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Development and validation of a sensitive liquid chromatography–tandem mass spectrometry method for the determination of paeoniflorin in rat brain and its application to pharmacokinetic study

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Abstract

A sensitive and specific method was developed and validated for the determination of paeoniflorin in rat brain with liquid chromatography–tandem mass spectrometry. Sample pretreatment involved protein precipitation following solid-phase extraction. Paeoniflorin and geniposide (internal standard) were separated isocratically on a Waters Symmetry C18 column (150 mm × 2.1 mm i.d., 5 μm), using a mobile phase of methanol/water with 0.1% formic acid (50:50, v/v) at a flow-rate of 200–300 μL/min in 4 min. A Finngan LTQ tandem mass spectrometer equipped with electrospray ionization source was operated in the positive ion mode. Selective reaction monitoring was performed to quantify paeoniflorin and the internal standard at *m/z* transitions of 503 → 381 and 411 → 231, respectively. A good linearity was found in the range of 2–500 ng/mL ($R^2 = 0.9939$). The intra- and inter-batch assay precisions (coefficient of variation, CV) at 5, 50 and 400 ng/mL ($n = 5$) ranged from 6.3% to 9.7% and 1.2% to 7.2%, respectively, and the accuracies were from 95.9% to 101.6% and 99.4% to 102.9%, respectively. The mean recoveries of paeoniflorin were 81.2%, 80.9% and 82.3% at 5, 50 and 400 ng/mL ($n = 5$), respectively, and the mean recovery of the internal standard was 76.7% with a concentration of 50 ng/mL ($n = 5$). Stability studies showed that paeoniflorin was stable in different conditions. Finally, the method was successfully applied to the pharmacokinetic study of paeoniflorin in rat brain following a single subcutaneous administration (10 mg/kg) to rats.

Keywords: LC–MS/MS; Paeoniflorin; Brain distribution; Matrix effects; Pharmacokinetics

1. Introduction

Paeoniflorin (PF) is a monoterpene glucoside (Fig. 1) isolated from the root of *Paeony Radix*, one of the traditional Chinese medicines [1]. It has been reported that PF has many clinical indications such as anti-inflammatory, anti-allergic [2], anti-hyperglycemic [3], anti-thrombosis [4], neuromuscular blocking [5–9], releasing of noradrenaline [10], enhancing glucose uptake [11], cognition-enhancing [12–18], and the neuroprotective [19,20] effects.

Liu et al. [19,20] reported, that *in vivo* subcutaneous (s.c.) administration of PF (2.5 and 5 mg/kg) at 5 min and 6 h after ischemic produced a dose-dependent decrease in both neurological impairment and the histologically measured brain infarction volume in both transient and permanent rat ischemia models [19,20], which indicated its potential as an anti-stroke agent. This neuroprotective effect of PF during cerebral ischemia is related to the activation of adenosine A_1 receptors [19].

The determination of PF in different biological matrices like serum [23,24], urine [25], plasma [26], and hippocampus [21,22] have been well established by using high performance liquid chromatography with ultra-violet spectroscopy detection (HPLC/UV). Although these methods present adequate linearity, precision and recovery, they show a series of limitations including lack of sensitivity which results in the lower limit of quantification (LLOQ) ranged from 10 ng/mL to 3.8 μg/mL

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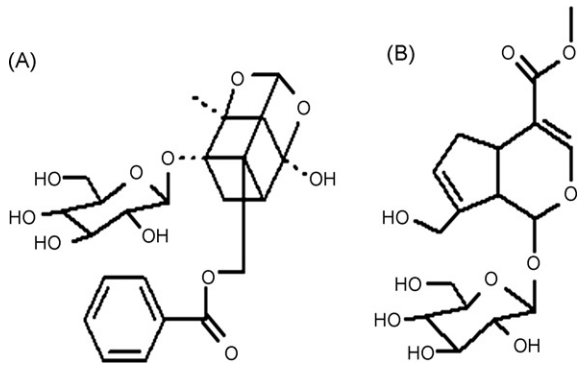

Fig. 1. Chemical structure of PF (A) and geniposide (IS) (B).

and long chromatographic times (≥15 min). Wang et al. [27] reported a specific and rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) (triple-quadrupole) method for the quantitative determination of PF in rat plasma. But to our knowledge, there were only HPLC/UV methods described for quantification in solid matrices (hippocampus) with a LLOQ at 1 μg/mL, and this may not meet the requirements for tissue distribution studies if administered with a relatively low dose of PF.

