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A fast and sensitive HPLC–MS/MS analysis and preliminary pharmacokinetic characterization of cudratricusxanthone B in rats

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ABSTRACT

A method for the quantitative analysis of cudratricusxanthone B (CXB) in rat plasma by high performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) has been developed and validated. The method involved liquid–liquid extraction from plasma, simple chromatographic conditions on a Venusil XBP-PH C_{18} column with the mobile phase of 0.5% formic acid in methanol, and mass spectrometric detection using an API-3000 instrument. Multiple reaction monitoring (MRM) mode was used to monitor precursor/product ion transitions of *m/z* 397.1/285.0 for CXB and *m/z* 381.6/269.2 for the internal standard (I.S.) cudraxanthone H. The standard curves were linear over the concentration range of 1–500 ng/mL for CXB in rat plasma. The intra- and inter-batch accuracy for CXB at four concentrations was 89.4–99.5% and 89.4–100.8%, respectively. The RSDs were less than 7.92%. The lower limit of quantification for CXB was 1.0 ng/mL using 100 μL of plasma. The average extraction recoveries of CXB ranged from 80.1 to 95.4% at the concentrations of 2, 50 and 500 ng/mL, respectively. This method was successfully applied to the pharmacokinetic study after an intravenous administration of CXB in male Sprague–Dawley (SD) rats.

1. Introduction

Cudrania tricuspidata (Carr.) Bur. is a deciduous shrub or tree distributed over China, Korea, and Japan. Its roots are applied in clinic for the treatment of digestive apparatus tumor, especially gastric carcinoma [1]. The pharmacological studies showed that the extract of *C. tricuspidata* had anti-tumor effects *in vivo* and *in vitro* [2]. Investigations on the bioactive constituents indicated that most of the isoprenylated xanthones possessed significant cytotoxicity against four kinds of human digestive apparatus tumor cell lines, including human colon carcinoma (HCT-116), hepatocellular carcinoma (SMMC-7721), and gastric carcinoma (SGC-7901 and BGC-823) [3,4]. Among the active compounds, cudratricusxanthone B (CXB, Fig. 1) with high content is a promising one [3]. Furthermore, isoalvaxanthone, an isoprenylated xanthone isolated from *Cudrania cochinchinensis* (Lour.) by our group, was found to inhibit colon cancer cell proliferation, migration and invasion through inactivating Rac1 and AP-1 [5]. An array of studies shows that isoprenylated xanthones are a kind of important plant secondary metabolites with anti-tumor activity [6].

The evaluation of the pharmacokinetics of herbal medicines can link data from pharmacological assays to clinical effects and also help the design of rational dosage regimens [7]. To the best of our knowledge, the method for the determination of CXB and its analogs in plasma has not been reported yet. In order to evaluate the pharmacokinetic characteristics of CXB after administration to animals, we developed a high performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) method for the determination of CXB in rat plasma. The method was successfully applied to the pharmacokinetic study after an intravenous administration of CXB in Sprague–Dawley (SD) rats.

2. Experimental

2.1. Chemicals and reagents

CXB (99.0%, purity) and cudraxanthone H (98.7%, purity, Fig. 1) as the internal standard (I.S.) were isolated in our laboratory. HPLC-grade acetonitrile, formic acid and methanol were supplied by Tedia Company Inc. (Fairfield, OH, USA). HPLC-grade methyl tert-butyl ether (TBME) was supplied by J.T. Baker (Denventer, Netherlands). Analytical-grade ethyl acetate, diethyl ether, cyclohexane and methylene dichloride were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, PR China). Double distilled

