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A sensitive LC–MS/MS method to quantify loureirin B in rat plasma with application to preclinical pharmacokinetic studies

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ABSTRACT

A novel method for the quantification of loureirin B in rat plasma using high-performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) was developed. Loureirin B and internal standard (buspirone) were extracted by liquid–liquid extraction and separated on a Agilent XDB C_{18} column (50 mm × 4.6 mm, 5 μm). As mobile phase a binary mixture of methanol (containing 0.1% formic acid)–water (containing 0.1% formic acid) was delivered by a Shimadzu LC-20AD pump in gradient mode at a flow rate of 0.4 ml/min in a run time of 5.0 min. The detector was a Q-trap™ mass spectrometer with an electrospray ionization (ESI) interface operating in the multiple reaction monitoring (MRM) mode. The calibration curve of loureirin B in plasma showed good linearity over the concentration range of 0.08–100 ng/ml. The limit of detection and limit of quantification were 0.03 ng/ml and 0.08 ng/ml, respectively. Intra- and inter-day precisions (as relative standard deviation) in all samples were both within 15%. The validated method was successfully applied to a preliminary pharmacokinetic study of loureirin B in rats. After oral administration of 16 g/kg longxuejie to rats, the main pharmacokinetic parameters t_{max}, C_{max}, $t_{1/2}$, K_e and AUC_{0-T} were 0.8 h, 7.99 μg/l, 1.94 l h, 0.365/h, and 22.21 μg h/l, respectively.

1. Introduction

Longxuejie (resina isolated from *Dracaena cochinchinensis* (Lour.) S.C. Chen) is one rare traditional Chinese medicinal herbal drug and has been used almost for 1500 years since ancient China [1]. Longxuejie, also called "Dragon's blood" as another Chinese name, distributes mainly in Yunnan, Guanxi and Hainna province in China [2]. There are many kinds of chemical constituents in longxuejie including flavones, saponines, steroids and volatile oils [3–6]. Pharmacological studies show that longxuejie could inhibit platelet aggregation, venous thrombosis and bacterial activity. It also shows anti-inflammatory, analgesic and homeostatic effects [7,8]. Recently, longxuejie has been clinically used for treatment of cerebral arterial thrombosis, ischemic heart disease and peptic ulcer [8,9]. Loureirin B is one single active component isolated form longxuejie and its chemical structure was shown in Fig. 1. The content of loureirin B reaches approximately 0.4% in longxuejie crude herb. It has been listed in National drug standards as a quality control index for longxuejie crude herb by State Food and Drug Administration of China [10]. Literature reports that loureirin B could restrain the formation of thrombus in rat, and reduce the viscosity of whole blood in the model of acute blood stasis rats [11]. Loureirin B also shows significantly analgesic effects in rats and mice [12,13]. Thus, loureirin B has been used as one of marker compounds for quality control of longxuejie crude herb or its preparations now.

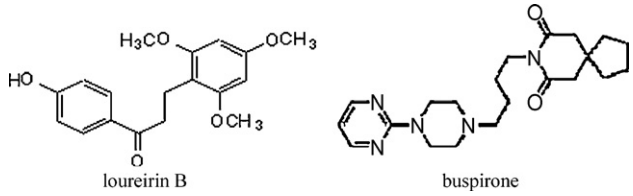

Fig. 1. Chemical structures of loureirin B and buspirone.

HPLC-UV methods for the determination of loureirin B in longxuejie crude herb or preparations containing longxuejie have been found in many papers [14–17], while reports on assay of loureirin B in bio-samples or pharmacokinetic study of loureirin B in experimental animals are unavailable until now. For further clinical application of longxuejie, it is necessary to develop one sensitive method for the determination of loureirin B in bio-samples and to obtain pharmacokinetic information of loureirin B in experimental animals.

In the present study, a sensitive and rapid method for quantitation of loureirin B in rat plasma was developed by high-performance

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liquid chromatographic separation with tandem mass spectrometric detection for the first time. This well validated LC/MS/MS method was successfully applied for pharmacokinetic study of loureirin B after oral administration of longxuejie to rats.

2. Experimental

2.1. Chemicals and reagents

Longxuejie was kindly supplied by Yulin Pharmacy Company (Xishuangbanna, China). Reference standard of loureirin B (98.7%, purity) and buspirone (used as an internal standard, I.S.) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structure of buspirone was also shown in Fig. 1. Methanol of HPLC grade was purchased from Yili Chemical Co. (Beijing, China). Distilled water, prepared by a Milli-Q water purification system from Millipore (Molsheim, France), was used throughout the study. All other chemicals were of analytical grade. Blank rat plasma (drug free and anti-coagulated with heparin sodium) was prepared in our laboratory.

