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Simultaneous UFLC–ESI–MS/MS determination of piperine and piperlonguminine in rat plasma after oral administration of alkaloids from *Piper longum* L.: Application to pharmacokinetic studies in rats

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ABSTRACT

The alkaloids from *Piper longum* L. showed protective effects on Parkinson's disease models in our previous study and piperine and piperlonguminine were the two main constituents in the alkaloids. The present study aimed at developing a rapid, sensitive, and accurate UFLC–ESI–MS/MS method and validating it for the simultaneous determination of piperine and piperlonguminine in rat plasma using terfenadine as the internal standard. The analytes and internal standard (IS) were extracted from rat plasma using a simple protein precipitation by adding methanol/acetonitrile (1:1, v/v). A Phenomenex Gemini 3 u C18 column (20 mm × 2.00 mm, 3 μm) was used to separate the analytes and IS using a gradient mode system with a mobile phase consisting of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) at a flow rate of 0.4 mL/min and an operating column temperature of 25 °C. The total analytical run time was 4 min. The detection was performed using the positive ion electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode with transitions at *m*/*z* 286.1–201.1 for piperine, *m*/*z* 274.0–201.1 for piperlonguminine, and *m*/*z* 472.4–436.4 for the IS. The calibration curves were both linear ($r > 0.995$) over a concentration range of 1.0 to 1000 ng/mL; the lower limit of quantification (LLOQ) was 1.0 ng/mL for both piperine and piperlonguminine. The intra-day and inter-day precisions (RSD %) were <12.1%, accuracies ranged from 86.6 to 120%, and recoveries ranged from 90.4 to 108%. The analytes were proven stable in the short-term, long-term, and after three freeze–thaw cycles. The method was successfully applied to pharmacokinetic studies of piperine and piperlonguminine in rats after oral administration of alkaloids from *P. longum* L.

1. Introduction

Piper longum L., a Piper plant species, is used as a traditional medicine in Asia and the Pacific islands [1]. It is widely used as an analgesic and a treatment for stomach disease in China [2]. Recently, multiple biological properties have been reported, including anti-inflammatory and antioxidant activity in many preclinical studies [3–5].

Piperine and piperlonguminine are two of the major alkaloids in *P. longum* L. [6]. Piperine behaves as a central nervous system depressant, an antipyretic, an analgesic, and displays anti-inflammatory activities [7]. Moreover, it exhibits a wide variety of biological effects, including bioenchancer and antioxidant properties, anti-platelet, antihypertensive, and hepatoprotective effects, as well as antithyroid, antitumor activity, etc. [8]. Piperlonguminine has gained considerable attention in recent years because of its low toxicity and significant anti-hyperlipidemia effects [9,10]. In addition, it has also shown antitumor effects in vivo [11].

Because of these multiple biological effects, bioanalysis and pharmacokinetic studies of piperine and piperlonguminine have become a focus of research. Previous assays have described several methods for the determination of piperine in biological fluids, including HP-TLC [12], HPLC [13–16], LC–NMR–MS [17], LC–MS–MS [18], and UFLC [19]. These methods do not meet modern drug development needs with respect to an efficient extraction procedure, shorter run time and high sensitivity. Recently, Sachin et al. developed an UPLC-qTOF-MS method for the determination of a piperine analogue (PA-1) in mice [20]. However, only the piperine and its metabolite were analyzed qualitatively or quantitatively by these methods. There is no published method for the assay of piperlonguminine. In addition, until now, there have been no simultaneous quantification methods reported for the determination of piperine and piperlonguminine in biological fluids.

Because plasma concentrations of piperine and piperlonguminine are usually very low (ng/mL) after oral administration, the HPLC–UV method was not sensitive enough to monitor drug

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delivered in this manner. Methodological development and validation for the simultaneous quantitative analysis of piperine and piperlonguminine in biological fluids are therefore required to support preclinical and clinical trials aimed at determining oral bioavailability, defining pharmacokinetic and safety profiles, and establishing optimal doses for maximum efficacy.