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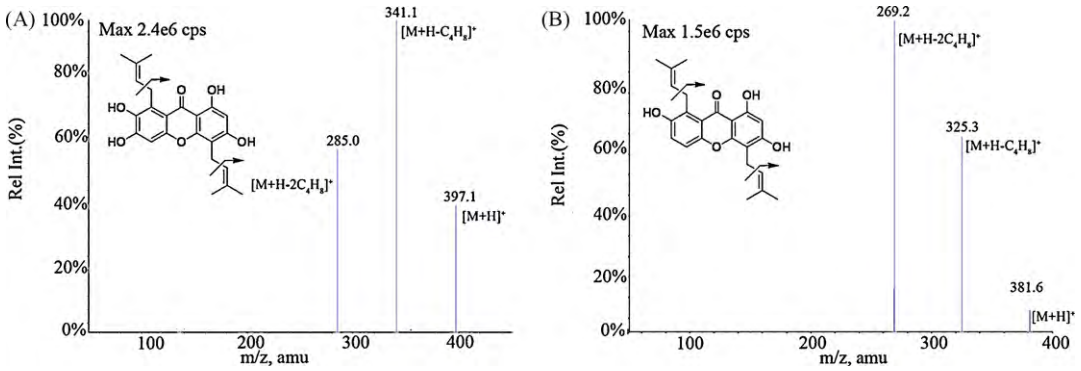

Fig. 1. Full-scan product ion scans of $[M+H]^+$ and structures for (A) CXB and (B) I.S.

water was purified using a Millipore Simplicity System (Millipore, Bedford, MA, USA).

2.2. Animals

The animal experimental protocol was approved by the ethic committee of School of Pharmacy of Fudan University. All animal studies were carried out according to the Guide for Care and Use of Laboratory Animals.

Male SD rats, weighing 250 ± 10 g, were supplied by Shanghai Lab. Animal Resources Center.

2.3. Standard solutions and plasma samples

Stock solution of CXB with a concentration of 100 μg/mL was prepared by dissolving 5.0 mg CXB in methanol. The working standard solutions (10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL) of CXB were prepared by diluting the stock solution with methanol–water (50:50, v/v). The working solution of I.S. (1000 ng/mL) was prepared in the same way as CXB. All standard solutions were stored at 4 °C in the dark when not in use.

Drug-free rat plasma containing sodium heparin as the anticoagulant was obtained from male SD rats. Plasma calibration standards of CXB (1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL) were prepared by adding 10 μL working standard solutions into 100 μL drug-free rat plasma. Quality control (QC) samples of 2, 50 and 500 ng/mL were prepared in the same way as calibration standards.

2.4. Sample preparation

A 100 μL of plasma sample was mixed with 10 μL of the I.S. working solution. Then, the sample was extracted with 1000 μL of TBME by vortex-mixing for 1 min and centrifuged at 10,600 × *g* for 10 min at 10 °C. The organic layer was transferred and evaporated to dryness at 45 °C under a stream of nitrogen. The residue was dissolved in 100 μL of methanol by vortex-mixing for 1 min and then 5 μL was injected onto LC–MS/MS system.

2.5. Chromatographic and apparatus conditions

An Agilent 1100 system consisting of a G1312A quaternary pump, a G1379A vacuum degasser, a G1316A thermostatted column oven (Agilent, Waldbronn, Germany) and a HTS PAL autosampler (CTC Analytics, Switzerland) was used. The chromatographic separation was performed on a Venusil XBP-PH C_{18} column (5 μm, 2.1 mm × 100 mm, Agela Technologies Inc.) coupled with a Phenomenex C_{18} guard column (5 μm, 4.0 mm × 3.0 mm), and the temperature was set at 40 °C. The mobile phase was 0.5% formic acid in methanol at a flow rate of 0.3 mL/min.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (Applied Biosystems, Toronto, Canada) in multiple reaction monitoring (MRM) mode. A TurboIonSpray ionization interface in positive ionization mode was used. Data processing was performed with Analyst 1.4.1 software package (Applied Biosystems). The precursor/product ion transitions selected were *m/z* 397.1/285.0 for CXB and *m/z* 381.6/269.2 for I.S., respectively. Quadrupoles Q1 and Q3 were set on unit resolution. The dwell time was set at 200 ms.

