown below. The spray voltage was 4.5 kV and the temperature of the heated capillary was set at 200 °C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set (in arbitrary units min^{-1}) to 20, 5, and 4, respectively. Capillary voltage was −18 V, tube lens was −80 V, split lens was 11 V, and the front lens was 6.25 V. All parameters were optimized by infusing the analyte in mobile phase (acetonitrile/methanol/0.1% formic acid (25/25/50)) at a flow rate of 5 μL min^{-1}. The SRM detection setup for the peurarin was m/z 415.3@41 → 295.2.

2.5. Method validation

2.5.1. Specificity

The interference of endogenous compounds in plasma was assessed by analyzing standard puerarin, drug-free plasma, plasma spiked with puerarin, and plasma samples obtained from subjects given puerarin–phospholipid complex capsule. All peaks showing mass spectrogram of puerarin were analyzed with MS.

2.5.2. Linearity

The linearity of the calibration curve for purarin was assessed by analyzing six standard solutions by triplicate in the range of

Table 1
Time schedule of on-line SPE switching LC–MS system

Time (min)	Valve position	Pump 1		Pump 2	
		ACN (%)	Flow rate (mL min^{-1})	ACN/MeOH (%)	Flow rate (mL min^{-1})
0	A	5	1	50	0.2
1.5		5	1		
1.7	B	5	0.2	50	0.2
3.4		98	0.2		
3.5	A	98	1	50	0.2
5.0		98	1		
5.1		5	1		
6.5		5	1		

0.39–400.00 ng mL^{-1} in plasma. Peak area was plotted against plasma concentrations. The straight-line regression equation was weighted (weighting factor: 1/concentration^2) and presented with its correlation coefficient.

2.5.3. Precision and accuracy

In order to assess the intra- and inter-batch precision and accuracy of the assay, QC samples were prepared at concentrations of 0.39, 25.00, and 400.00 ng mL^{-1}. The intra-batch precision of the assay was assessed by calculating the coefficients of variation (CV) for the analysis of QC samples in five replicates, and inter-batch precision was determined by the analysis of QC samples on five consecutive days. Accuracy was assessed by calculating the relative error (RE), which compared the calculated and known concentrations.

The evaluation of precision was based on the criteria that CV for each concentration level should not be more than ±15% except for LLOQ, for which it should not exceed ±20%. Similarly, for accuracy, the mean value should not deviate by ±15% of the nominal concentration except for the LLOQ, where the limit was ±20%.

2.5.4. Sensitivity

The LLOQ was defined as the lowest non-zero calibration samples yielding a precision with less than 20% CV and RE with less than 20% of the theoretical value.

2.5.5. Stability

The short-term stability of puerarin in plasma was determined by assessing replicate QC samples at the concentrations of 0.39, 25.00, and 400.00 ng mL^{-1}, which were kept at room temperature (25 °C) for 24 h. Freeze–thaw stability was studied after three cycles (−20 °C/room temperature) to the QC samples on three consecutive days. The long-term stability was done by assessing QC samples stored at −20 °C for 1 month.

2.6. Application of the assay and sample collection

Specific-pathogen-free male Beagle canines were provided by Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). On the day of the study, the canines were orally administrated with puerarin–phospholipid complex capsules (containing 220 mg kg^{-1} of puerarin). Blood samples were collected at 0 min (pre-dose), 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12 h after dosing. The blood was then transferred into heparinized microfuge tube and centrifuged at 10 000 × g for 10 min. The supernatant plasma was transferred into test tubes and frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

3.1.1. General consideration

Non-selective SPE, typically alkyl-bonded silica or copolymer sorbents was widely used for analyte extraction and enrichment from aqueous samples, primarily in pharmaceutical and biomedical analysis [19]. In most literatures [20], when extracting puerarin, the sorbents was C_{18}-bonded silica and the sample solution and solvents were slightly acidified to prevent ionization of the puerarin.

During the method development, protein precipitation was firstly tested according to Ren et al. [21] using perchloric acid. But the results were not as good as expected. Peak shape of puerarin was poor and the recoveries of plasma samples were low. These phenomena might be owing to two reasons. First, after precipitation, the pH of the supernatant was not fitted to load puerarin onto the C_{18} sorbents, and further, the ClO_4^- probably prevented the ionization of puerarin even though in very low concentration. Finally, the epactal ions were not introduced into the plasma before SPE step, the types of SPE sorbents, ratios and pH of the mobile phase, and MS conditions were optimized in order to obtain both the good extraction on SPE column and good ionization on ESI-MS.