2.2. Stock solutions

All concentrations of loureirin B refer to the free base. A stock solution of loureirin B was prepared in methanol at a concentration of 1.0 mg/ml. Standard solutions (0.80, 2.00, 4.00, 20.0, 40.0, 80.0, 200 and 1000 ng/ml) and quality control solutions (2.00, 40.0 and 800 ng/ml) were prepared by serial dilution of the stock solution with methanol–water (50:50, v/v). A stock solution of buspirone (1.0 mg/ml) was also prepared in methanol and then diluted with methanol–water (50:50, v/v) to get a final concentration of 100 ng/ml. All solutions were stored at 4 °C and used within one month after preparation.

2.3. LC–MS/MS analysis

A Shimadzu LC-20AD series HPLC system (Shimadzu, Japan) coupled to an Applied Biosystems Sciex Q-trap™ mass spectrometer (Concord, Ontario, Canada) via an electrospray ionization (ESI) source was used for analysis. Applied Biosystems Analyst software version 1.3.2 package was used to control the LC–MS/MS system and for data acquisition and processing. Loureirin B and I.S. were separated on an Agilent XDB-C_{18} column (4.6 mm × 50 mm, 5 μm, Agilent Technologies, USA) maintained at 20 °C. The mobile phase consisted of A: water (containing 0.1% formic acid) and B: methanol (containing 0.1% formic acid) and was delivered at a flow rate of 0.4 ml/min. The gradient was as follows: 0 min 20% B, 0.5 min 20% B, 2 min 95% B, 3 min 95% B, 3.01 min 20% B, 5 min stop. A two phase switching valve was used to divert the pre-eluent from entering the ion source.

The mass spectrometer was operated in the 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 activated dissociation (CAD) gas. The precursor-to-product ion transitions were monitored at m/z 317 → 181 for loureirin B and at m/z 386 → 122 for buspirone. Mass spectrometer instrumental parameters were tuned to maximize the generation of precursor and fragment ions by infusion of a solution of loureirin B and I.S. into the ESI source at 10 μl/min. Optimum parameters were as follows: nebulizer (GS1), heater (GS2) and curtain gas flow rates 60, 55 and 20 units, respectively; ionspray needle voltage 5000 V; heater gas temperature 450 °C; collision gas (N_2) medium; declustering potential 31 V for loureirin B and 91 V for buspirone; collision energies 15 eV for loureirin B and 12 eV for buspirone.

2.4. Sample preparation

After thawing at room temperature for about 30 min, plasma samples were vortexed. 50 μl plasma sample, 10 μl I.S. solution and 5 μl methanol–water (50:50, v/v) were transferred into 2.0 ml tubes. The mixture was vortexed for 10 s and extracted with 500 μl methyl *tert*-butyl ether (MTBE). After vortexing for 1 min and centrifuging at 3500 rpm for 10 min, aliquots of 300 μl upper organic phase were carefully transferred to another tube and evaporated at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl methanol–water (50:50, v/v) and 10 μl injected into the LC–MS/MS system. Serial standard and QC samples were prepared following above method.

2.5. Assay validation

Blank rat plasma samples were spiked with standard solutions loureirin B to get the final concentration ranges (0.08, 0.2, 0.4, 2, 4, 8, 20 and 100 ng/ml). Linearity was assessed by weighted linear regression ($1/x^2$) of analyte–internal standard peak area ratios based on three independent calibration curves prepared on each of three separate days. x was the concentration of lourerin B in spiked samples. The limit of detection (LOD) was defined as signal/noise ratio of 3. The limit of quantitation (LOQ) was defined as the lowest drug concentration that can be determined with a relative error (RE) and precision (relative standard deviation, R.S.D.) of less than 20%.

Quality control samples (QC samples, 0.2, 4 and 80 ng/ml) were prepared to evaluate the accuracy, precision, recovery of the assay. Accuracy (expressed as relative error, RE) and intra- and inter-day precisions (expressed as relative standard deviation, R.S.D.) were assessed by assay of six replicate QC samples on three different days. Recovery of loureirin B was determined by comparing peak areas of extracted QC samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations. Matrix effects for loureirin B were 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. Stability of loureirin B was evaluated using QC samples subjected to three freeze–thaw cycles, stored at −20 °C for one month and at room temperature for 12 h. Stability in stock solutions (4 °C) and mobile phase on storage in plastic autosampler vials under autosampler conditions for 12 h were also assessed.

