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A Rapid and Sensitive UPLC-MS/MS Method for Quantification of Bruceantinol in Rat Plasma and Its Application to a Pharmacokinetic Study

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ABSTRACT

Bruceantinol (BOL), a quassinoid compound isolated from the fruits of *Brucea javanica*, has been reported to have cytotoxic and antibacterial effects. In this study, a rapid, sensitive, and specific ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method was developed for the quantitative determination of BOL in rat plasma. The samples were treated by simple liquid-liquid extraction with ethyl acetate and separated on an UPLC BEH C18 column (2.1 mm × 50 mm) using a 3-min gradient elution scheme, which consists of water (0.1% v/v, formic acid) and methanol (0.1%, v/v, formic acid) to achieve the separation of BOL and sinomenine (IS) with high selectivity. The electrospray ionization source was used in positive ion mode; the multiple reaction monitoring quantified the target fragment ions *m/z* 629.6 → 569.5 for BOL and *m/z* 330.5 → 207.3 for IS. This work was evaluated with regards to the specificity, extraction recovery, matrix effect, linearity, accuracy, precision, stability, and dilution integrity. This approach was used to examine the pharmacokinetics of BOL in rats after oral (0.3 mg/kg) and intravenous (0.15 mg/kg) administration. BOL presented fast excretion and very low oral bioavailability.

Introduction

As an evergreen shrub, *Brucea javanica* (L.) Merr. (Simaroubaceae) is widespread in northern Australia and Southeast Asia [1]. In Southeast Asia, all parts of *B. javanica* are used as an antimalarial agent, and the fruits of this plant (such as "ya dan zi," "ya tan tzu," "kho-sam," "Macassar kernals," and "Makassaarse pitjes") are also employed as folk herbs for the treatment of various ailments, including cancer, amoebic dysentery, and skin disorders [2]. In China, *B. javanica* seed oil has long been used as an available Chinese patented medicine for the treatment of malignant tumors, such as hepatoma, lung carcinoma, prostate cancer, and breast tumor [3]. It has been observed that plants in the genus *Brucea* are rich in quassinoids that have a broad spectrum of biological activities, including potential antimalarial, anti-HIV, anti-tuberculosis, anti-tumor, cancer chemoprophylaxis, and cytotoxic activities [4].

Bruceantinol (BOL, $C_{30}H_{38}O_{13}$, molecular weight 606.2, ▸ **Fig. 1**) is a typical member of the quassinoid group of compounds and was isolated from the fruit of *B. javanica* and the stem bark of *Brucea antidysenterica* Mill. (Simaroubaceae) [5]. Notably,

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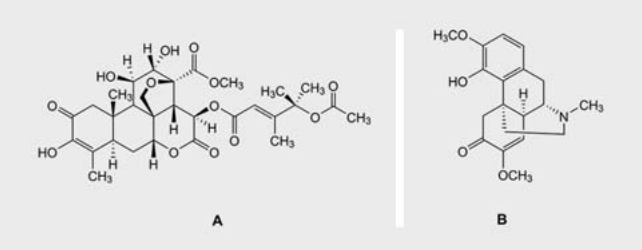

▶ **Fig. 1** The chemical structure of BOL (**A**) and IS (**B**).

BOL possesses antitrypanosomal, antibabesial, and anti-tumor effects [6–9]. Additionally, its analogs, including bruceantin, bruatol, bruceine A, and bruceine D, have exhibited different biological effects, including antitrypanosomal, anthelmintic, antibabesial, hypoglycemic, and cytotoxic activities [10–14]. Several quassinoid compounds have been evaluated as novel drug candidates in preclinical or clinical trials in the 1980s [15, 16]. However, not enough research has been performed to understand the pharmacokinetic properties of these compounds in proper animal models. To date, no quantitative analytical methods are available to determine BOL in biological fluids. Pharmacokinetic parameters and the bioavailability of BOL remain unknown. Thus, it is important to propose a highly reliable and accurate analytical method for quantifying BOL in biological samples.

