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ORIGINAL ARTICLE

Pharmacokinetic Study on Piplartine and Piperine after Oral Administration of *Piper chaba* Root by Liquid Chromatography-mass Spectrometry/mass Spectrometry

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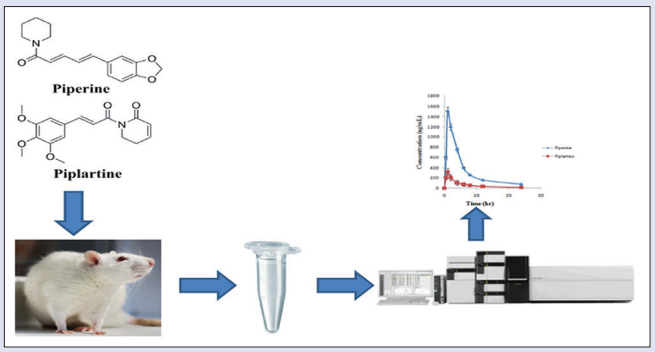
ABSTRACT

Background: Piperaceae family are a well-known source of structurally diverse amides with the wide range of bioactivities such as cytotoxic, stomach aches, insect repellents, anti-inflammatory, insecticidal, and antifeedant activities. It has been reported that piplartine and piperine, alkaloid/amide compounds from *Piper* species, show antitumor activities. **Objective:** A rapid, sensitive liquid chromatography-tandem mass spectrometry method has been developed and validated for the determination of piplartine and piperine from *Piper chaba* extract. **Materials and Methods:** The two analytes, together with internal standard (IS, trichostachine), were separated on a Waters Acquity ethylene bridged hybrid C_{18} (2.1 mm × 100 mm, 1.9 μ) column using a mobile phase of acetonitrile with 0.1% formic acid and water with 0.1% formic acid (70:30, v/v) with isocratic elution. The detection was performed using the positive ion electrospray ionization in multiple reaction monitoring mode with transitions at m/z 318→221 for piplartine, m/z 286→201 for piperine, and m/z 272→201 for the IS. **Results:** The calibration curves were both linear (r^2 > 0.995) over a concentration range of 1.0–2000 ng/mL; the lower limit of detection quantification was 1.0 ng/mL for both piplartine and piperine. The intra-day and inter-day precisions (relative standard deviation %) were <10.9%, and recoveries ranged from 90.3% to 103.0%. **Conclusions:** 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 piplartine and piperine in rats after oral administration of *P. chaba* extract.

Key words: *Piper chaba* extract, piperine, piplartine

SUMMARY

- The aim of this study was to establish the pharmacokinetic profiles for the bioactive compounds, i.e., piplartine and piperine through the oral route of administration performed on animal (Rat) model. The performed compounds were in major amount from *Piper chaba* roots. Results: the established method shows good agreement with the validation parameters to understand the pharmacological effects.



Abbreviations used: AUC: Area under the curve; BEH: Ethylene bridged hybrid; CDER: Centre for drug evaluation and research; CID: Collision-induced dissociation; C_{max}: Maximum concentration; CTO: Column Temperature Oven; DGU: Degassing Unit; ESI: Electrospray ionization; eV: Electron volt; FCV: Flow control valve; HPLC: High-pressure liquid chromatography; HPTLC: High performance thin layer chromatography; IS: Internal standard; LLOQ: Lower limit of quantitation; LC: Liquid chromatography; LC-MS: Liquid chromatography-Mass Spectrometry; LC-MS/MS: Liquid chromatography-Mass Spectrometry/Mass Spectrometry; LC-HRMS: Liquid chromatography-High resolution mass Spectrometry; LC-NMR-MS: Liquid chromatography-Nuclear magnetic resonance-Mass Spectrometry; MRM: Multiple reaction monitoring; MC: Methyl cellulose; N_2: Nitrogen; RSD: Relative standard deviation; RE: Relative error; r^2: Regression coefficient; $t_{1/2}$: Half-life; T_{max}: Time to maximum effect; QC: Quality control; UFLC: Ultrafast liquid chromatography; UPLC-qTOF-MS: Ultra pressure liquid chromatography-Time of flight-Mass spectrometry; USFDA: United states Food and Drug Administration

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INTRODUCTION

Herbal medicine plays an important role owing to its effectiveness in public health. It is widely accepted that the multiple constituents in herbal medicine have led to their remarkable clinical application.[1] To ensure the quantity and efficiency in clinical use, the quality control (QC) of herbal medicine should be reflected by phytoequivalence and pharmacological effects.[2] Thus, a huge quantity of work has to be performed to develop

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various analytical methods for the identification, quantification, and QC of the active components in raw plant materials, extracts, and the final products. However, studies on the absorption, distribution, metabolism, and excretion of herbal drugs are rarely reported.[3] One of the main reasons lies in the lack of sensitive, specific, and reliable analytical methods for pharmacokinetic studies of active constituents present in herbal medicines.