In this paper, a rapid, sensitive, and accurate UFLC–ESI–MS/MS method was developed and validated for the simultaneous quantitative determination of piperine and piperlonguminine in rat plasma. The validated LC/MS/MS method was successfully applied to determine plasma concentrations of piperine and piperlonguminine after an oral administration of alkaloids from *P. longum* L.

2. Experimental

2.1. Chemicals and reagents

A reference standard for piperine (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Alkaloids of *P. longum* L. (containing 54.10% piperine and 3.74% piperlonguminine) and a reference standard for piperlonguminine (>98% purity) were prepared by the Laboratory for Chemistry of Chinese Material of Chinese Capital Medical University (Beijing, China). Terfenadine (Internal standard, IS) was purchased from Sigma–Aldrich. HPLC grade acetonitrile and methanol were obtained from the TEDIA Company, USA. All other chemicals were of analytical reagent grade. Distilled water was prepared by a Milli-Q water purification system from Millipore (Molsheim, France).

2.2. Instrumentation and analytical conditions

A Shimadzu LC-20AD series UFLC system (Shimadzu, Japan) coupled to an Applied Biosystems Sciex Q-trap^{TM} mass spectrometer (Concord, Ontario, Canada) via an electrospray ionization (ESI) source was used for analysis. Applied Biosystems Analyst software version 1.5.1 was used to control the LC–MS/MS system and for data acquisition and processing. Piperine, piperlonguminine, and IS were separated on a Phenomenex Gemini 3 u C18 110A column (20 mm × 2.00 mm, 3 μm, USA). The mobile phase consisting of a 0.1% formic acid aqueous solution (A)/0.1% formic acid acetonitrile solution (B) was run at a flow rate of 0.4 mL/min at an operating temperature of 20 °C. The gradient was as follows: 0 min 10% B, 0.6 min 10% B, 0.8 min 98% B, 2.5 min 98% B, 2.51 min 10% B, 4 min stop. A two-phase switching valve was used to divert the pre-eluent from entering the ion source.

A mass spectrometer was operated in positive ESI mode with multiple reaction monitoring (MRM) at unit resolution. Nitrogen was used as the nebulizer, heater, and curtain gas, as well as the collision activation dissociation (CAD) gas. The precursor-to-product ion transitions were monitored at m/z 286.1 → 201.1 for piperine, m/z 274.0 → 201.1 for piperlonguminine, and m/z 472.4 → 436.4 for terfenadine. ESI parameters were selected to maximize the generation of precursor and fragment ions by infusion of two solutions of piperine and piperlonguminine into the ESI source at 25 μL/min. Optimum parameters were as follows: nebulizer (GS1), heater (GS2), and curtain gas flow rates were set at 40, 40, and 12 units, respectively; ionspray needle voltage was 5500 V; heater gas temperature was 550 °C; collision gas (N_2) 12 units; a declustering potential of 61 V for piperine, 56 V for piperlonguminine, and 66 V for terfenadine; collision energies of 27 eV for piperine, 23 eV for piperlonguminine, and 37 eV for terfenadine.

2.3. Preparation of stocks, calibration standards, and quality control samples

The stock solutions of piperine and piperlonguminine were separately prepared in methanol at a concentration of 1 mg/mL. Standard solutions (10, 20, 50, 200, 1000, 2000, 5000, and 10,000 ng/mL) and QC solutions (20.0, 1000, and 8000 ng/mL) containing both piperine and piperlonguminine were prepared by serial dilution of the stock solution with methanol. A stock solution of terfenadine (1 mg/mL) was also prepared in methanol and then diluted with methanol/acetonitrile (1:1, v/v) to a final concentration of 10 ng/mL. All solutions were stored at 4 °C and used within one month of preparation.