A simple, selective, and highly sensitive ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method was proposed and proven for the quantitative measurement of BOL in rat plasma. The chromatography run time was 3 min/sample. This approach was proposed and successfully applied in BOL pharmacokinetic studies after oral and intravenous administration to rats. It is expected that the use of this analytical method and BOL's PK (pharmacokinetics) data could be useful for future studies on BOL and its analogs.

Results and Discussion

The extraction efficiency from both the liquid-liquid extraction and protein precipitation was compared while preparing the samples. The results from the liquid-liquid extraction method were much better, as this process can remove many endogenous substances, thus improving the BOL detection limits. Several organic reagents, such as ethyl acetate, dichloromethane, methyl tert-butyl ether and diethyl ether, were examined as potential extract solvents. Ethyl acetate was chosen as the optimal solvent because it supplied more sensitive and stable extraction recoveries for BOL and sinomenine (IS) and displayed lower toxicity during sample preparation [17].

Optimization of the ionization conditions for the analysis of BOL and IS was carefully performed by adapting the UPLC-MS/MS operating parameters. Using a full-range scan in positive and negative ionization modes, the signal intensities of BOL and IS were evaluated through the flow injection of their authentic solutions. No signals of BOL were observed in negative ionization mode. In the mass spectrometer, BOL and IS yielded major $[M + Na]^+$ ions at *m/z* 629.6 and $[M + H]^+$ ions at *m/z* 330.5, respectively. Thus, in multiple reaction monitoring (MRM) positive mode, BOL showed the most intense transition $[M + Na]^+$ at *m/z* 629.6 → 569.5 (as a quantifier) and a second transition at *m/z* 629.6 → 461.3 (as a qualifier); the most intense transition for IS was at *m/z* 330.5 → 207.3. UPLC-MS/MS conditions and representative mass spectra of BOL and IS, as well as NMR spectra (^{1}H, ^{13}C, HSQC, and HMBC) and NMR data of BOL, are available as Supporting Information.

To achieve optimal chromatographic behaviors (with high resolution and reproducibility, symmetrical peaks, good baseline, and shorter retention times), various liquid chromatographic conditions (sorts of column; composition, pH value, flow rate, and proportion of mobile phase; and temperature sets) were investigated for the BOL pharmacokinetics study. Finally, a gradient mobile phase of 0.1% formic acid in water and methanol was determined because it exhibited better reproducibility and separation during the process. Under these chromatographic conditions, the retention times of BOL and IS were 2.16 and 0.54 min, respectively, in a run time of 3 min. No endogenous peaks were observed at the retention times for either BOL or IS. The specificity was determined using representative chromatograms from blank plasma, lower limit of quantification (LLOQ), and the rat plasma samples. ▶ **Fig. 2** shows that our assay was free of endogenous interference from all plasma analytes in the corresponding BOL and IS retention times.

The extraction recovery and matrix effects of BOL and IS in the plasma are summarized in ▶ **Table 1**. The absolute extraction recovery (%) of BOL for quantification from the four quality control (QC) concentration levels was 82.3 ± 4.7%, 86.2 ± 5.6%, 90.9 ± 6.1%, and 85.4 ± 5.8%. Considering the matrix effects, the ratios of the MS peak response for BOL were 80.8 ± 4.9%, 92.9 ± 7.0%, 83.0 ± 3.9%, and 81.4 ± 6.8% at 2, 20, 200, and 1000 ng/mL, respectively. These results demonstrated that co-eluting plasma interference did not significantly inhibit BOL ionization and that the influence of the plasma matrix was consistent in our measurements. The BOL calibration curve exhibited nice linearity in the concentration range from 1.0–2000 ng/mL. The typical standard curve was demonstrated by $y = 0.001606x + 0.000287$, where y represents the peak area ratio of the analytes to IS, and x is the BOL plasma concentration. The BOL LLOQ (▶ **Fig. 2B**) was achieved at 1.0 ng/mL, and at that concentration level, both the accuracy and precision were within acceptable limits ($n = 6$, $S/N \geq 10$, and RE = 7.3%). In the method for quantifying of BOL, the accuracy and precision were evaluated by six replicates at each QC level. The experimental data (▶ **Table 2**) demonstrated that the intra-day and inter-day precision were 5.9–12.5% and 3.3–11.6%, respectively, while accuracy was −5.8 to 6.4% and −3.4 to 6.7%, respectively. The stability test on BOL in stored samples and during preparation was conducted by analyzing four QC sample levels. All the experimental results suggested that no significant stability problems were encountered during the analysis of the plasma samples after maintaining the samples at ambient temperature for 2 h, in the −80 °C for 30 d, after three freeze-thaw cycles, or at 4 °C in the autosampler for 24 h (▶ **Table 3**). Six repetitions with the dilution integrity samples at 5000 ng/mL were examined and recalculated as 4530.7 ± 228.1 ng/mL (rela-