3.1.2. The choose of the SPE sorbents

An Agilent Zorbax C_{18} spherical SPE stationary phase was finally chosen for sample on-line preparation purposes in this study. Agilent AcutBOND^{II} C_{18} and Waters Sep-Vac C_{18} SPE stationary phase were also considered, but did not show the desired extraction behaviors. Independent of pH value for sample loading and washing, recovery were low with the latter two stationary phases. Furthermore, instrument failures resulted from pressure build-up, probably caused by the irregularity of latter two sorbents. The Agilent Zorbax C_{18} spherical SPE stationary phase could effectively eliminate the interfering materials in plasma and provide a quantitative extraction of puerarin in canine plasma.

3.1.3. HPLC condition

The HPLC conditions for analysis of puerarin were optimized according to two aspects, i.e., the elution capability on the SPE column and separation capability on the analytical col-

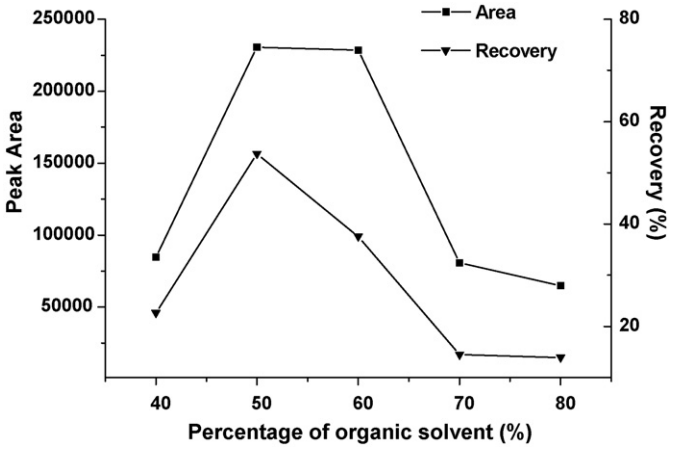

Fig. 3. The influence of different percentage organic solvent on the area and recovery of puerarin. Recovery was defined as the percentage value of the peak area ratio of puerarin in beagle plasma, compared to the value for water solution containing the same concentrations of puerarin.