This paper details a rapid (chromatographic time: 4 min), sensitive (LLOQ = 2 ng/mL) and reliable LC–MS/MS (ion trap) method for the determination of PF in rat brain using protein precipitation with solid-phase extraction (SPE) for sample preparation, which was more sensitive compared with existing methods. This method was successfully applied to determine the brain concentration of PF following a single s.c. administration (10 mg/kg) in rats.

2. Experimental

2.1. LC–MS/MS analysis

2.1.1. Materials and reagents

PF and geniposide (internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was from Tedia (Fairfield, OH, USA). Ultrapure water was obtained from a Millipore (Milford, MA, USA) MilliQ apparatus.

2.1.2. Instrumentation

The HPLC–MS system consists of a Surveyor MS pump, a Surveyor auto-sampler and a Thermo Finnigan LTQ plus ion trap mass spectrometer equipped with electrospray ionization (ESI) source (ThermoElectron, San Jose, CA, USA). Xcalibur 1.4 software was used for data acquisition and analysis (ThermoElectron, San Jose, CA, USA). The data processing was carried out using Thermo Finnigan LCQquan 2.0 data analysis program.

2.1.3. LC–MS/MS conditions

The LC separation was carried out on a Symmetry C18 column (150 mm × 2.1 mm i.d., 5 μm; Waters, Milford, MA, USA) with an Alltima RP18 guard column (7.5 mm × 4.6 mm i.d., 5 μm; Alltech, Deerfield, IL, USA). The mobile phase consisted of methanol/water with 0.1% formic acid (50:50, v/v) at a flow rate of 200–300 μL/min (Table 1), and the column temperature was maintained at 40 °C. The mass spectrometer was operated in the positive ion detection mode. Nitrogen was used as the sheath gas (60 L/min), auxiliary gas (30 L/min), and sweep gas (1.46 L/min). The I spray voltage was 4.50 kV, and the spray current was 0.14 μA. The voltage and the temperature of the capillary were 42 V and 300 °C, respectively, and the tube lens was 140 V. The selected reaction monitoring (SRM) mode was used. PF and the IS were monitored at *m/z* transitions of 503 → 381 and 411 → 231, respectively. The optimized collision energy of 27 V was chosen for PF and IS with argon as the collision gas.

Table 1
LC–MS/MS mobile phase gradient program for the determination of PF and IS

Time (min)	Methanol%	Water% (0.1% formic acid)	Flow rate (μL/min)
0	50	50	200
2.5	50	50	200
2.6	50	50	300
3.5	50	50	300
3.6	50	50	200
4.0	50	50	200

2.1.4. Working solutions preparation

Stock solutions of PF and IS were prepared in water to result in final concentrations of 256 and 410 μg/mL, respectively. They were stored at −20 °C until use. A series working solutions of PF with concentrations of 24, 60, 120, 240, 600, 1200, 2400, 4800 and 6000 ng/mL were obtained by further dilution of the stock solution with water. The working solution of IS with a concentration of 600 ng/mL was obtained in the same way. Calibration standard samples were prepared at concentrations of 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL by spiking 25 μL of PF working solutions into 300 μL blank brain homogenate, and "zero" standard sample was prepared by spiking 25 μL of water. Each standard sample was also spiked with 25 μL of IS working solution to give a final concentration of 50 ng/mL. In the same manner, quality control (QC) samples with IS at 50 ng/mL and PF at low (5 ng/mL), medium (50 ng/mL), and high (400 ng/mL) concentrations were prepared to evaluate accuracy and precision of this LC–MS/MS method.