Piper is well reputed in the Indian Ayurvedic system of medicine.[4,5] Plants of the Piperaceae family are a well-known source of structurally diverse amides with the wide range of bioactivities such as cytotoxic, stomach aches, insect repellents, anti-inflammatory, insecticidal, and antifeedant activities.[6-9] The fascinating structural features and multiple biological activities of amides isolated from different species of piper from our laboratory[10-13] have encouraged us to continue the study of this family.[14] The dried roots and fruits of *Piper chaba* have been used to treat asthma, bronchitis, fever, pain in abdomen, and as a stimulant in hemorrhoidal afflictions.[15,16]

Piplartine and piperine are two of the major alkaloids in *P. chaba*. Piperine behaves as a central nervous system depressant, an antipyretic, an analgesic, and displays anti-inflammatory activities.[17] Moreover, it exhibits a wide variety of biological effects, including bioenhancer and antioxidant properties, antiplatelet, antihypertensive, and hepatoprotective effects, as well as antithyroid, antitumor activity, etc.[18] Piplartine has several biological activities, such as antifungal, anti-platelet aggregation, insecticidal, antiparasitic, anxiolytic, and antidepressant activities.[19-21]

Due to these multiple biological effects, bioanalysis and pharmacokinetic studies of piplartine and piperine have become a focus or research. Previous assays have described several methods for the determination of piperine alone or in combination with other bioactive compounds in biological fluids, including high-performance thin layer chromatography,[22] high-pressure liquid chromatography (HPLC),[23-25] liquid chromatography (LC) nuclear magnetic resonance-mass spectrometry (MS),[26] LC-MS,[27-29] LC-MS/MS,[30,31] LC-high resolution mass spectrometry,[32] Ultrafast LC,[33,34] and Ultra pressure LC-time of flight-MS.[35] To the best of our knowledge, no studies have been reported for the determination of piperine and piplartine in biological fluids.

In this paper, a rapid, sensitive, and accurate LC-MS/MS method was developed and validated for the simultaneous quantitative determination of piperine and piplartine in rat plasma. The validated LC/MS/MS method was successfully applied to determine plasma concentration of piperine and piplartine after an oral administration of *P. chaba* extract.

MATERIALS AND METHODS

Plant material, chemicals, and standards

Roots of *P. chaba* were collected from the forest area Tirupati, Chittoor, Andhra Pradesh, India were identified and authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati , India. Voucher specimens of the plant material PC-NPL-08 were kept at Natural Products laboratory of Indian Institute of Chemical Technology, Hyderabad. Piplartine, piperine, and trichostachine were isolated in our laboratory as described earlier.[36] HPLC grade methanol, acetonitrile, and formic acid were obtained from Merck Specialties Pvt., Ltd., (Mumbai, India). Water used in the entire analysis was prepared on a Milli-Q water purification system procured from Millipore (Bangalore, India). All the chemicals were of analytical reagent grade.

Instrumentation and analytical conditions

The LC was a Shimadzu (Japan) Nexera X2 system with a SIL-30AC autosampler, an LC-30AD high-pressure gradient pump system, a Degassing Unit-20A5R vacuum degasser, and a column temperature oven-20AC column oven. A flow control valve-32AH six-port flow changeover valve and an auxiliary LC-20AD pump were added for sample pretreatment. Piperine, piplartine, and internal standard (IS) were separated on a Waters Acquity ethylene bridged hybrid (BEH) C_{18} (2.1 mm × 100 mm 1.9 µm). The mobile phase consisting of a 0.1% formic acid aqueous solution (A)/0.1% formic acid acetonitrile solution (B) using an isocratic system at a flow rate of 0.3 mL/min at an operating temperature of 25°C.