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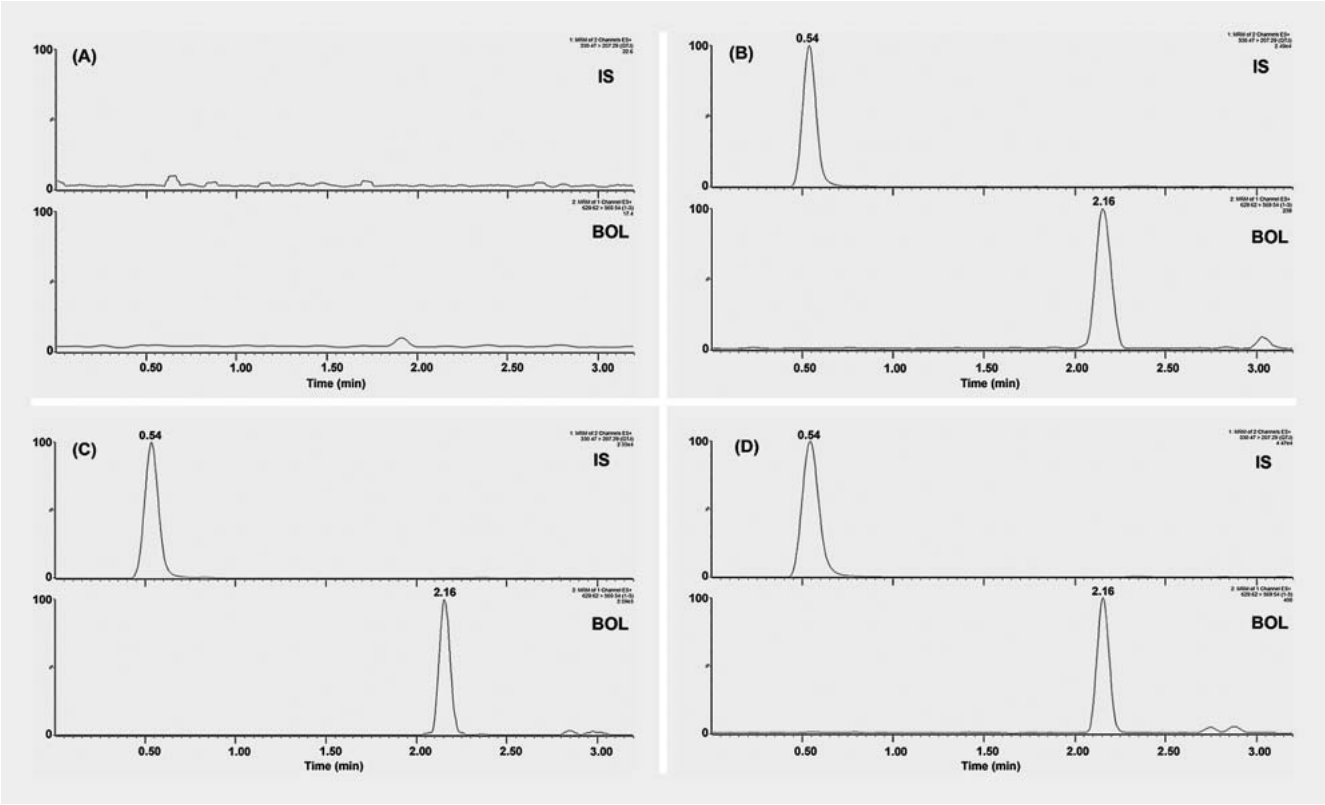