2.1.5. Sample preparation procedures

For the determination of PF in brain, weighted whole brain (blank or samples) were thawed and then homogenized in precooled water (1 g brain:2 mL water). Each of blank brain homogenate (300 μL) samples was spiked with 50 μL water, and dosed brain homogenate (300 μL) samples were spiked with 25 μL water and 25 μL IS (600 ng/mL). Then, the samples were precipitated with 1200 μL acetonitrile. After vortex-mixing for 60 s and centrifuging at 20,000 × *g* for 15 min, the supernatant was dried under a stream of nitrogen at 45 °C. And the residue was redissolved with 900 μL water, then transferred into Sep-

Pak Vac 1 cc cartridges (100 mg, 1 mL; Waters, Milford, MA, USA). Each cartridge was pre-conditioned with 2 × 1 mL of methanol followed by 2 × 1 mL ultrapure water. After sample (total redissolved sample) loaded, the cartridge was washed with 2 × 0.5 mL of 15% methanol. Then, PF and IS were eluted with 0.5 mL of 90% methanol. A 20 μL aliquot of elute solvent was injected into the LC–MS/MS system for analysis.

2.2. LC–MS/MS method validation

Assays were validated according to the U.S. FDA guidance on bioanalytical method validation [28].

2.2.1. Specificity and selectivity

The specificity of the method was demonstrated by comparing chromatograms of blank brain homogenate samples (from six different drug-free rats), brain homogenate samples spiked with PF and IS, and brain homogenate samples obtained from rats administrated with PF.

2.2.2. Sensitivity

The LLOQ was determined during the evaluation of the linear range of calibration curve. LLOQ was defined as the lowest concentration yielding precision with coefficient of variation (CV) less than 20% and accuracy within 20% of the theoretical value for both intra- and inter-batch analysis.

2.2.3. Construction of calibration curve

The calibration curves for PF were constructed by plotting measured peak area ratios of analyte to IS against nominal concentration of PF in brain homogenate using a $1/X$ weighted linear least-squares regression model. The minimally acceptable correlation coefficient (R^2) for the calibration curve was 0.99 or greater.

2.2.4. Precision and accuracy

In order to assess the intra- and inter-batch precision and accuracy of the assay, PF QC samples at low, medium, and high concentrations were prepared as described above. The intra-batch precision of the assay was assessed by calculating the CV for the analysis of QC samples in five replicates, and inter-batch precision was determined by the analysis of QC samples in three batches. Accuracy was calculated by comparing the averaged measurements and the nominal values, and was expressed in percent. The criteria for acceptability of precision was that CV for each concentration level should not exceed ±15% with the exception of the low level, for which it should not exceed ±20%. Similarly, for accuracy, the averaged value should be ranged from within ±15% of the nominal concentration except for the low level within ±20%.

2.2.5. Recovery and matrix effect

Three sets of extraction methods were prepared to evaluate the recovery and the matrix effect (ME) [29], including both absolute and relative matrix effects.

Set 1. Analytes were added into five different lots of blank brain homogenate and then extracted.
Set 2. Analytes were added into five different lots of pre-extracted blank brain homogenate.
Set 3. Analytes were dissolved in matrix component-free eluent solvent.

The peak areas obtained in set 1 were indicated as A, the corresponding peak areas in set 2 as B, and in set 3 as C. The recovery, absolute matrix effect, and relative matrix effect were calculated as follows:

$$\text{Recovery}(\%) = \frac{\text{Mean } A}{\text{Mean } B} \times 100$$

$$\text{Absolute ME}(\%) = \frac{\text{Mean } B}{\text{Mean } C} \times 100$$

$$\text{Relative ME} = (\text{CV of } B) - (\text{CV of } C)$$

2.2.6. Stability

The stability of PF was investigated. Freezing stability of PF in rat brain homogenate was assessed by analyzing QC samples stored at −70 °C for 45 days. The in-autosampler (10 °C) stability of PF in the elute solvent from SPE was evaluated by reinjecting QC samples 24 h after the initial injection. The normalized concentrations of PF and IS in different QC levels were used as references to determine the stability of PF and IS in the experiments.

2.3. Pharmacokinetics of PF in the rat brain

Animal experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica.

Male Sprague–Dawley rats (180–200 g, $n = 78$) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). They were group housed under a 12 h light/dark cycle in an environmentally controlled breeding room for 3 days and fed with standard laboratory food and water *ad libitum*, and fasted overnight before the experiment. Rats were administered subcutaneously with PF in saline at 10 mg/kg body weight or the same volume of saline only for 0 time point. Six rats for each time point were sacrificed by cervical dislocation immediately at 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 180,and 300 min (12 timepoints) and the whole brain tissues samples were dissected, then stored in refrigerator at −70 °C until use. Six rats without any treatment were sacrificed and brains were collected in the same way for method development and validation.