2.4. Animals

Male Sprague-Dawley rats (220–250 g) were obtained from the Beijing Military Medical Sciences Experimental Animal Co., Ltd. (Beijing, China). All experimental procedures were approved by the Experimental Animal Care and Use Committee at the Capital Medical University (Beijing, China) and carried out in accordance with the corresponding guidelines. The rats were housed under standard conditions; water was provided *ad libitum*; and a standard laboratory diet was provided throughout the experiments. Two days before the experiment, polyethylene cannula was implanted in the femoral vein of rats anesthetized with pentobarbital (50 mg/kg, i.v.). The cannula were externalized at the back of the neck and filled with heparinized saline (20 units/mL).

2.5. Pretreatment of plasma samples

After thawing the plasma samples at room temperature for about 30 min, they were vortexed and aliquots (50 μL) mixed with 150 μL of IS solution [10 ng/mL terfenadine in methanol/acetonitrile (1:1, v/v)] and 5 μL methanol (or a standard or QC solution) were placed in 1.1 mL tubes. After vortexing for 1 min and centrifuging at 5000 × g for 10 min, a 10-μL aliquot of each supernatant was injected into the LC–MS/MS system. Samples with concentrations exceeding that of the highest standard (1000 ng/mL) were diluted with blank rat plasma prior to analysis.

2.6. Method validation

The analytical method was performed according to US FDA guidelines for industry for bioanalytical method validation (CDER). The method was validated for selectivity, linearity, lower limits of quantification (LLOQs), accuracy, precision, recovery, and stability.

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with corresponding spiked plasma samples. The matrix effects for piperine and piperlonguminine were also evaluated by comparing peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions.

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. Plasma samples were quantified using the ratio of the peak area of each analyte to that of terfenadine as the assay response. The peak area ratio (y) and concentration of each analyte (x) were subjected to a weighted ($1/x^2$) least squares linear regression analysis to calculate the calibration equation and correlation coefficients. The LLOQ, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing six replicate samples that were prepared by spiking 50 μL of blank rat plasma with 5 μL of the standard solution containing appropriate concentrations of each analyte.

Accuracy (relative error, RE) and intra-assay and inter-assay precision (% relative standard deviation or RSD) were assessed by assaying six replicate QC samples on three different days. Recovery of piperine and piperlonguminine was determined by comparing peak areas of extracted QC samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations.

The extraction recoveries of piperine, piperlonguminine, and terfenadine were calculated by comparing the analytical results of extracted QC samples with samples at the same analyte concentrations obtained by spiking extracted blank rat plasma samples with analyte working standard solutions.

The stability of piperine and piperlonguminine in rat plasma was investigated under a variety of storage and process conditions. Its storage stability at −20 °C was evaluated for at least 30 days. The freeze–thaw stability of piperine and piperlonguminine was assessed by analyzing QC samples at three concentrations (2.0, 100, and 800 ng/mL) subjected to three freeze (−20 °C)–thaw (20 °C) cycles. The stability of the reconstituted solution was investigated by testing QC samples at three concentrations under ambient conditions for 24 h. The results were compared with results for freshly prepared QC samples, and the percentage concentration deviation was calculated.

2.7. Pharmacokinetic study

Five male Sprague-Dawley rats (220–250 g) were enrolled to investigate the pharmacokinetics of piperine and piperlonguminine. After a 2-day recovery from femoral vein cannulated surgery, the animals were administered a 100 mg/kg dose of the alkaloids from *P. longum* L. by oral gavage. The doses of piperine and piperlonguminine that were converted according to their contents in the alkaloids were 54.1 mg/kg and 3.74 mg/kg. The alkaloid solutions were dissolved in 0.5% methylcellulose (MC), 0.1% Tween 80 aqueous solution to obtain a final concentration of 10 mg/mL. After oral administration, aliquots of 0.3 mL blood samples were collected in heparinized 1.5 mL polythene tubes at different time intervals post-dosing (0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h). Plasma was separated by centrifugation at 6750 rpm for 5 min and stored at −20 °C until analysis. Pharmacokinetic parameters including half-life ($t_{1/2}$), maximum plasma time (T_{max}) and concentration (C_{max}), area under the concentration–time curve (AUC_{last} and AUC_{Inf}), and mean residence time (MRT) of piperine and piperlonguminine were analyzed using a non-compartmental method with WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, USA).