▶ **Fig. 2** **A** Chromatograms of blank rat plasma; **B** LLOQ (1.0 ng/mL); **C** a rat plasma sample at 2 h after intravenous administration of BOL (0.15 mg/kg); **D** a rat plasma sample at 15 min after oral administration of BOL (0.3 mg/kg).

▶ **Table 1** Matrix effect and extraction recovery for determination of BOL in plasma (n = 6, mean ± SD).

Concentration (ng/mL)	Extraction recovery (mean ± SD [%])	Matrix effect (mean ± SD [%])
2	82.3 ± 4.7	80.8 ± 4.9
20	86.2 ± 5.6	92.9 ± 7.0
200	90.9 ± 6.1	83.0 ± 3.9
1000	85.4 ± 5.8	81.4 ± 6.8

▶ **Table 2** Intra-day (n = 6) and inter-day (n = 18) precision and accuracy for assay of BOL in rat plasma.

	Nominal concentration (ng/mL)	Calculated concentration (ng/mL, mean ± SD)	RSD (%)	RE (%)
Intra-day	2	2.0 ± 0.2	12.5	− 2.5
	20	18.8 ± 1.4	7.4	− 5.8
	200	201.3 ± 11.6	5.7	6.4
	1000	967.2 ± 57.1	5.9	− 4.6
Inter-day	2	2.1 ± 0.1	6.4	6.7
	20	21.0 ± 2.4	11.6	4.9
	200	193.3 ± 6.4	3.3	− 3.4
	1000	983.7 ± 54.7	5.6	− 1.6

tive error [RE%], − 9.6%). Thus, the stability and dilution integrity are satisfactory for routine pharmacokinetics studies

The UPLC-MS/MS analytical method described here was constructed to study the pharmacokinetics of BOL in rats. After oral (0.3 mg/kg) and intravenous (0.15 mg/kg) administration of BOL, the mean plasma concentration-time profile is shown in ▶ **Fig. 3**. The main pharmacokinetic parameters from the non-compartment model analysis are listed in ▶ **Table 4**. After a single intravenous dose, the plasma elimination half-life ($t_{1/2}$) of BOL was computed as 2.7 ± 0.4 h, and the total plasma clearance (CL_z) was 0.4 ± 0.1 L/h/kg, while the mean volume of distribution (V_z) was 1.5 ± 0.4 L/kg. After oral administration with twice the intravenous dose, the BOL plasma concentration was very low, with a C_{max} of 7.1 ± 1.2 ng/mL, CL_z (clearance) of 27.3 ± 11.9 L/h/kg, and $t_{1/2}$ of 0.7 ± 0.3 h. Based on the AUC values, the oral absolute bioavailability was $F = (AUC_{p.o.} \times Dose_{i.v.})/(AUC_{i.v.} \times Dose_{p.o.}) \times 100\%$. The absolute oral BOL bioavailability was 1.82 ± 0.62% in rats, which means that it displays poor oral absorption efficiency or strong degradation and metabolism via gastrointestinal administration.