2.4. Data analysis

Data were expressed as mean ± SD. The obtained PF brain concentrations were analyzed using noncompartmental model

with DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society) to obtain the relative pharmacokinetic parameters.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

In this study, liquid–liquid extraction (LLE) by ethyl acetate and protein precipitation by acetonitrile and methanol were tested. The recoveries of PF and IS extracted by ethyl acetate were 54% and 23%, respectively, and the recovery of IS was lower than a half of PF. The signals of PF and IS in brain homogenate precipitated by acetonitrile and methanol were very low in the LC–MS/MS determination because of ion suppressing effect. Therefore, the SPE technique was developed. To get a higher recovery and avoid the clogging of the column, protein precipitation with acetonitrile was applied before loading. In previous test, the impurities were eluted out with the analytes when 50% acetonitrile was used as elute solvent. It was shown that PF and IS could be eluted out by 0.5 mL of 90% methanol completely instead of 0.5 mL × 2 of 15% methanol and both higher extraction recoveries and better selectivity were obtained with PF and IS.

3.1.2. LC–MS/MS method optimization

The electrospray ionization (ESI) source was chosen because better sensitivity, fragmentation and linearity were obtained for PF as compared to the atmospheric pressure ionization (APCI) source. Both the analyte and IS contain terpene and glucose moiety. Thus, they could be detected under either negative or positive electrospray ionization (ESI) conditions. However, it was found that positive ESI could offer higher sensitivity and better peak reproducibility as compared with negative ESI. In the full mass spectrum of PF and IS, molecular $[M+Na]^+$ ions (*m*/*z* 503 for PF and *m*/*z* 411 for IS) were the most intensive ions so they were chosen as precursor ions for the analytes. The ESI interface and mass spectrometer parameters were opti-

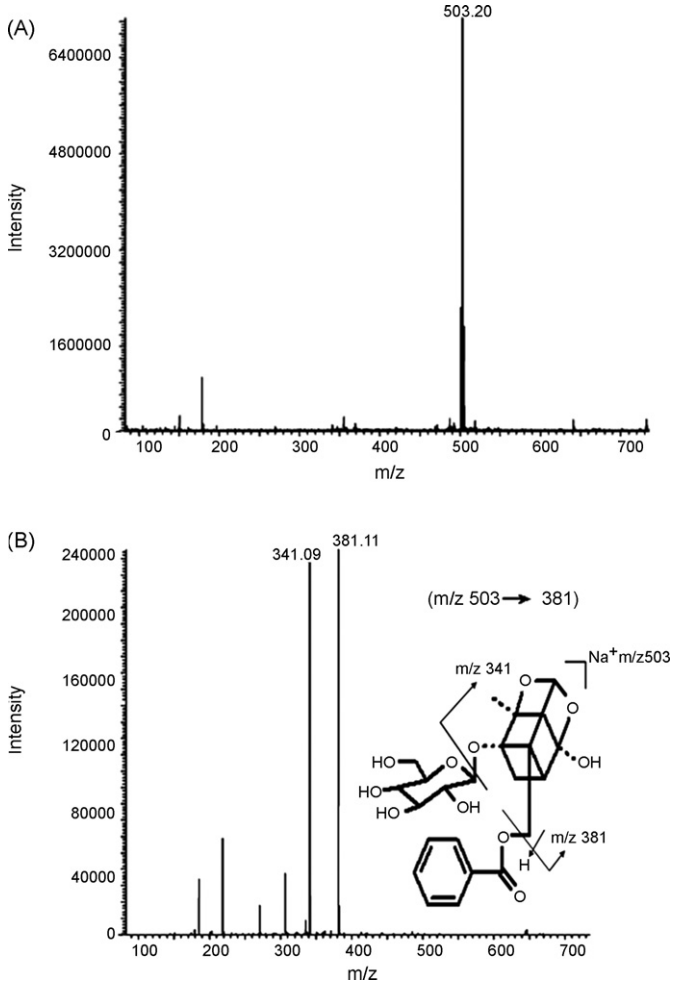

Fig. 2. Typical electrospray ionization mass spectra (A) and product ion mass spectra (B) of PF. The singly charged molecule plus sodium ion at *m*/*z* 503 was selected as precursor ion for PF, and the possible cleavage reactions for the formation of the product ions 341^+ and 381^+ were presented, and the ion 381^+ was selected in SRM acquisition.