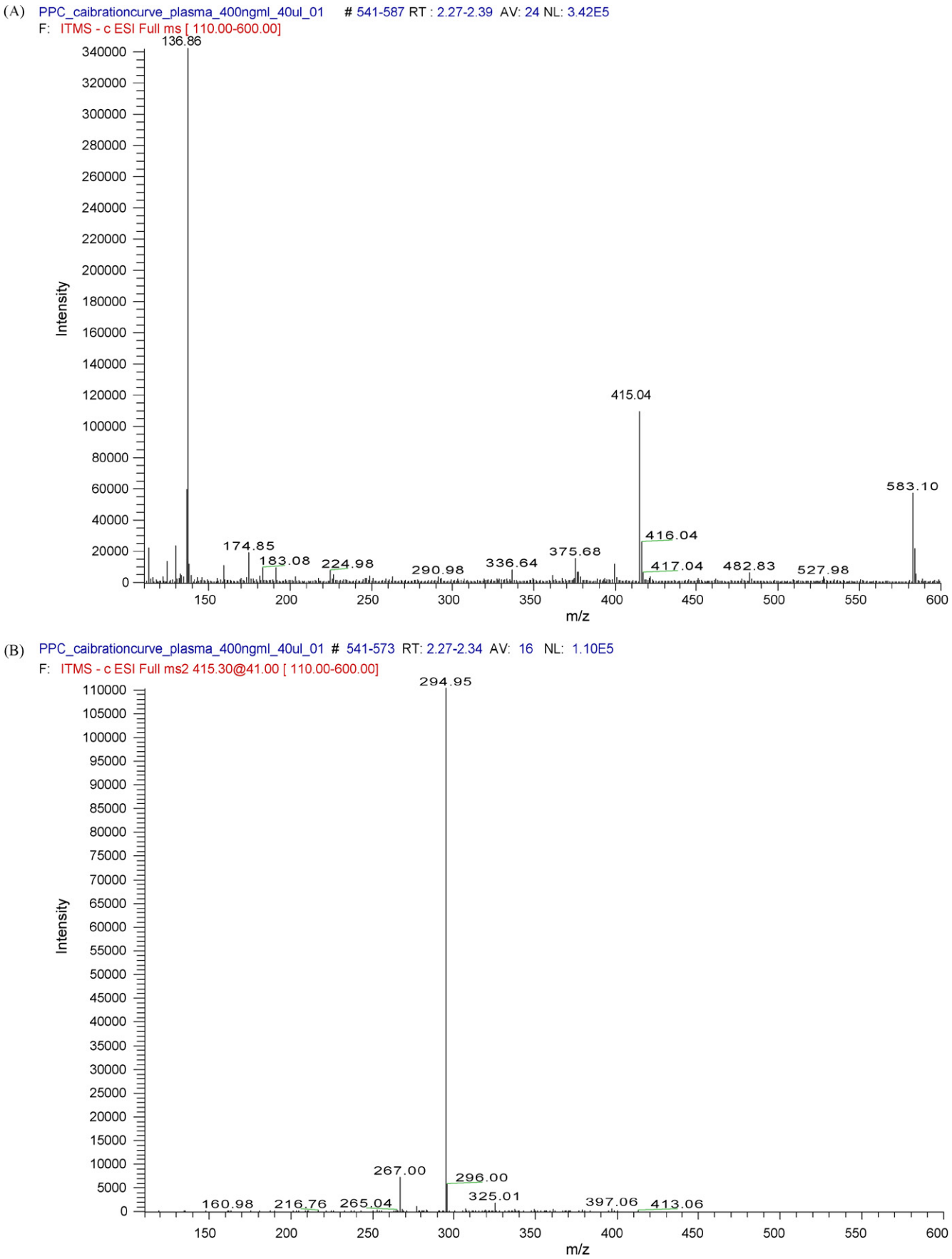

Fig. 4. The negative ion electrospray full scan mass spectra (A) and product ion spectra (B) of puerarin at 400 ng mL^{-1}.