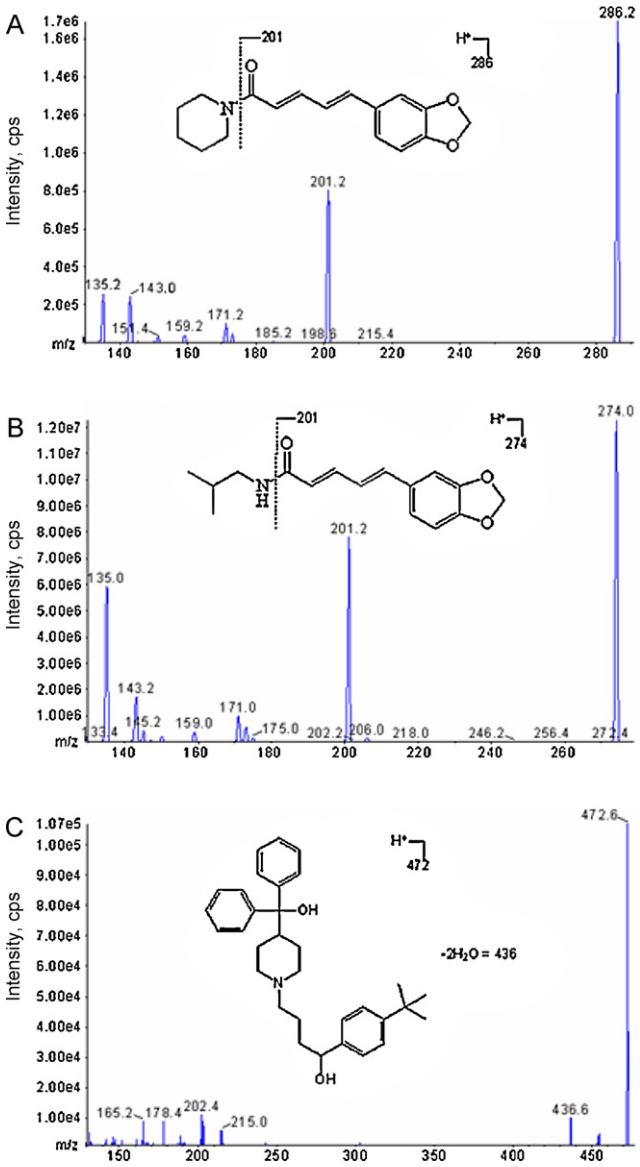

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation schemes for (A) piperine, (B) piperlonguminine and (C) terfenadine (IS).

3. Results and discussion

3.1. Chromatographic and mass spectrometric conditions

A number of commercially available reversed phase HPLC columns and various mobile phases were evaluated for chromatographic behavior and the ionization responses of piperine, piperlonguminine, and IS. Preliminary results and comparisons were obtained using a Phenomenex Gemini 3 u C18 column (20 mm × 2.00 mm, 3 μm) to separate these two analytes and IS using a gradient mode system with a mobile phase consisting of water with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.4 mL/min at an operating temperature of 25 °C. Under these chromatographic conditions, the retention times were 2.28, 2.26, and 2.09 min for piperine, piperlonguminine, and terfenadine, respectively. The total analytical run time was 4 min.

As regard to the mass spectrometer detection, both piperine and piperlonguminine produced strong signals in the positive ion mode due to the presence of an amide group in their structures. The ion spray voltage was set at 5500 V and the source temperature was increased to 550 °C to improve the response of piperine and piperlonguminine. Other parameters were adjusted appropriately to optimize ionization. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways for piperine, piperlonguminine, and terfenadine are shown in Fig. 1. The precursor-to-product ion transitions were monitored at m/z 286.1 → 201.1 for piperine, m/z 274.0 → 201.1 for piperlonguminine, and m/z 472.4 → 436.4 for terfenadine.