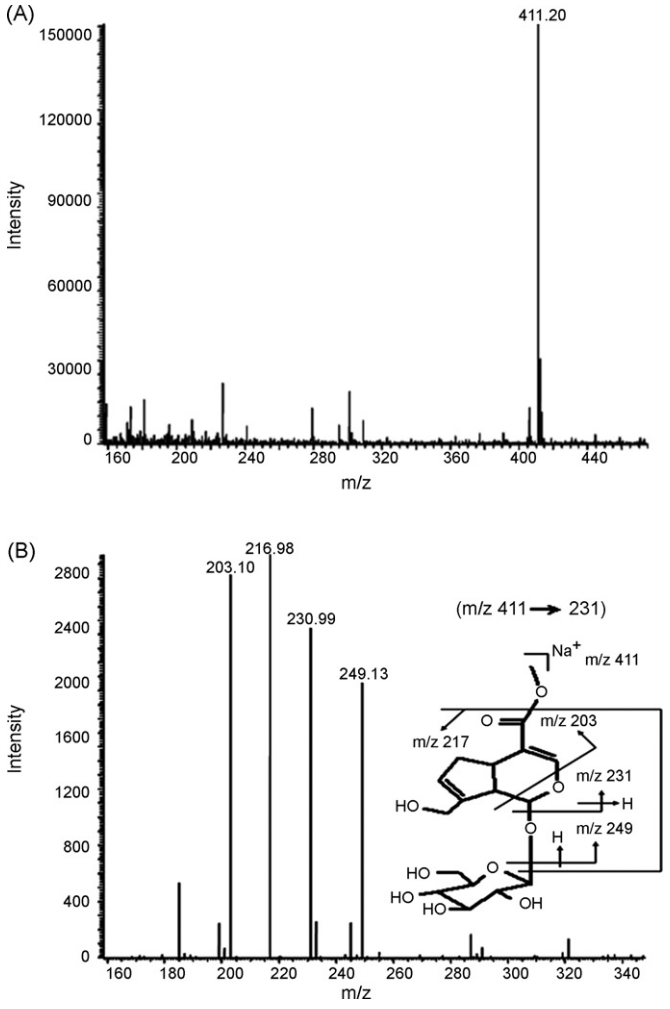

Fig. 3. Typical electrospray ionization mass spectra (A) and product ion mass spectra (B) of IS. The singly charged molecule plus sodium ion at *m*/*z* 411 was selected as precursor ion for IS, and the possible cleavage reactions for the formation of the product ions 203^+, 217^+, 231^+ and 249^+ were presented, and the ion 231^+ was selected in SRM acquisition.

mized in order to obtain maximum sensitivity of $[M+Na]^+$. In addition, methanol instead of acetonitrile was employed as mobile phase as it could provide more intensive response. It was also found that the organic additive formic acid in the mobile phase provided a higher peak response and better peak shape. In the product ion spectrum, several fragment ions were obtained, and the possible cleavage reactions for the formation of the product ions 341^+ and 381^+ of PF (*m/z* 503) are presented in Fig. 2(B); and the possible cleavage reactions for the formation of the product ions 203^+, 217^+, 231^+ and 249^+ of IS (*m/z* 411) are presented in Fig. 3(B). And the ions 381^+ and 231^+ were selected for PF and IS in SRM acquisition, respectively, because they displayed best stability and intensity in all the sample analysis. The full scan and product ion spectrum of the analyte and IS are shown in Figs. 2 and 3, respectively. In the developed method, the SRM mode was used to carry out the quantitative analysis, and it could detect the precursor ion and product ions at the same time and provided high selectivity and sensitivity. Therefore, the development of the chromatographic system was focused on short retention times instead of chromatographic separation. Finally, methanol–water (50:50) was employed to separate and quantify the analyte in the presence of endogenous species and the matrix effect was low.

3.2. LC–MS/MS method validation

3.2.1. Specificity and selectivity

Fig. 4 represents chromatograms of PF and IS from rat brain homogenate after SPE. The typical retention times for PF and IS were 2.71 and 2.64 min, respectively. No interference of endogenous peaks were observed with PF or IS at their respective retention times in blank rat brain homogenate.