2.6. Pharmacokinetic study

Six Wistar rats (200–220 g, three males and three females, purchased from Experimental Animal Institute of the Medical Science Academy, Beijing, China) were used in pharmacokinetic studies. The rats were fasted for 12 h and had free access to water before dosing. After oral administration of 16 g/kg longxuejie to rats through gavage, serial blood samples (0.3 ml) were collected by retro-orbital puncture at 0, 0.083, 0.17,0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 18 h, and then centrifuged at 5000 rpm for 5 min immediately. All plasma samples were stored at −20 °C until analysis. The plasma concentrations of loureirin B at different time points were expressed as mean ± S.D. (standard deviation, S.D.). The concentration versus time curve was plotted. The main pharmacokinetic parameters $t_{1/2}$ (the biological half-life), K_e (elimination rate constant) and AUC_{0-T} (area under curve) were calculated by DAS (Drug and Statistics) 2.0 statistical software (Mathematical Pharmacology Professional Committee of China). The parameter t_{max} (the time to reach peak concentration) was obtained from the time point corresponding to peak concentration of loureirin B in rat plasma. C_{max} (the peak concentration) was determined from peak concentration of loureirin B in rat plasma after oral administration.

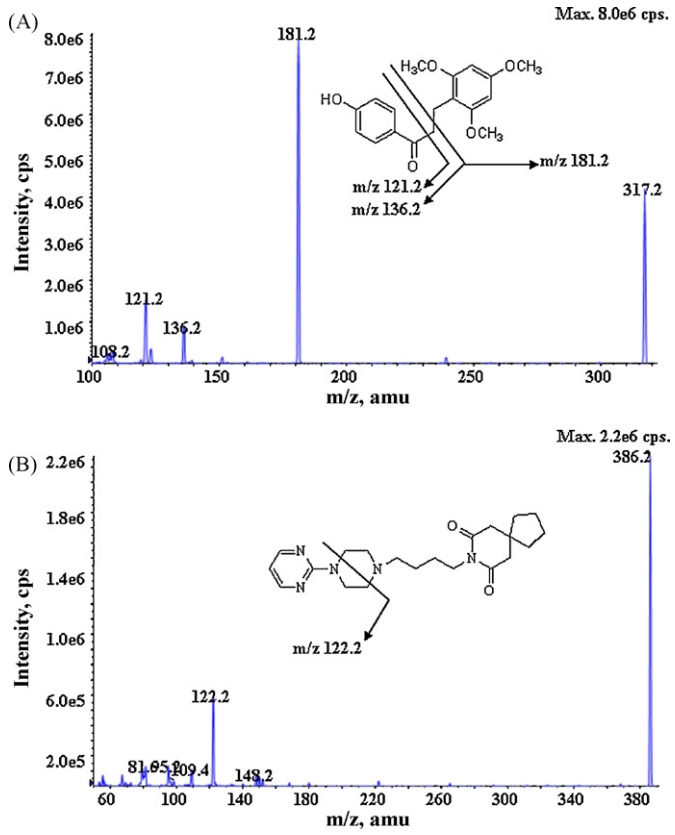

Fig. 2. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways for (A) loureirin B and (B) buspirone. Intensity cps, ion intensity is in counts per second.

3. Results and discussion

3.1. Method development

Several possible internal standards were tested under present experimental conditions including propranolol, buspirone and spinosin. Propranolol was first considered as I.S. but the signal suppression of loureirin B relative to that of propranolol increased with increasing loureirin B concentration, potentially affecting the linearity of the assay. Spinosin could not give satisfactory retention time under such experimental condition. Buspirone was selected as the I.S. because its chromatographic behavior and extraction efficiency were similar to those of loureirin B.

As regards mass spectrometer detection, both loureirin B and buspirone produced strong signals in the positive ion mode due to the presence of functional groups in their structures. The ion spray voltage was set at 5000 V to reduce in-source dissociation and the source temperature for GS2 to 450 °C. Other parameters were adjusted to optimize ionization. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways of loureirin B and buspirone are shown in Fig. 1. The transition m/z 317 → 181 was chosen for quantitation of loureirin B and m/z 386 → 122 was used as the qualifier.