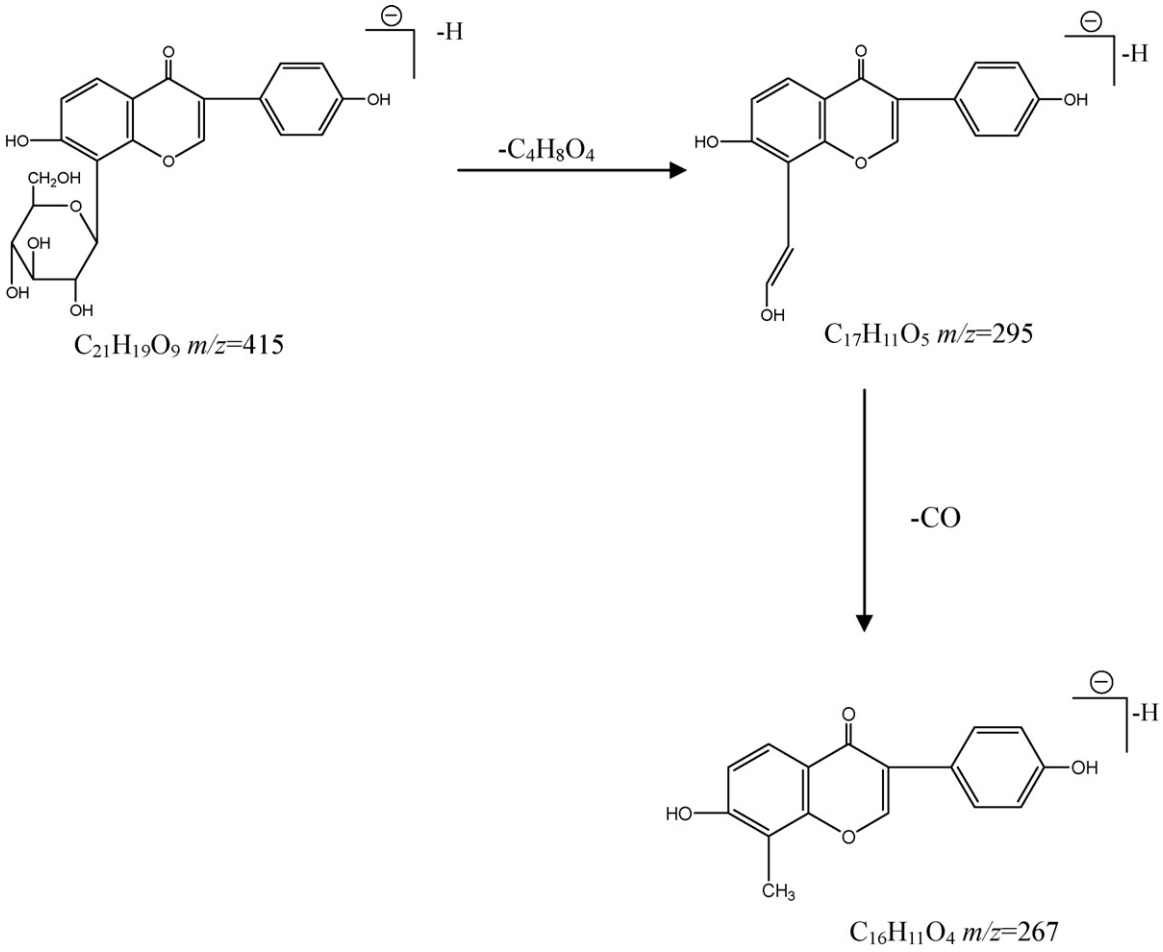

Fig. 5. Fragmentation schedule of puerarin in negative mode.

umn. Methanol/acetonitrile (50:50, v/v) was found to be the best solvent to elute puerarin from the Agilent Zorbax C_{18} spherical SPE sorbents. In order to obtain optimum HPLC conditions we compared different percentage of organic modifier. When the percentage of methanol/acetonitrile was 50% in the mobile phase, the peak shape and recovery of analyte showed good results (Fig. 3). Furthermore, we found that the solvent system at a flow rate of 0.2 mL min^{-1} provided good chromatographic profiles of puerarin for canine plasma.

3.1.4. MS conditions

Under above-HPLC conditions, standard solution (400 ng mL^{-1}) was introduced into mass spectrometer using syringe pump at a flow rate of 5 μL min^{-1}. Because puerarin is an acidic compound, negative ionization mode was preferred. ESI parameters such as capillary voltage, cone voltage and nebulizer gas were optimized to obtain strong signal of [*M*–H] at *m/z* 415. High specific SRM scan was used, and the most strong fragment ion at *m/z* 295 was selected as the product ion used in SRM acquisition (Fig. 4). Ion *m/z* 295 corresponding to $C_{17}H_{11}O_5^-$, which resulted from $[M–H]^-$ by the lost of $C_4H_8O_4$ group of 6-*C*-glycosyl. This fragment ion subsequently lost CO yielded ion at *m/z* 267 (Fig. 5). Collision energy and helium gas flow were determined when observing maximum response of the product ion.

3.2. Method validation

3.2.1. Specifity

The resulting chromatograms were essentially free from endogenous interferences (Fig. 6), which included a blank plasma sample, a plasma sample spiked with puerarin 25.00 ng mL^{-1} and a plasma sample obtained 4 h after orally dose of puerarin–phospholipid complex capsule containing 220 mg kg^{-1} of puerarin. Retention times were approximately 2.3 min for puerarin, and the peak shape was satisfactory and suitable.

3.2.2. Linear range and limit of quantification

The calibration curve of puerarin in canine plasma was generated automatically by LCQuan software. The linear dynamic range evaluated was between 0.39 and 400.00 ng mL^{-1}. The linear regression equation was $y = 4853.89x + 1073.21$ ($r^2 = 0.9994$). The lower limit of quantification (LLOQ) of puerarin in plasma was 0.39 ng mL^{-1}.