3.2.2. Sensitivity

The LLOQ of PF extracted from 300 μL rat brain homogenate was found to be 2 ng/mL after injection of 20 μL of the 500 μL elute solvent from SPE. The intra and inter-batch accuracies

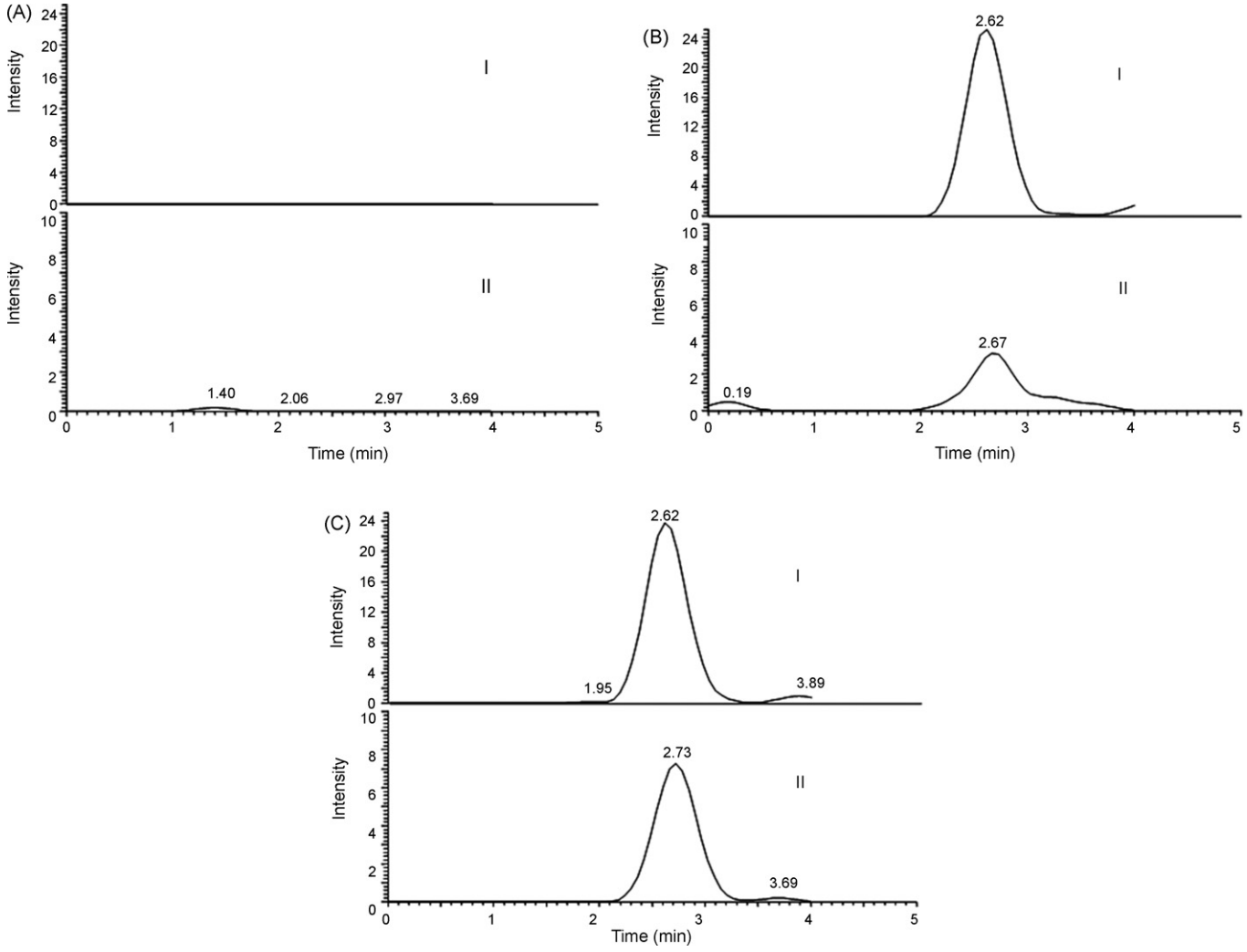

Fig. 4. Representative SRM chromatograms for the determination of PF in rat brain homogenate samples by LC–MS/MS method: (A) a blank rat brain homogenate sample; (B) a blank rat brain homogenate sample spiked with PF at the LLOQ of 2 ng/mL and IS (50 ng/mL); and (C) a rat brain homogenate sample at 120 min after s.c. administration of PF at dose of 10 mg/kg and spiked with 50 ng/mL IS. Peak I, IS; peak II, PF.