In general, matrix effects are a significant problem in LC–MS/MS analysis of biological samples, but in this assay, comparisons were performed using piperine and piperlonguminine standard solutions in methanol and post-extraction blank plasma samples spiked with each analyte stock solution. It was demonstrated that no significant signal suppression or enhancement were found under these conditions.

An internal standard is usually required in a LC–MS/MS analysis. In this study, terfenadine, a readily available compound, was selected as the IS; its chromatographic behavior and extraction efficiency were similar to those of the two analytes, and in addition,

Table 1
The intra- and inter-day precisions and accuracies of piperine and piperlonguminine in rat plasma ($n = 5$).

Sample	Spiked (ng/mL)	Measured (mean ± SD)	Accuracy (RE%)	Precision (RSD%)
Piperine				
Intra-day	2	2.02 ± 0.25	1.00	12.10
	100	92.9 ± 5.01	−7.10	5.39
	800	730 ± 22.6	−8.70	3.09
Inter-day	2	2.10 ± 0.09	5.00	4.31
	100	97.9 ± 4.50	−2.00	4.59
	800	759 ± 24.8	−5.10	3.26
Piperlonguminine				
Intra-day	2	2.01 ± 0.15	1.00	7.56
	100	93.3 ± 4.14	−6.70	4.44
	800	721 ± 25.5	−9.90	3.54
Inter-day	2	2.04 ± 0.04	2.30	2.10
	100	97.9 ± 4.47	2.10	4.56
	800	740 ± 19.0	7.50	2.57

there were no interferences from the two analytes and endogenous substances.

3.2. Method validation

3.2.1. Selectivity

Blank rat plasma samples were prepared to the same as the plasma samples and analyzed to detect the potential interferences co-eluting with these two analytes and IS. As shown in Fig. 2, there were no significant interferences at retention times of 2.09 (IS), 2.28 (piperine), and 2.26 min (piperlonguminine). The detection of piperine, piperlonguminine, and terfenadine by MRM was highly selective with no significant interferences. Typical chromatograms are shown in Fig. 2. The run time was set at 4 min because full chromatographic separation was also necessary to avoid a potential matrix effect.

3.2.2. Linearity

The calibration curves calculated in the range 1–1000 ng/mL were linear to analyze piperine and piperlonguminine from rat plasma. The slopes, intercepts, and correlation coefficients of the regression equations were determined by least squares linear regression using an eight-factor of $1/x^2$. Typical equations for the standard curves were $y = 0.0654x + 0.00380$ ($r = 0.9982$) for piperine and $y = 0.0672x + 0.00620$ ($r = 0.9966$) for piperlonguminine. Deviations were within ±15% for all regression equations. The lower limit of quantification (LLOQ) was 1 ng/mL for both piperine and piperlonguminine.

3.2.3. Precision and accuracy

The intra-day and inter-day precisions and accuracies of rat plasma were evaluated at three QC concentrations: 2, 100, and 800 ng/mL. The results for piperine and piperlonguminine are summarized in Table 1. The precisions of the low-level QC samples were all less than 20% (RSD) and the precisions of the high and medium levels were all less than 15% (RSD); the accuracies of the three QC samples ranged from 80 to 120%. In summary, the precision and accuracy results were satisfactory at the three concentrations studied.