MS was conducted using a Shimadzu LC-MS 8040 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in positive-ion mode and multiple reaction monitoring (MRM) with following parameters: desolvation line temperature, 250°C; heater block, 400°C; nebulizing gas (N_2) flow, 3 L/min; dry gas (N_2) flow 15 L/min; probe voltage - 3.5 kV, and Argon as collision-induced dissociation gas. The MRM parameters, such as the precursor ion m/z, collision energy, and production m/z for piperine and piplartine were optimized by automatic MRM optimization function. Data acquisition and peak processing were automatically performed with LCMS Lab solutions (Japan, Tokyo) software V5.65.

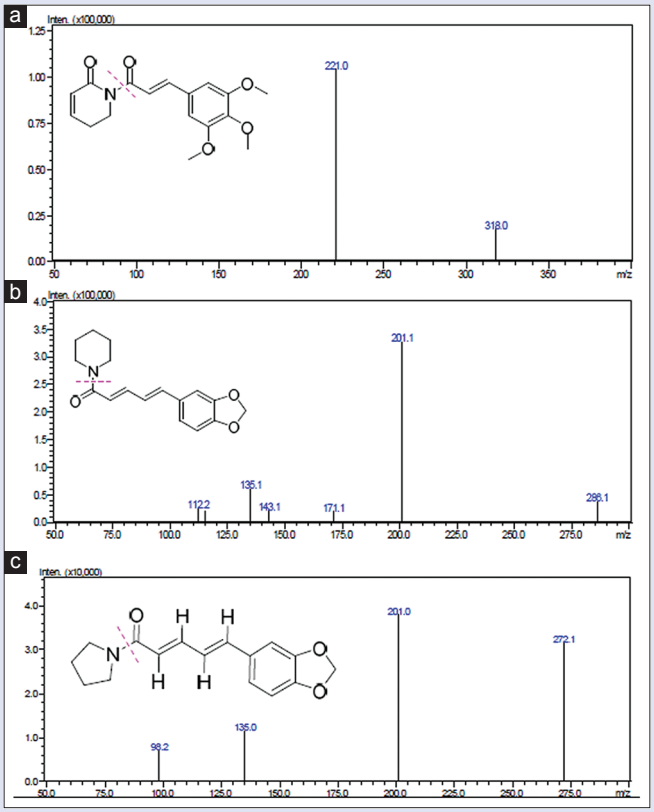

Figure 1: Full-scan product ion spectra of $(M + H)^+$ ions and fragmentation schemes for (a) piplartine, (b) piperine and (c) trichostachin

Table 1: The mass spectrometry/mass spectrometry parameters of the multiple reaction monitoring method of the monitored compounds

Compound	Precursor ion	MRM transition	CE
Piplartine	318.00	318.00>221.0	20.0
Piperine	286.10	286.10>201.10	18.0
Trichostachin	272.10	272.10>201.00	18.0

MRM: Multiple reaction monitoring; CE: Collision energy

Preparation of the extract

Twenty grams of *P. chaba* roots were powdered and extracted with 200 mL methanol for 2 h in a reflux condenser. The filtrate was collected, and the residue was reextracted with 200 mL methanol. Then, the solvent was removed under reduced pressure in a rotary evaporator.

Preparation of stocks, calibration standards, and quality control samples

The stock solution of piplartine and piperine were separately prepared in methanol at a concentration of 1 mg/mL. Standard solutions (1, 2, 10, 50, 100, 200, 400, 800, 1000, and 2000 ng/mL), and QC solutions (2, 100, and 800 ng/mL) containing both piplartine and piperine were prepared by serial dilution of the stock solution with methanol. The IS was prepared in methanol at a concentration of 100 μg/mL and was further diluted to 100 ng/mL as a working solution.

Plasma sample preparation

The plasma sample was thawed to room temperature. A 90 μL aliquot of rat plasma, 10 μL IS solution (100 ng/mL), and 1000 μL methanol was added. After vortex-mixing for 1 min and centrifugation (14000 × g) for 10 min, the supernatant was separated out and evaporated to dryness under vacuum at 45°C. Then, the residue was reconstituted in 100 μL methanol, vortex-mixed briefly, and finally, 10 μL of the sample solution was injected for LC/MS/MS analysis.

Method validation

The analytical method was performed according to the United states Food and Drug Administration guidelines for the industry for bioanalytical method validation (centre for drug evaluation and research). The method was validated for selectivity, linearity, lower limits of quantification, accuracy, precision, recovery, and stability.[37]

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 piplartine were also evaluated by comparing peak areas of postextraction blank plasma spiked at concentration of QC samples with the areas obtained by direct injection of corresponding standard solutions.