2.6. Method validation

A thorough and complete method validation of CXB in rat plasma was done following the USFDA guidelines [8]. The validation of the bioanalytical method includes specificity, linearity, lower limit of quantification (LLOQ), accuracy, precision, extraction recovery, matrix effect, stability, and dilution integrity.

2.6.1. Specificity

The specificity was determined by analyzing six blank plasma samples. There should be no interference from endogenous or exogenous materials observed at the retention time in the ion channel of either the analyte or the I.S.

2.6.2. Linearity and LLOQ

Each calibration curve consisted of nine calibration points (1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL). Linearity was evaluated by constructing linear regression equation fitted with the peak area ratios of CXB to I.S. versus the CXB concentrations using a $1/x^2$ weighting. The sensitivity of the method was expressed as the LLOQ that can be quantitatively determined with acceptable accuracy and precision and should be at least 10 times of signal to noise (S/N).

2.6.3. Accuracy and precision

The accuracy and precision were assessed by determining QC samples at three concentration levels on three different validation batches. The QC samples for intra-batch were prepared for six duplicates together with calibration samples. The accuracy and precision of the LLOQ samples were also assessed.

2.6.4. Matrix effect and extraction recovery

Matrix effect was assessed by comparing the peak areas of the neat CXB standards with those of standards spiked after extraction in three different lots of plasma at three concentration levels.

The extraction recoveries of CXB were assessed by comparing the peak areas of CXB standards spiked before extraction with those of standards spiked after extraction in three different lots of plasma at three concentration levels. The extraction

recovery of I.S. was assessed in the same manner in the QC samples.

2.6.5. Stability

The long-term and short-term stability studies of CXB in plasma were evaluated by assaying samples after storage for 7 days at −20 °C and storage at room temperature (about 20 °C) for 2 h, respectively. Stability was also investigated during three successive freeze–thaw cycles from −20 to 37 °C and after placing processed QC samples in the autosampler at 4 °C for 12 h.

2.6.6. Dilution integrity

If the concentration in a real sample was above the upper limit of quantification (ULOQ), the sample was analyzed after dilution with blank plasma. The dilution integrity experiment was carried out at 5 times of the ULOQ concentration (2500 ng/mL) and also at ULOQ level (500 ng/mL). Six replicate samples each of 1/10 of 5 × ULOQ (250 ng/mL) and ULOQ concentration (50 ng/mL) were prepared and their concentrations were calculated, by applying the dilution factor against the freshly prepared calibration curve for CXB. The %change from the comparison sample should be within ±15%.

2.7. Application to pharmacokinetic study

The method was successfully applied to the determination of CXB in plasma obtained from six rats following a single intravenous administration of CXB. Rats were housed in an environmentally controlled breeding room (temperature 24 ± 2 °C, relative humidity 45–60%) for one week before the experiments, and were fasted for 12 h but were allowed water before drug administration. Then, 5.0 mg/kg CXB was administered to the rats via the tail vein. Blood samples (about 50 μL at 0.083 and 0.25 h, and about 200 μL at other points) were drawn from the retro-orbital plexus before dosing and at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 9, 15 and 24 h after dosing. The blood sample was centrifuged at 1700 × *g* for 10 min at 10 °C and the separated plasma was stored at −20 °C until analysis.

The pharmacokinetic parameters for CXB were processed by the computer program 3P97 (Chinese Pharmaceutical Association, 1997), and the appropriate type of the compartmental model was chosen according to the lowest Akaike's information criterion (AIC) value. The areas under the plasma concentration–time curve from zero to the last quantifiable sampling point (AUC_{0-t}) and infinity ($AUC_{0-\infty}$) were calculated using the linear trapezoidal rule with a non-compartment model, and other parameters were estimated with a three-compartment model.