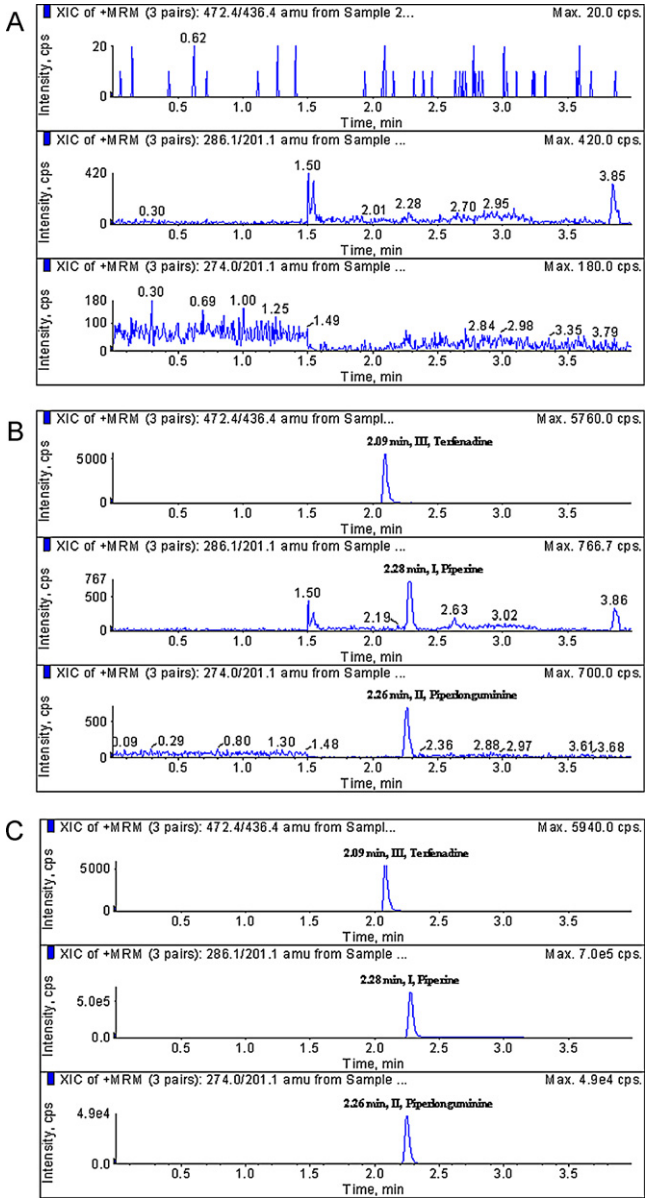

Fig. 2. Typical chromatograms of (A) blank rat plasma, (B) blank rat plasma spiked with piperine (1.0 ng/mL), piperlonguminine (1.0 ng/mL) and I.S. (1.0 ng/mL) and (C) an unknown rat plasma sample collected at 30 min after an oral administration of 100 mg/kg alkaloids. Peak I, piperine; Peak II, piperlonguminine; Peak III: Terfenadine.

3.2.4. Recovery

As shown in Table 2, the recoveries of rat plasma after protein precipitation ranged from 108 to 90.4% for piperine and from 98.9

Table 2
The recoveries of piperine and piperlonguminine from rat plasma ($n = 5$).

Compound	No.	Recovery (%)		
		2.00 ng/mL	100 ng/mL	800 ng/mL
Piperine	Measured (mean ± SD)	108 ± 11.4	90.4 ± 7.67	91.5 ± 1.50
	RSD (%)	10.6	8.49	1.64
Piperlonguminine	Measured (mean ± SD)	98.9 ± 4.91	91.2 ± 4.77	90.9 ± 1.27
	RSD (%)	4.96	5.23	1.40

Table 3
Stability of piperine and piperlonguminine in rat plasma at different conditions determined by LC–MS/MS method ($n = 6$).

Conditions	Spiked (ng/mL)	Piperine		Piperlonguminine	
		Measured (mean ± SD)	Recovery (%)	Measured (mean ± SD)	Recovery (%)
Room temperature (24 h)	2	1.97 ± 0.12	98.5 ± 6.12	2.11 ± 0.23	105 ± 11.7
	100	95.2 ± 1.80	95.2 ± 1.80	96.1 ± 0.90	96.1 ± 0.90
	800	720 ± 8.19	90.0 ± 1.03	7 13 ± 10.0	89.2 ± 1.25
Storage at −20 °C (30 days)	2	1.89 ± 0.14	94.9 ± 7.05	1.91 ± 0.08	95.7 ± 4.11
	100	102 ± 13.1	102 ± 13.1	103 ± 13.5	103 ± 13.5
	800	754 ± 18.9	94.2 ± 2.40	737 ± 10.7	92.1 ± 1.33
Three freeze–thaw cycles	2	19.6 ± 0.13	98.3 ± 6.43	19.4 ± 0.06	97.4 ± 3.31
	100	95.7 ± 2.30	95.7 ± 2.30	96.5 ± 4.19	96.5 ± 4.19
	800	733.7 ± 36.0	91.7 ± 4.50	715 ± 23.2	89.4 ± 2.94

to 90.9% for piperlonguminine at the three QC concentration levels. The recoveries were both within the criteria for acceptability. These results suggested that the method was free from the matrix effect.