Calibration curves were constructed by plotting peak-area ratios of each analyte to IS versus plasma concentration using a $1/X^2$ weighted linear least-squares regression model in duplicate on three consecutive days. The lower limit of quantitation (LLOQ) is defined as the lowest concentration point of the calibration curve at which an acceptable accuracy within ± 20% and precision below 20% can be obtained. Analyte response at the level of LLOQ should be at least five times the blank plasma.

Precision was expressed as the relative standard deviation (RSD%), and accuracy was calculated as the relative error (RE). Three levels of QC samples in six replicates were analyzed during the same day using the same calibration curve to determine the intra-day precision. Three

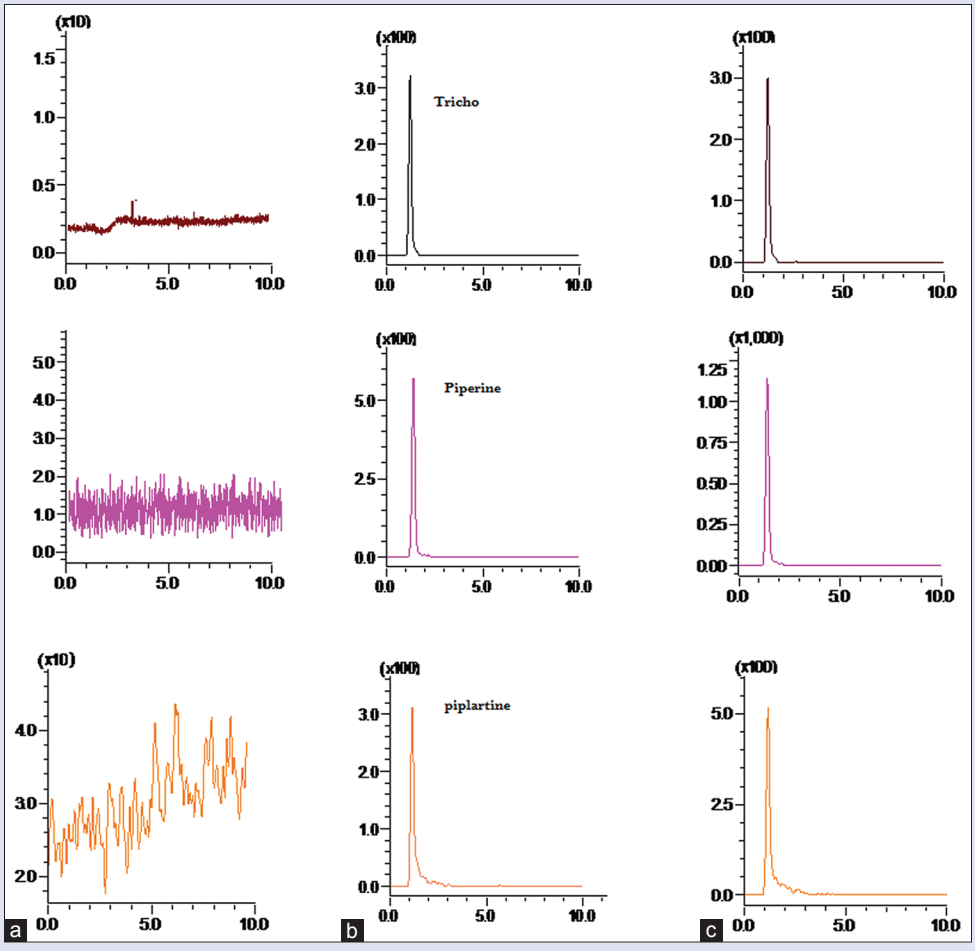

Figure 2: Chromatograms of (a) blank rat plasma, (b) blank rat plasma spiked with analytes and internal standard (c) Unknown rat plasma sample collected at 30 min after an oral administration