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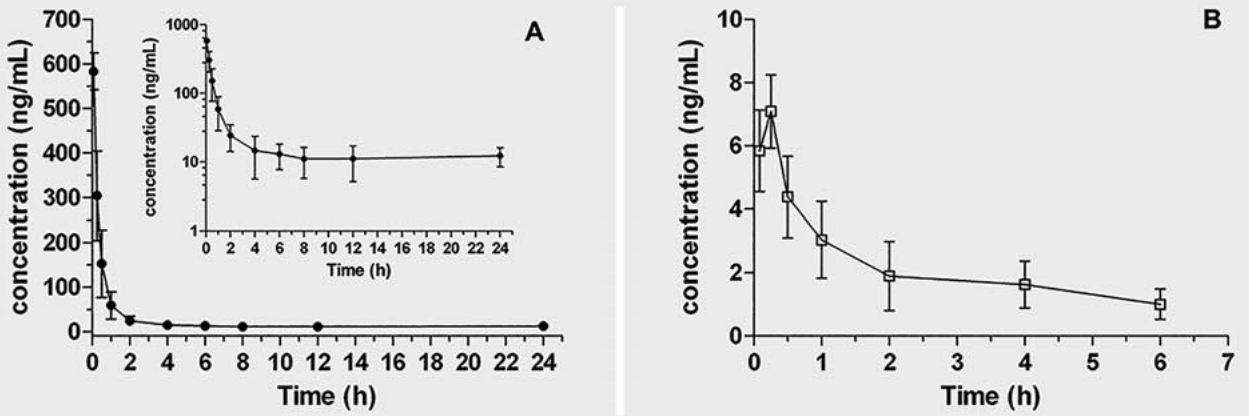
▶ **Table 3** Summary of stability of BOL in rat plasma under different storage conditions (n = 6).

Concentration (ng/mL)	Room temperature (2 h)		− 80 °C (30 d)		Three freeze and thaw cycles		4 °C (24 h)	
	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
2	9.5	− 9.2	8.6	− 14.2	9.5	− 1.7	8.2	− 8.3
20	7.3	− 2.9	6.4	− 9.2	9.4	− 3.9	4.7	− 1.8
200	3.1	1.8	4.8	− 3.7	2.8	0.7	8.2	4.7
1000	4.7	− 3.3	4.0	− 3.8	2.1	− 3.3	− 2.4	1.9



▶ **Fig. 3** Mean plasma concentration-time curve of BOL after intravenous (**A**) and oral administration (**B**) of 0.15 and 0.3 mg/kg, respectively (n = 6).

In conclusion, a rapid, sensitive method based on UPLC-MS/MS was proposed and evaluated in this pharmacokinetics study of BOL in rats. For simplicity during operations, the plasma samples were pretreated before liquid-liquid extraction with ethyl acetate. Chromatographic separation was conducted on a C18 column with a gradient elution with methanol-water (0.1%, v/v, formic acid), and a turnover rate of 3 min per sample. After oral (0.3 mg/kg) and intravenous (0.15 mg/kg) administration in rats, the quantitative analysis for plasma samples was applied to determine the BOL pharmacokinetics profiles. Following a single intravenous administration, BOL was rapidly eliminated from rat plasma and reached a low concentration level; however, there were tiny amounts of BOL detected in the plasma by the oral route. Its fast excretion and low bioavailability suggested that extensive metabolism, low trans-membrane transport, or bulk degradation occurred *in vivo*, likely due to the ester bonds and rigid rings in the BOL chemical structure. Moreover, the esterases present in rat plasma can decompose compounds such as BOL through ester functions, which potentially led to the loss of BOL in the blood. The results described in this manuscript provide helpful information for pharmacokinetics study on BOL in other animal models.

▶ **Table 4** The pharmacokinetic parameters of BOL in rat plasma after oral and intravenous administration (n = 6, mean ± SD).

Parameters	Intravenous (0.15 mg/kg)	Oral (0.3 mg/kg)
$AUC_{0\text{-}t}$ (ng/mL h)	322.2 ± 85.7	11.7 ± 4.0
$AUC_{0\text{-}\infty}$(ng/mL h)	400.8 ± 98.0	12.7 ± 4.3
$MRT_{0\text{-}t}$ (ng/mL h)	10.0 ± 0.2	2.8 ± 0.3
$MRT_{0\text{-}\infty}$ (ng/mL h)	6.1 ± 2.1	2.1 ± 0.5
$t_{1/2}$ (h)	2.7 ± 0.4	0.7 ± 0.3
T_{max} (h)	0.083 ± 0	0.194 ± 0.086
V_z (L/kg)	1.5 ± 0.4	24.8 ± 7.6
CL_z (L/h/kg)	0.4 ± 0.1	27.3 ± 11.9
C_{max} (ng/mL)	616.8 ± 40.8	7.1 ± 1.2
Absolute bioavailability		1.82 ± 0.62%