3.2.5. Stability

The detailed results for the stabilities of piperine and piperlonguminine in rat plasma are shown in Table 3. Piperine and piperlonguminine in rat plasma were stable for 30 days when stored at −20 °C. The relative error (% RE) of piperine and piperlonguminine in rat plasma between the initial concentrations and the concentrations following the three freeze–thaw cycles was ±15.0%. The processed samples were also stable in the reconstituted solution for 24 h at 20 °C.

3.3. Pharmacokinetic study

This method was successfully applied in pharmacokinetic studies of piperine and piperlonguminine in Sprague-Dawley rats after oral gavage of 100 mg/kg of alkaloids from *P. longum* L. PK parameters for piperine and piperlonguminine are shown in Table 4.

Mean plasma concentration–time profiles for piperine and piperlonguminine after oral gavage of 100 mg/kg of alkaloids from *P. longum* L. are shown in Fig. 3. The main pharmacokinetic parameters for piperine and piperlonguminine are listed in Table 4, respectively. These parameters indicated that piperine and piperlonguminine were rapidly absorbed after administration and cleared slowly from rats.

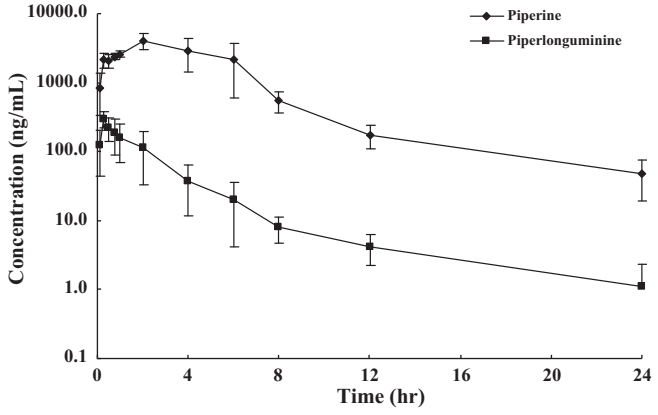

Fig. 3. Mean plasma concentration–time profile of piperine and piperlonguminine determined by LC–MS/MS method after oral administration of 100 mg/kg alkaloids from *Piper longum* L. to rats. Each point represents mean ± SD ($n = 5$).

Table 4
Pharmacokinetic parameters of piperine and piperlonguminine after oral administration of alkaloids from *Piper Longum* L. to rats ($n = 5$, mean ± SD).

PK parameters	Unit	Values (mean ± SD)	
		Piperine	Piperlonguminine
$t_{1/2}$	h	4.10 ± 0.94	4.69 ± 2.05
t_{max}	h	2.45 ± 2.12	0.35 ± 0.22
C_{max}	ng/mL	4292 ± 967	299 ± 79.3
AUC_{last}	h ng/mL	22,803 ± 7062	628 ± 289
AUC_{Inf}	h ng/mL	23,107 ± 7189	643 ± 286
MRT	h	4.63 ± 0.71	3.52 ± 1.25

4. Conclusion

A rapid, sensitive, and accurate UPLC–ESI–MS/MS method with simple protein precipitation was developed and validated for the simultaneous determination of piperine and piperlonguminine in rat plasma. The method was successfully applied in pharmacokinetic studies of piperine and piperlonguminine in rats after oral administration of alkaloids from *P. longum* L. with excellent sensitivity, good linearity of responses, and high precision and accuracy. The total analytical run time was 4 min.

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