Table 2: Precision and accuracy of piplartine and piperine in rat plasma

Components	Concentration	Intra-day (n=6)			Inter-day (n=6)		
		Measured concentration	RSD (%)	RE (%)	Measured concentration	RSD (%)	RE (%)
Piplartine	2	2.01±0.22	10.99	0.50	2.04±0.12	5.92	2
	100	93.53±4.56	4.88	−6.47	97.25±4.36	4.49	−2.75
	800	722.50±20.81	2.88	−9.68	740.03±19.94	2.69	−7.49
Piperine	2	2.03±0.05	2.79	1.50	2.10±0.11	5.23	5.00
	100	93.13±3.79	4.07	−6.87	97.83±4.25	4.34	−2.17
	800	737.50±22.54	3.05	−7.81	751.39±24.12	3.21	−6.07

RSD: Relative standard deviation; RE: Relative error

Table 3: The recoveries of piplartine and piperine from rat plasma (n=6)

Analytes	Spiked concentration	Mean recovery (%)±SD	RSD
Piplartine	2	97.66±2.51	2.57
	100	92.33±8.25	8.93
	800	90.33±1.52	1.69
Piperine	2	103.00±10.14	9.85
	100	91.28±4.79	5.25
	800	91.50±1.32	1.44

RSD: Relative standard deviation; SD: Standard deviation

batches of QC samples were analyzed on three consecutive days to evaluate the inter-day precision and accuracy.

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

The stability of piplartine and piperine in rat plasma was investigated under a variety of storage and process condition. Its storage stability at −20°C was evaluated for at least 30 days. The freeze-thaw stability of piplartine and piperine was assessed by analyzing QC samples at three concentrations 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.

Pharmacokinetic studies

The experimental protocol was approved by the Animal Ethical Committee of Indian Institute of Chemical Technology, Hyderabad. The extract was suspended in 0.5% methylcellulose and five rats were orally administrated at the dose of 100 mg/kg body weight. After oral administration, aliquots of 0.3 mL blood samples were collected in heparinized Eppendorf tubes at different time intervals postdosing (0, 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 −80°C until analysis. Pharmacokinetic parameters including half-life ($t_{1/2}$), maximum plasma time (T_{max}) and maximum plasma concentration (C_{max}) and area under the concentration-time curve (AUC_{last} and AUC_{inf}) of piplartine and piperine were subjected to noncompartmental pharmacokinetic analysis using linear trapezoidal rule.

RESULTS AND DISCUSSIONS

Chromatographic and mass spectrometric conditions

The chromatographic condition, especially the composition of mobile phase, plays a critical role in achieving good chromatographic behavior and appropriate ionization. Different mobile phases (methanol-water, acetonitrile-water with or without formic acid or ammonium acetate) were investigated using Waters Acquity BEH C_{18} (2.1 mm × 100 mm 1.9 µ) column to optimize the analytical performance. It was observed that acetonitrile was found to be better regarding resolution and peak shapes as compared with methanol. Using 70% of acetonitrile with 0.1% formic acid in 0.1% formic acid in water with good peak shape, considerable response and baseline separation were achieved. The mobile phase was operated at a flow rate of 0.3 mL/min allowing a short run time of 10 min.

As regards the mass spectrometer detection, both piplartine and piperine produced strong signals in the positive ion mode due to the presence of an amide group in their structures. The collision energy values for piperine and piplartine were 18 and 20 (eV). Other parameters were adjusted appropriately to optimize ionization. Full-scan product ion spectra of $(M + H)^+$ ions and fragmentation pathways for piplartine, piperine, and trichostachine are shown in [Figure 1]. The precursor-to-product ion transitions were monitored at m/z 286→201 for piperine, m/z 318→221 for piplartine and m/z 272→201 for trichostachine [Table 1].

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

Selection of appropriate IS is an important aspect to deal with sample matrix effects. An ideal IS should be a structurally similar analog or stable isotope-labeled compound. Trichostachine was chosen as the IS because of its similarity in structure, ionization response and extraction recovery in ESI-MS and a similar elution pattern.

Method validation

Selectivity

The typical chromatograms of a blank sample, a plasma sample spiked with the two analytes at LLOQ and IS, and a plasma sample from a rat 1 h after oral administration of *P. chaba* extract are shown in Figure 2, there were no significant interferences at retention time of (IS), (piplartine) and (piperine). The detection of piplartine, piperine, and trichostachine by MRM was highly selective with no significant interferences. The runtime was set at 10 min because full chromatographic separation was also necessary to avoid a potential matrix effect.