Table 2
Precision and accuracy of the LC–MS/MS method for the determination of PF in rat brain homogenate (mean ± SD, $n = 5$)

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Accuracy (%)	CV (%)
Intra-batch			
2	1.92 ± 0.18	95.9	9.0
5	5.08 ± 0.39	101.6	7.8
50	49 ± 3.1	98.5	6.3
400	400 ± 28	100.1	7.0
Inter-batch			
2	2.06 ± 0.15	102.9	7.2
5	5.07 ± 0.06	101.3	1.2
50	50 ± 0.9	99.7	1.8
400	397 ± 5	99.4	1.3

for brain homogenate samples were 95.9% and 102.9%, respectively, and precisions were 9.0% and 7.2%, which were below 20% at the LLOQ (Table 2). This limit was sufficient to determine the concentration–time profile of PF in rat brain following a single s.c. administration (10 mg/kg) of PF.

3.2.3. Construction of calibration curve

The calibration curves for PF were linear in the concentration range of 2–500 ng/mL in rat brain homogenate. A typical equation of calibration curve was as follows: $Y = 0.107271 + 0.0505832X$ ($R^2 = 0.9939$), where Y is the peak area ratio of PF to IS, and X is the concentration of PF in brain homogenate sample. Concentrations of analyte in unknown samples or quality control samples were subsequently determined by interpolation from these regressions.

3.2.4. Precision and accuracy

As shown in Table 2, at the concentrations of 5, 50, 400 ng/mL, intra- and inter-batch accuracy ranged from 98.5% to 101.6% and 99.4% to 102.9%, respectively. The intra- and inter-day assay precisions (CV) ranged from 6.3% to 7.8% and 1.2% to 1.8%, respectively. These data suggested that this method was accurate and reproducible for the determination of PF in rat brain.

3.2.5. Extraction efficiency and matrix effect

The results (Table 3) showed that the recoveries of PF with protein precipitation following solid-phase extraction were 81.2%, 80.9% and 82.3% at concentrations of 5, 50 and 400 ng/mL ($n = 5$), respectively, while the mean recovery of the IS was 76.7% at the concentration used in the assay procedure (50 ng/mL) ($n = 5$).

A value of 100% ME indicated that the response in the mobile phase and in brain homogenate extracts was the same and no matrix effect was observed [29]. The absolute matrix effects of PF were 94.5%, 82.6% and 95.4% at concentrations of 5, 50 and 400 ng/mL ($n = 5$), respectively, while IS (50 ng/mL) ($n = 5$) was 90.4%. The relative matrix effects of PF were 8.55%, 0.05% and 1.5% at concentrations of 5, 50 and 400 ng/mL ($n = 5$), respectively, while IS (50 ng/mL) ($n = 5$) was 6.06%. Without significant difference of the sets B and C, the matrix effect in the quantitative analysis could be ignored.

3.2.6. Stability

The stabilities of PF under various conditions are summarized in Table 4. PF stock solution (256 μg/mL in water) was stable for at least 3 months (data not shown) at −20 °C. The analyte was also shown to be stable in the elute solution of 90% methanol–water for at least 24 h at 10 °C and in rat brain homogenate at −70 °C for at least 45 days.

3.3. Application to pharmacokinetic study of PF

This LC–MS/MS method was applied to the quantitation of PF in rat brain. The mean brain concentration–time profile after s.c. administration of PF (10 mg/kg) in the rats is shown in Fig. 5. The basic pharmacokinetic parameters of PF in the rats are summarized in Table 5.