MRT: mean residence time

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Materials and Methods

Materials

BOL (purity > 98.0%, ▸ **Fig. 1**) and IS (purity > 98.0%) were supplied by Beijing Oriental Xintai Biological Technology Co., Ltd. HPLC-grade methanol and acetonitrile were purchased from Merck. Calcium heparin was from Tianjin Biochem Pharmaceutical Co., Ltd. LC/MS-grade formic acid was bought from Fisher Scientific. All the other reagents were of analytical grade. Ultra-pure water came from a Milli-Q water purification system (Millipore Corp.).

UPLC-MS/MS condition

The UPLC-MS/MS consisted of an Acquity ultra-performance liquid chromatography system with an XEVO TQD triple quadrupole mass spectrometer, which was equipped with an electrospray ionization (ESI) source for mass spectrometry (Waters Corp.). Chromatography was performed on an Acquity BEH column (C18 Waters, 1.7 µm, 2.1 mm × 50 mm) attached to a stainless steel frit filter (0.2 µm). The temperature of column was controlled at 35 °C.

During separation, a gradient elution program was conducted using the following mixtures of organic A (methanol, 0.1%, v/v, formic acid) and aqueous B (water, 0.1%, v/v, formic acid) in media: 0–0.5 min (40–60% A), 0.5–1.0 min (60–98% A), 1.0–2.4 min (98–98% A), 2.4–2.6 min (98–40% A), and 2.6–3.0 min (40–40% A). The rate of flow was set as 0.3 mL/min. A 10 µL aliquot of the sample solution was applied for analysis. Mass spectrometry was conducted with a +ESI method with the following optimal settings: nitrogen as the nebulizer gas, cone gas: 50 L/h, and desolvation gas: 800 L/h, source temperature: 150 °C, desolvation temperature: 400 °C, cone voltage: 78 V, capillary voltage: 3.30 kV, argon as collision gas, collision voltage: 30 eV and collision-induces dissociation. Detection and quantification of all the analytes was conducted under the MRM condition using the unit mass resolution for the characteristic precursor ion → product ion transition pairs. The MRM transition pairs were *m/z* 629.6 → 569.5 and *m/z* 330.5 → 207.3 for BOL and IS, respectively. The data acquisition and system control software was Masslynx 4.1 (Waters Corp.).

Preparation of calibration standards and QC samples

A stock solution of BOL (1 mg/mL) and IS (200 µg/mL) was prepared in methanol. Then, fresh BOL working solutions with different concentrations were prepared by diluting the primary stock solution with methanol. A calibration standard and a QC sample were obtained by adding 10 µL of the corresponding BOL working solution to 90 µL of blank rat plasma. Thus, the final concentrations of the calibration standards were 1, 5, 10, 50, 100, 500, 1000, and 2000 ng/mL for BOL, and the QC samples were 2, 20, 200, and 1000 ng/mL for BOL. The IS working solution (2 µg/mL) was prepared by diluting the IS stock with methanol. All the experimental samples were stored at − 80 °C until analysis.

Sample preparation

Before analysis, all samples previously stored at − 80 °C were thawed at room temperature. In a 1.5 mL v-bottom Eppendorf tube, a 10-µL aliquot of the IS working solution (2 µg/mL) was added to 100 µL of each sample. The mixture was immediately homogenized with a vortex-mixer for 1 min and extracted with 1 mL of ethyl acetate by vortex-mixing for approximately 5 min. Next, each sample was centrifuged (5 min at 16 000 × g), the resulting supernatant (approximately 1 mL) was carefully transferred into a new 2 mL tube, and the organic solvent was discarded at 40 °C with a stream of nitrogen. The residues were reconstituted with 100 µL of methanol : water (4 : 6, v/v) and then vortexed for 5 min. Finally, the mixture was centrifuged at 16 000 × g for 8 min and the resulting supernatant (10 µL) injected for continuous UPLC-MS/MS analysis.