Linearity

The calibration curves calculated in the range 1–2000 ng/ml were linear to analyze piperine and piplartine from rat plasma. Calibration curves were $y = 122.8x + 512.5$ ($r^2 = 0.998$) for piperine and $y = 65.33x - 1137$($r^2 = 0.998$) for piplartine. The lower limit of quantification (LLOQ) was 1 ng/mL for both piperine and piplartine.

Precision and accuracy

The intra-day and inter-day precision and accuracies of rat plasma were evaluated at three QC concentrations as follows: 2, 100, and

Table 4: Stability of piplartine and piperine in rat plasma at different conditions determined by liquid chromatography-mass spectrometry/mass spectrometry

Components	Concentration	Piplartine			Piperine		
		Measured concentration	RSD (%)	RE (%)	Measured concentration	RSD (%)	RE (%)
Room temperature (24 h)	2	2.16±0.21	9.72	−0.50	1.96±0.15	7.76	2.00
	100	96.11±1.93	2.01	−3.45	95.93±1.83	1.91	−2.75
	800	713.03±12.95	1.81	−10.87	732.66±8.14	1.11	−7.49
Storage at−20°C (30 days)	2	1.91±0.04	2.28	−4.50	1.85±0.17	9.42	−7.50
	100	103.35±14.17	13.71	3.35	102.53±13.35	13.02	2.53
	800	716.00±27.71	3.87	−10.50	752.44±18.95	2.51	−5.94
Three freeze (thaw cycle)	2	1.94±0.04	2.36	−3.00	1.96±0.15	8.01	−2.00
	100	96.57±3.52	3.65	−3.43	95.13±2.27	2.38	−4.87
	800	718.00±17.52	2.44	−10.25	737.16±30.63	4.15	−7.80

RSD: Relative standard deviation; RE: Relative error

800 ng/ml. The results for piplartine and piperine are summarized in Table 2. The precisions of the low-level QC samples were all <15% (RSD), and the precisions of the high and medium levels were all <10% (%RSD); the accuracies of three QC samples ranged from 80% to 120%. The precision and accuracy results were satisfactory at the three concentrations studied.

Recovery

As shown in Table 3, the recoveries of rat plasma after protein precipitation ranged from 103% to 91.2% for piperine and from 97.6% to 90.3% for piplartine at the three QC concentration levels. The recoveries were both within the criteria for acceptability. These results suggested that the method was free from matrix effect.

Stability

The detailed results for the stabilities of piplartine and piperine in rat plasma are shown in [Table 4]. Piperine and piplartine in rat plasma were stable for 30 days when stored at −20°C. The %RE of piplartine and piperine 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.

Pharmacokinetic study

The mean concentration-time data were subjected to noncompartmental pharmacokinetic analysis using linear trapezoidal rule. Figure 3 shows the mean plasma concentration-time profile of piplartine and piperine. The pharmacokinetic parameters such as C_{max}, $T_{max,}$ $t_{1/2,}$ $AUC_{0\text{-}t,}$ and $AUC_{0\text{-}\infty}$ for piperine and piplartine are summarized in Table 5.

Table 5: Pharmacokinetic parameters for piplartine and piperine in rat plasma after oral administration of *Piper chaba* root extract (mean±standard deviation, *n*=5)

Pharmacokinetic parameters	Mean±SD	
	Piplartine	Piperine
C_{max} (ng/mL)	323±86.75	1502±80
T_{max} (h)	0.38±0.10	0.95±0.2
$t_{1/2}$ (h)	2.82±0.46	2.65±0.5
AUC_{0-t} (ng/mL h)	623.51±228	5154±208
$AUC_{0-\infty}$ (ng h/mL)	640±216	5027±216

T_{max}: Time to maximum effect; C_{max}: Maximum concentration; AUC: Area under curve; $t_{1/2}$: Half life; SD: Standard deviation

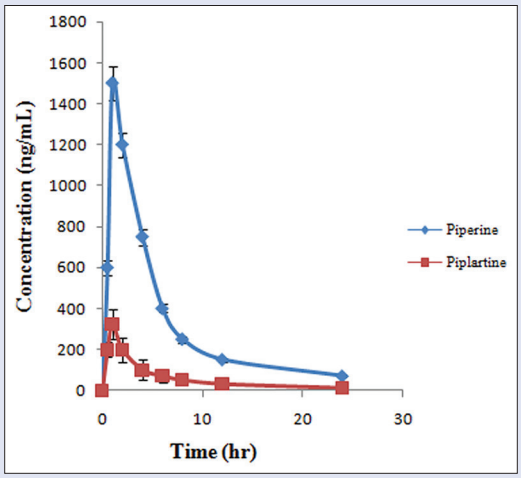

Figure 3: The concentration-time curves of piplartine and piperine in rat plasma after oral administration of *Piper chaba* root extract