The maximum brain concentration (C_{max}) of PF reached 153 ± 26.7 ng/mL at 20 min after dosing, and biological half-life ($t_{1/2}$) was 271 ± 109 min. The area under brain concentration–time curve ($AUC_{0\text{-}t}$) was 11127 ± 3091 μg/L min and $AUC_{0\text{-}\infty}$ was 19565 ± 10802 μg/L min. The mean residence time ($MRT_{0\text{-}t}$) was 96 ± 6.5 min and $MRT_{0\text{-}\infty}$ was 338 ± 152 min. The total body clearance (TBCL) was 0.62 ± 0.26 L/min/kg, and the apparent volume of distribution (V_d) was 216 ± 72 L/kg. This suggests that PF was rapidly absorbed and penetrated into the brain

Table 3
Matrix effect (ME) and recovery of PF and IS in rat brain homogenate ($n = 5$)

Nominal concentration (ng/mL)	Recovery (%)		Absolute ME (%)		Relative ME (%)	
	PF	IS	PF	IS	PF	IS
5	81.2		94.5		8.55	
50	80.9	76.7	82.6	90.4	0.05	6.06
400	82.3		95.4		1.50	

Table 4
Stability of PF at different experimental conditions (mean ± SD, $n = 3$)

Nominal concentration (ng/mL)	Stability condition	% remaining
5	45 days storage at −70 °C	102 ± 10.2
	24 h in autosampler at 10 °C	97.2 ± 10.9
50	45 days storage at −70 °C	104 ± 12.9
	24 h in autosampler at 10 °C	95.9 ± 14.1
400	45 days storage at −70 °C	93.7 ± 9.2
	24 h in autosampler at 10 °C	93.8 ± 10.6

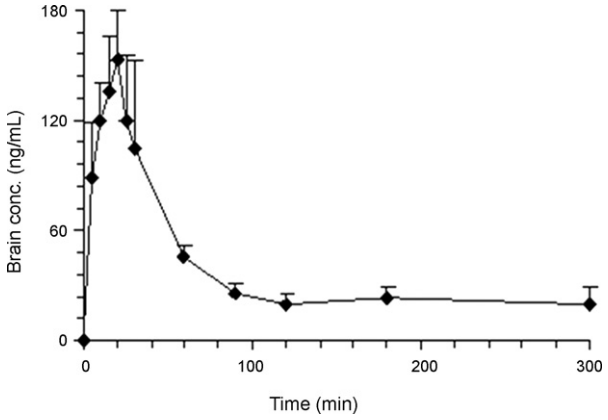

Fig. 5. Brain concentration–time profile of PF in rat brain after a single s.c. administration of 10 mg/kg boy weight of PF (mean ± SD, $n = 6$).

Table 5
Pharmacokinetic parameters of PF (10 mg/kg) in rat brain after s.c. administration to rats (mean ± SD, $n = 6$)

Parameters	Value
$t_{1/2}$ (min)	271 ± 109
C_{max} (ng/mL)	153 ± 26.7
T_{max} (min)	20 ± 0
$AUC_{(0\text{-}t)}$ (μg/L min)	11127 ± 3091
$AUC_{(0\text{-}\infty)}$ (μg/L min)	19565 ± 10802
TBCL (L/min/kg)	0.62 ± 0.26
V_d (L/kg)	216 ± 72
$MRT_{(0\text{-}t)}$ (min)	96 ± 6.5
$MRT_{(0\text{-}\infty)}$ (min)	338 ± 152

with a relative low concentration after s.c. administration.

4. Conclusions

A selective LC–MS/MS method was presented and validated for the determination of PF in rat brain. This is the first report using LC–MS/MS to quantify PF in solid biological samples such as rat brain. The method was sensitive and rapid with a LLOQ of 2 ng/mL using 300 μL rat brain homogenate. The short chromatographic time of 4 min allowed high-throughput analysis and more than 300 samples could be assayed daily. Indeed, the present method was successfully applied to determine the brain concentration of PF after s.c. administration of PF with a relatively very low concentration penetrated through blood–brain barrier. The basic pharmacokinetic parameters of PF in rat brain such as clearance, $t_{1/2}$, steady state volume of distribution, etc. were obtained, and which would be helpful to elucidate of its neuroprotection effects. The established LC–MS/MS method for studying the Pharmacokinetics of PF in rat brain including chromatographic conditions as well as sample preparation procedures is very useful for future study in PF related drug development. It also could facilitate, with minor modification, the development and validation of LC–MS/MS analytical assays to analyze PF in other biological matrixes such as urine, plasma and other tissue homogenates.

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