3.2.3. Accuracy and precision

Accuracy and precision were determined by five replicates of intra-batch and five inter-batch canine plasma samples containing puerarin at the QC levels. The results showed (Table 2)

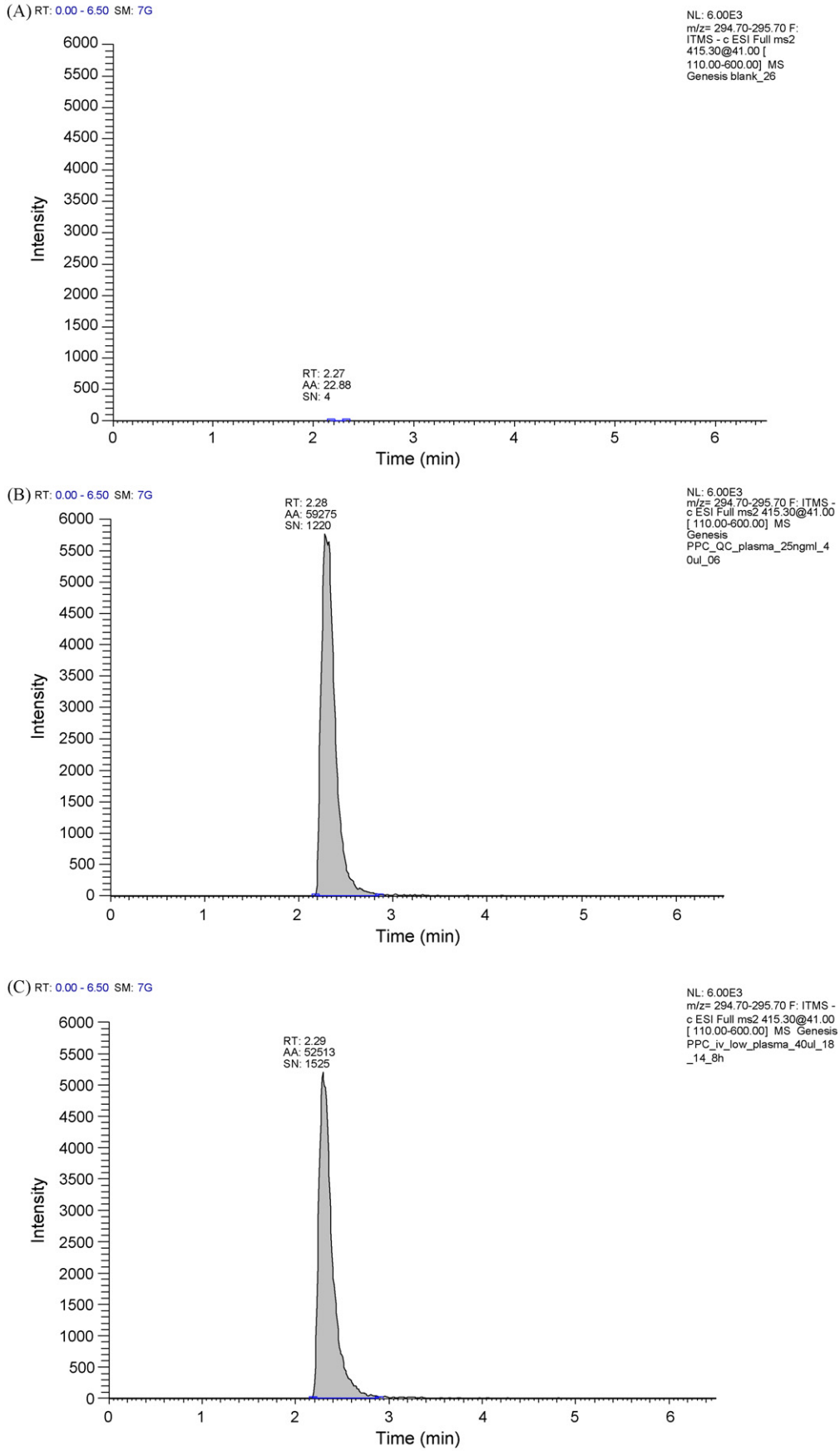

Fig. 6. Representative chromatograms of extracted blank plasma (A), plasma spiked with 25 ng mL^{-1} of puerarin (B), and test plasma at 8 h after the dose of 220 mg kg^{-1} (op) (C).

Table 2
Results of precision and accuracy for puerarin in beagle plasma ($n = 5$)

	Concentration (ng mL^{-1})		
	0.39	25.00	400.00
Intra-batch ($n = 5$)			
Mean ± S.D. (ng mL^{-1})	0.37 ± 0.03	23.90 ± 1.48	381.25 ± 10.03
CV (%)	7.61	6.13	2.49
RE (%)	−1.54	−0.16	−1.21
Inter-batch ($n = 5$)			
Mean ± S.D. (ng mL^{-1})	0.40 ± 0.03	25.80 ± 0.50	363.42 ± 18.10
CV (%)	6.42	1.88	5.02
RE (%)	−0.51	1.20	−2.31

S.D.: standard deviation; CV: coefficients of variation; RE: relative error, RE (%) = 100 × ((mean concentration − nominal concentration)/nominal concentration).

that intra- and inter-batch CV ranged from 1.88% to 7.61% and accuracy ranged from −2.31% to 1.20%. These results indicated that the method was accurate, reliable and reproducible.