CONCLUSIONS

A rapid and sensitive LC/MS/MS method has been developed, for the simultaneous determination of piplartine and piperine in rat plasma after oral administration of 100 mg/kg *P. chaba* extract. The pharmacokinetic results may help to better understand the pharmacological actions of the herb *P. chaba*. The method had excellent sensitivity, good linearity of response, and high precision and accuracy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 2006;71:1397-421.
2. Zheng Y. Effects of Chinese medicine on poliovirus and other intestinal virus. Chin Med J 1964;50:521.
3. Liu CX. The understanding drug metabolism and pharmacokinetics of traditional Chinese medicines. Asian J Drug Metab Pharmcokine 2005;5:25-36.
4. Kirtikar KR, Basu BD. Indian Medicinal Plants. Vol. 3. New Delhi, India: Periodical Experts Book Agency; 1933. p. 2128.
5. The Wealth of India, Raw Materials. Vol. 8. New Delhi: NISCAIR, CSIR; 1969. p 83.
6. Geran RI, Greenberg NH, Macdonald MM. Protocols for screening chemical agents and natural products against tumor and other biological systems. Cancer Chemother Rep 1972;3:1-103.

7. Kirtikar KR, Basu BD. Indian Medicinal Plants. Vol. 3. New Delhi, India: Periodical Experts Book Agency; 1933. p. 2130.
8. Jacobson M, Crosby DG. Naturally Occurring Insecticides. Vol. 144. New York: Dekker; 1971. p. 226.
9. Kuniaki M, Katsura M. The structure of piperenone, a new insect antifeeding substance from *Piper futokadzura*. Tetrahedron Lett 1975;16:1905-8.
10. Sumathykutty MA, Rao JM. 8-Hentriacontanol and other constituents from *Piper attenuatum*. Phytochemistry 1991;30:2075-6.
11. Reddy SV, Srinivas PV, Praveen B, Kishore KH, Raju BC, Murthy US, *et al.* Antibacterial constituents from the berries of *Piper nigrum*. Phytomedicine 2004;11:697-700.
12. Srinivas PV, Rao JM. Isopiperolein B: An alkamide from *Piper nigrum*. Phytochemistry 1999;52:957-8.
13. Pullela SV, Tiwari AK, Vanka US, Vummenthula A, Tatipaka HB, Dasari KR, *et al.* HPLC assisted chemobiological standardization of alpha-glucosidase-I enzyme inhibitory constituents from *Piper longum* linn – An Indian medicinal plant. J Ethnopharmacol 2006;108:445-9.
14. Boonen J, Bronselaer A, Nielandt J, Veryser L, De Tré G, De Spiegeleer B, *et al.* Alkamid database: Chemistry, occurrence and functionality of plant N-alkylamides. J Ethnopharmacol 2012;142:563-90.
15. Domínguez XA, Alcorn JB. Screening of medicinal plants used by huastec mayans of Northeastern Mexico. J Ethnopharmacol 1985;13:139-56.
16. Morikawa T, Matsuda H, Yamaguchi I, Pongpiriyadacha Y, Yoshikawa M. New amides and gastroprotective constituents from the fruit of *Piper chaba*. Planta Med 2004;70:152-9.
17. Virinder SP, Subhash CJ, Kirpal SB, Rajini J, Poonam T, Amitabh J, *et al.* Phytochemistry of the genus *Piper*. Phytochemistry 1997;46:597-73.
18. Singh A, Duggal S. Piperine – A review of pharmacological properties. Int J Pharm Sci Nanotech 2009;2:615-62.
19. Bezerra DP, Moura DJ, Rosa RM, de Vasconcellos MC, e Silva AC, de Moraes MO, *et al.* Evaluation of the genotoxicity of piplartine, an alkamide of *Piper tuberculatum*, in yeast and mammalian V79 cells. Mutat Res 2008;652:164-74.
20. Moraes JD, Nascimento C, Lopes PO, Nakano E, Yamaguchi LF, Kato MJ, *et al.* Schistosoma mansoni: *In vitro* schistosomicidal activity of piplartine. Exp Parasitol 2011;127:357-64.
21. Rodrigues RV, Lanznaster D, Longhi Balbinot DT, Gadotti Vde M, Facundo VA, Santos AR, *et al.* Antinociceptive effect of crude extract, fractions and three alkaloids obtained from fruits of *Piper tuberculatum*. Biol Pharm Bull 2009;32:1809-12.