Bioanalytical method validation

Development and validation of the method for quantifying BOL was conducted in line with the U. S. Food and Drug Administration Guidelines [18].

The selectivity of the approach was tested by analyzing blank plasma samples from six different rats to test the potential interference of endogenous substances with BOL and IS. The procedure for handling the blank plasma samples was the same as described in the "Sample preparation" section.

The matrix effect on ionization efficiency was investigated with blank plasma extracts (six rats) and water extracts spiked to equivalent QC concentration levels (2, 20, 200, and 1000 ng/mL). The extraction recovery of BOL was tested by comparing the BOL peak areas from the extracted QC samples and those from the aqueous standard solutions at four different concentration levels. The matrix effect and extraction recovery of IS were evaluated in a manner similar to BOL at a concentration of 200 ng/mL.

To evaluate the linearity, eight BOL (1–2000 ng/mL) concentrations were calibrated and tested for three consecutive days. The linearity of BOL was tested using ($1/x^2$ weighted) least squares linear regression based on the peak area ratios of BOL and IS versus the BOL nominal concentrations. For sensitivity purposes, the LLOQ was tested by assaying six independent plasma samples at the lowest concentrations on the calibration curve (1 ng/mL); the precision (relative standard deviation, RSD%) and accuracy (RE%) must be within ± 20% and the signal/noise (S/N) > 10.

The intra-day precision and accuracy of the UPLC-MS/MS method were measured by examining six individual spiked samples of BOL at each QC level (2, 20, 200, and 1000 ng/mL). The inter-day values were further verified by analyzing 24 QC samples in three different validation periods. The allowed precision and accuracy for the quantification of BOL were as follows: the RSD was within 15%, and the RE ranged between − 15% and 15%.

BOL stability in the biological matrix and in the reconstituted solution were assessed at four QC concentration levels under the following storage situations: short-term stability, maintained at room temperature for 2 h; long-term stability, in the − 80 °C for 30 d; freeze-thaw stability, after three freeze-thaw cycles; and post-preparation stability, in the 4 °C autosampler for 24 h. The dilution integrity was tested by analyzing six parallel samples spiked with 5000 ng/mL of BOL. The six analytes were diluted five-fold with blank rat plasma and assayed, and then the BOL concentrations, RSD%, and RE% were calculated.

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Animals and pharmacokinetic study

Twelve male Sprague-Dawley rats (270 ± 30 g) were obtained from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). All the experimental steps were supported by the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine (March 5, 2015; No: 201503051). For evaluating the bioavailability and pharmacokinetics behavior of BOL, the mice were randomly divided into two groups (oral and intravenous administration groups). Before the experiments, all the rats were fasted for 12 h, though water was supplied *ad libitum*. The dosages for the oral and intravenous administration groups were 0.3 and 0.15 mg/kg, respectively. After that, blood samples (approximately 300 µL) were drawn from the tail veins into heparinized Eppendorf tubes at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h. These tubes were centrifuge at 16,000 ×g for 5 min at 4 °C, and then each supernatant fraction (> 100 µL) was carefully transferred into another clean prelabeled Eppendorf tube and maintained at − 80 °C.

The pharmacokinetics parameters were computed with the Drug and Statistical software (Shanghai, China Pharmacology and Pharmacology Program version 2.1) with a non-compartmental model.

Supporting Information

UPLC-MS/MS conditions and representative mass spectra of BOL and IS, as well as NMR spectra (^{1}H, ^{13}C, HSQC, and HMBC) and NMR data of BOL, are available as Supporting Information.

Acknowledgements

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Conflict of Interest

We declare that we have no conflicts of interest.

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