3.2.4. Stability in canine plasma

Puerarin was found to be stable after three freeze–thaw cycles in plasma. It was also proved that puerarin could be stable in the canine plasma at room temperature for at least 24 h and at −20 °C for 1 month. Table 3 listed the accuracy for puerarin at each concentration, and ranged from −13.72% to 6.30%. So, it is expected that the present method would be applicable to the pharmacokinetic study of puerarin in canine.

3.3. Application to the analysis of pharmacokinetic study of puerarin in beagles

As an example of the application of this method, the concentrations of puerarin in plasma samples from six beagles which orally received puerarin–phospholipid complex capsules (containing 220 mg kg^{-1} of puerarin) were analyzed. A time course of plasma concentrations of puerarin measured by on-line SPE column switching HPLC–MS system was shown in Fig. 7. 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 provided a sensitive and reliable HPLC–MS method for the quantitative determination of puerarin in beagles' plasma and was currently being employed in a pharmacokinetic study of puerarin in canine.

Table 3
Results of stability for puerarin in beagle plasma ($n = 5$)

Concentration (ng mL^{-1})	RE (%)		
	Short-term	Freeze–thaw	Long-term
0.39	2.98	3.02	6.30
25.00	−0.78	−4.32	−13.72
400.00	−2.04	−7.27	−7.94

The short-term stability of puerarin in plasma was determined by keeping the samples at room temperature (25 °C) for 24 h. Freeze–thaw stability was studied after three cycles (−20 °C/room temperature) on three consecutive days. The long-term stability of pureranin in plasma was determined by storing samples stored at −20 °C for 1 month.

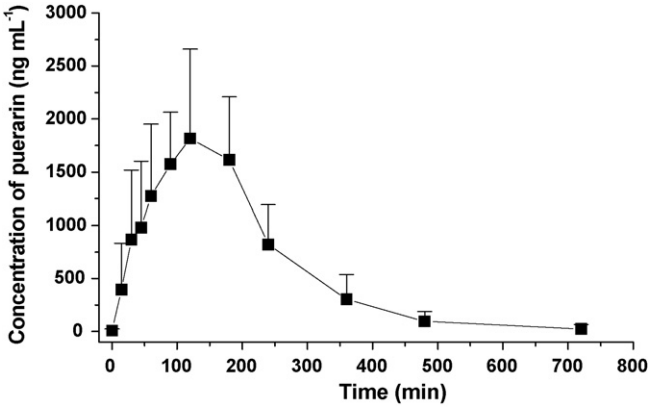

Fig. 7. The plasma concentration (±S.D.)–time profile of puerarin following oral administration at 220 mg kg^{-1} in beagles ($n = 6$).

3.4. Flexibility of the on-line SPE switching column HPLC–MS system

The flexibility of the on-line switching column HPLC–MS system was very important in respect of entire PK study and therapeutic drug monitoring (TDM). We studied the quantitative capability of our SPE HPLC–MS system on puerarin in human plasma. Results showed that the method could successfully apply to the analysis of human plasma samples without any change. The representative calibration curve was $y = 1260.31x + 4816.92$ ($r^2 = 0.9997$). It indicated that the on-line switching column HPLC–MS system we developed could be easily applied to clinical PK study.

4. Conclusion

In this paper we have developed a novel automatic on-line SPE column switching HPLC–tandem MS system. This system involved direct plasma injection into a liquid chromatograph and column switching, which was automated by software control and required only 6.5 min for a single analysis. The detailed procedures of the system were optimized, including the types of SPE sorbents, ratios and pH of the mobile phase and LTQ-MS conditions. The method behaved good characteristics of specificity, linearity, sensitivity and precision, which allowed for numerous samples to be processed in PK studies of puerarin

in canine plasma. Furthermore, this method was successfully applied to quantification of puerarin in human plasma. Thus, it should be a powerful method for preclinical and clinical PK study of puerarin and other isoflavonoids drugs.

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