22. Bhat BG, Chandrasekhara N. Determination of piperine in biological tissues by thin-layer chromatography and ultraviolet absorption densitometry. J Chromatogr 1985;338:259-63.
23. Bajad S, Singla AK, Bedi KL. Liquid chromatographic method for determination of piperine in rat plasma: Application to pharmacokinetics. J Chromatogr B Analyt Technol Biomed Life Sci 2002;776:245-9.
24. Bajad S, Johri RK, Singh K, Singh J, Bedi KL. Simple high-performance liquid chromatography method for the simultaneous determination of ketoconazole and piperine in rat plasma and hepatocyte culture. J Chromatogr A 2002;949:43-7.
25. Vadaparthi PR, Kumar K, Pawar AK, Babu KS. Matrix-solid phase dispersion extraction followed by HPLC-DAD method for the determination of major constituents in *Piper chaba* root. J Nat Prod Resour 2017;3:115-8.
26. Bajad S, Coumar M, Khajuria R, Suri OP, Bedi KL. Characterization of new rat urinary metabolite of piperine by LC/NMR/MS. Eur J Pharm Sci 2003;19:413-21.
27. Boonen J, Baert B, Burvenich C, Blondeel P, De Saeger S, De Spiegeleer B, *et al.* LC-MS profiling of N-alkylamides in *Spilanthes acmella* extract and the transmucosal behaviour of its main bio-active spilanthol. J Pharm Biomed Anal 2010;53:243-9.
28. Sharma V, Boonen J, Chauhan NS, Thakur M, De Spiegeleer B, Dixit VK, *et al. Spilanthes acmella* ethanolic flower extract: LC-MS alkylamide profiling and its effects on sexual behavior in male rats. Phytomedicine 2011;18:1161-9.
29. Boonen J, Sharma V, Dixit VK, Burvenich C, De Spiegeleer B. LC-MS N-alkylamide profiling of an ethanolic *Anacyclus pyrethrum* root extract. Planta Med 2012;78:1787-95.
30. Liu H, Luo R, Chen X, Liu J, Bi Y, Zheng L, *et al.* Tissue distribution profiles of three antiparkinsonian alkaloids from *Piper longum* L. in rats determined by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2013;928:78-82.
31. Basu S, Patel VB, Jana S, Patel S. Liquid chromatography tandem mass spectrometry method (LC-MS/MS) for simultaneous determination of piperine, cinnamic acid and gallic acid in rat plasma using a polarity switch technique. Anal Methods 2013;5:967-76.
32. Ramesh B, Rao Vadaparthi PR, Sukumar G, Manjula N, Suresh Babu K, Sita Devi P, *et al.* LC-HRMS determination of piperine on rat dried blood spots: A pharmacokinetic study. J Pharm Anal 2016;6:18-23.
33. Kakarala M, Dubey SK, Tarnowski M, Cheng C, Liyanage S, Strawder T, *et al.* Ultra-low flow liquid chromatography assay with ultraviolet (UV) detection for piperine quantitation in human plasma. J Agric Food Chem 2010;58:6594-9.
34. Liu J, Bi Y, Luo R, Wu X. 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. J Chromatogr B Analyt Technol Biomed Life Sci 2011;879:2885-90.
35. Sachin BS, Najar IA, Sharma SC, Verma MK, Reddy MV, Anand R, *et al.* Simultaneous determination of etoposide and a piperine analogue (PA-1) by UPLC-qTOF-MS: Evidence that PA-1 enhances the oral bioavailability of etoposide in mice. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:823-30.
36. Rao VR, Muthenna P, Shankaraiah G, Akileshwari C, Babu KH, Suresh G, *et al.* Synthesis and biological evaluation of new piplartine analogues as potent aldose reductase inhibitors (ARIs). Eur J Med Chem 2012;57:344-61.
37. Food and Drug Administration, Guidance for Industry. Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine; 2001. Available from: http://www.fda.gov/cder/guidance/index.htm. [Last accessed on 2018 May 01].