A number of commercially available reversed phase HPLC columns and various mobile phases were evaluated for chromatographic behavior and the ionization responses of loureirin B and I.S. The best response was obtained from gradient delivery of a mixture of water (containing 0.1% formic acid) and methanol (containing 0.1% formic acid). An Agilent XDB-C_{18} column (4.6 mm × 50 mm, 5 μm) with gradient delivery gave satisfactory chromatographic results with minimal matrix effects. In this assay, no significant signal suppression or enhancement was found under current conditions.

Table 1
Precision and accuracy for the determination of loureirin B in rat plasma by LC–MS/MS method ($n = 6$).

Nominal concentration (ng/ml)	Average measured concentration (ng/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy RE (%)
0.20	0.22	7.3	9.7	9.0
4.00	4.11	2.0	2.6	2.8
80.0	81.4	0.36	1.1	1.7

In terms of sample preparation, loureirin B and buspirone are sufficiently lipophilic to allow a one-step liquid–liquid extraction procedure and give satisfactory recovery. Several extraction solvents (diethyl ether, dichloromethane, ethyl acetate and methyl *tert*-butyl ether) were tested to optimize recovery (Fig. 2).

3.2. Assay validation

The detection of loureirin B and buspirone by MRM was highly selective with no significant interferences. Typical chromatograms are shown in Fig. 3. The run time was set to be 5 min because full chromatographic separation was also necessary to avoid matrix effect. The standard curve was linear over the range of 0.08–100 ng/ml with an LOQ of 0.08 ng/ml and an LOD of 0.03 ng/ml. The linear regression of the curve for the peak area ratio of loureirin B to I.S. (y) versus concentration of loureirin B (x) was plotted

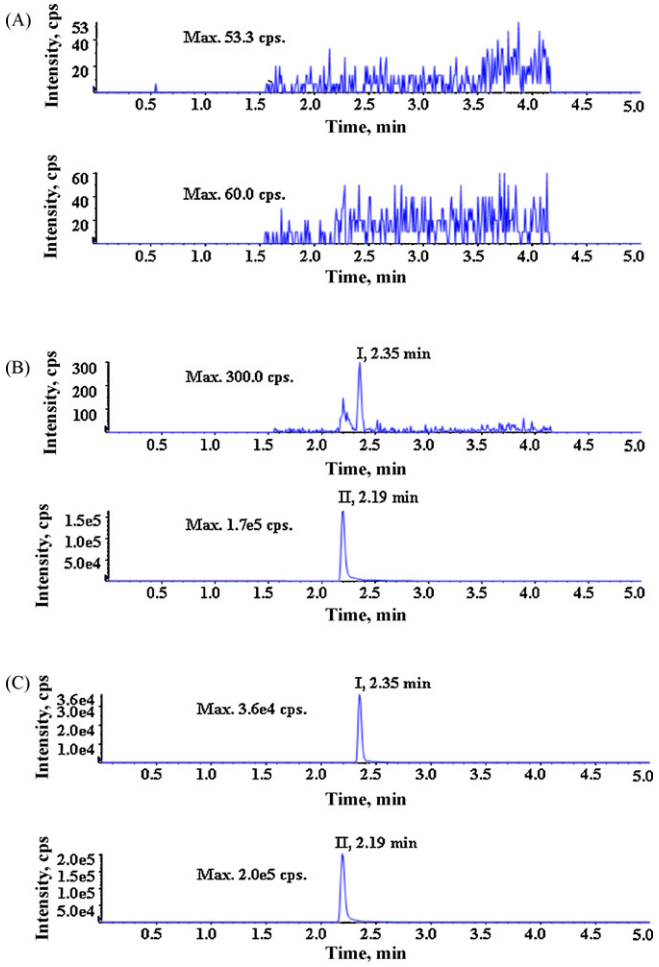

Fig. 3. Typical chromatograms of (A) blank plasma, (B) blank plasma spiked with loureirin B (0.08 ng/ml) and I.S. (2.0 ng/ml) and (C) a real plasma sample collected at 0.75 h after an oral dose of 16 g/kg longxuejie. Peak I, loureirin B; Peak II, buspirone. Intensity cps, ion intensity is in counts per second.

Table 2
The extraction recoveries of loureirin B and buspirone in rat plasma by LC–MS/MS method ($n = 6$).

Compound	Added concentration (ng/ml)	Extract recovery (%)	R.S.D. (%)
Buspirone	2.0	75	4.8
Loureirin B	0.2	87.0	3.1
	4.0	89.1	7.4
	80	90.4	4.6

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

Conditions	Spiked concentration (ng/ml)		
	0.2	4.0	80
Freeze–thaw stability (relative error (%))			
0 cycle	8.0	−1.3	2.1
3 cycles	−4.0	3.8	2.4
Storage stability at −20 °C (relative error (%))			
0 day	5.0	3.0	1.9
30 days	−6.7	0.63	2.5
Processed plasma samples at 20 °C (relative error (%))			
0 h	3.8	−2.9	4.9
24 h	9.1	7.2	1.4

for plasma sample. A typical equation of the standard curve was $y = 0.037, x \pm 0.00516, R = 0.9988$. Intra-day and inter-day precisions were less than 9.7%. Accuracy ranged from 1.7% to 9.0%. Precision and accuracy (shown in Table 1) were satisfactory at the three concentrations investigated.

The best recovery was obtained with methyl *tert*-butyl ether. Recovery result was presented in Table 2. Recoveries of loureirin B at 0.20, 4.00 and 80.0 ng/ml were 87.0%, 89.1% and 90.4%, respectively. Recovery of the I.S. was approximately 75%. The stability of loureirin B in drug-free plasma was found to be satisfactory (RE < ±8.0%, Table 3) under all the conditions examined. Loureirin B both in stock solutions kept at 4 °C and in mobile phase under autosampler conditions for 12 h were rather stable (>98% drug remaining, data not shown).

3.3. Pharmacokinetic study

Mean plasma concentration–time profile of loureirin B after oral administration of 16 g/kg longxuejie was shown in Fig. 4. The main pharmacokinetic parameters were listed in Table 4. Pharmacokinetic results showed that loureirin B reached peak concentration at 0.8 h in plasma of rats after oral administration and it was cleared little quickly from rats. In our previous study, effect of longxuejie on cerebral blood flow in rats with ischemic stroke was also evaluated. Those results indicated that significant increase in cerebral blood flow of rats was observed around 1 h after oral administration of longxuejie at a single dose (data not published). It could be concluded that pharmacokinetic result consisted with our previous findings in pharmacological experiments.

In the present study, only free loureirin B was determined in rat plasma samples. Conjugated metabolites of loureirin B such as sulphate and glucoside metabolites were detected in rat plasma samples. Metabolites of loureirin B in plasma still needs for further study.

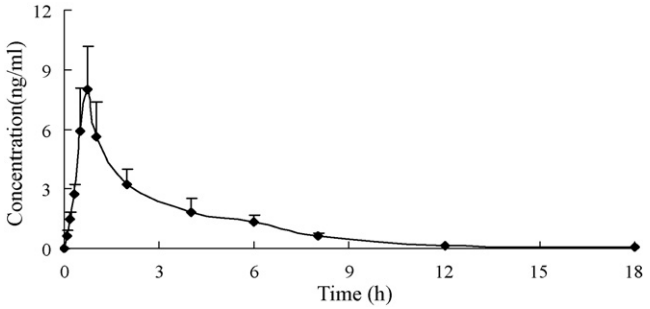

Fig. 4. Mean plasma concentration–time profile of loureirin B determined by LC–MS/MS method after oral administration of 16 g/kg longxuejie to rats. Each point represents the mean ± S.D. ($n = 6$).

Table 4
Pharmacokinetic parameters of loureirin B after oral administration of longxuejie (16 g/kg) to rats ($n = 6$, Mean ± S.D.).

Parameters	Values	Parameters	Values
K_e (/h)	0.365 ± 0.06	AUC_{0-T} (μg h/l)	22.2 ± 1.9
$t_{1/2}$ (h)	1.94 ± 0.27	$AUC_{0-\infty}$ (μg h/l)	22.6 ± 1.8
t_{max}(h)	0.81 ± 0.11	CL (l/h/kg)	978.2 ± 117.3
C_{max} (μg/l)	7.99 ± 1.2	Vc (l/kg)	2768.6 ± 751.5

4. Conclusions

A simple, sensitive and selective LC–MS/MS method for the determination of loureirin B in rat plasma has been developed for the first time. This validated method was shown to be suitable for pharmacokinetic study of loureirin B after oral administration of 16 g/kg longxuejie.

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