3. Results and discussion

3.1. Sample preparation

Compared with protein precipitation (PPT), liquid–liquid extraction (LLE) could produce a relatively clean sample and reduce the possibility of introducing highly polar materials into the column and MS system. The effects of sample pH from 3 to 7 on the extraction recoveries were evaluated, and the pH values of 7.0 and 4.0 showed good results. In consideration of the convenience of sample preparation, no buffer solution was added into the sample due to its pH values at about 7. Several kinds of organic extraction solvents were investigated, including cyclohexane, diethyl ether, methylene dichloride, ethyl acetate, and TBME. TBME produced the highest recovery. The extraction recoveries of CXB at the concentration of 50 ng/mL are summarized in Table 1.

LLE with TBME was finally applied to the extraction of CXB from rat plasma.

Table 1
LLE recoveries of CXB with different solvents.

Extraction solvent	Extraction recovery (%)
Cyclohexane	2.32%
Diethyl ether	56.3%
Methylene dichloride	32.1%
Ethyl acetate	42.3%
TBME	89.5%

3.2. Selection of internal standard

For HPLC–ESI-MS/MS quantification assay, a stable isotope-labeled CXB is the optimal I.S. However, sometimes it is difficult to obtain such a reference standard. In addition, an analog of CXB could be an ideal I.S. because it would exhibit behavior similar to CXB during the entire sample extraction, chromatographic elution, and mass spectrometric detection procedures. In our experiments, cudraxanthone H was chosen as the I.S. due to its similarity with CXB in structure, extraction condition, chromatographic behavior, and stability.

3.3. Optimization of LC–MS/MS conditions

The MS ionization was achieved using ESI mode by infusing a single standard solution of CXB. The instrument parameters were adjusted to maximize the responses for CXB and I.S. by direct injection of their standard solutions into the mass spectrometer. Acquisition of mass spectrometry data for CXB and I.S. standards was performed in positive ionization mode. CXB formed predominately protonated molecules at *m/z* 397.1 $[M+H]^+$ while I.S. formed predominately protonated molecules at *m/z* 381.6 $[M+H]^+$ in the Q1 full scan of the MS-scan mass spectra. Product ions were obtained in product ion scan (MS^2) by collisionally activated precursor ion fragmentations using nitrogen as the collision gas. The product ion scans of $[M+H]^+$ of CXB and I.S. are shown in Fig. 1. CXB exhibited a higher fragment ion signal at *m/z* 341.1 and 285.0, corresponding to $[M+H-C_4H_8]^+$ and $[M+H-2C_4H_8]^+$, respectively. I.S. exhibited a major fragment ion at *m/z* 269.2, produced from the loss of $2C_4H_8$ molecules [9]. The mechanism of the generation of ion fragmentations is shown in Fig. 1.

When assaying CXB in plasma samples at the MRM mode of precursor/product ion transition of 397.1/341.1 and 397.1/285.0, an intense peak with chromatographic retention time 1.13 min was found, but some internal interference at the retention time of 1.13 min was observed in the blank plasma at 397.1/341.1. Therefore, the mass transitions chosen for quantification were *m/z* 397.1/285.0 for CXB, and *m/z* 341.1/268.2 for I.S.

The compound-dependant parameters and gas conditions were optimized to produce the most intense MS signal. The spray voltage was set at 5000 V and the ion source temperature was 500 °C. The compound parameters, viz., collision energy (CE), declustering potential (DP), entrance potential (EP), focusing potential (FP), and collision exit potential (CXP) were 38.0, 30.0, 10.0, 200.0, 15.0 V for CXB and 40.0, 30.0, 10.0, 200.0, 15.0 V for I.S., respectively.

3.4. Method validation

3.4.1. Specificity

Representative MRM chromatograms of CXB and I.S. in rat plasma samples are shown in Fig. 2(A) blank plasma sample, (B) blank plasma sample spiked with CXB at the LLOQ of 1.0 ng/mL and I.S. (100.0 ng/mL), (C) blank plasma sample spiked with CXB 50.0 ng/mL and I.S. (100.0 ng/mL), and (D) plasma sample from a rat at 9 h after a single intravenous administration of 5.0 mg/kg CXB. No interfering peaks were observed at the retention times of interest.

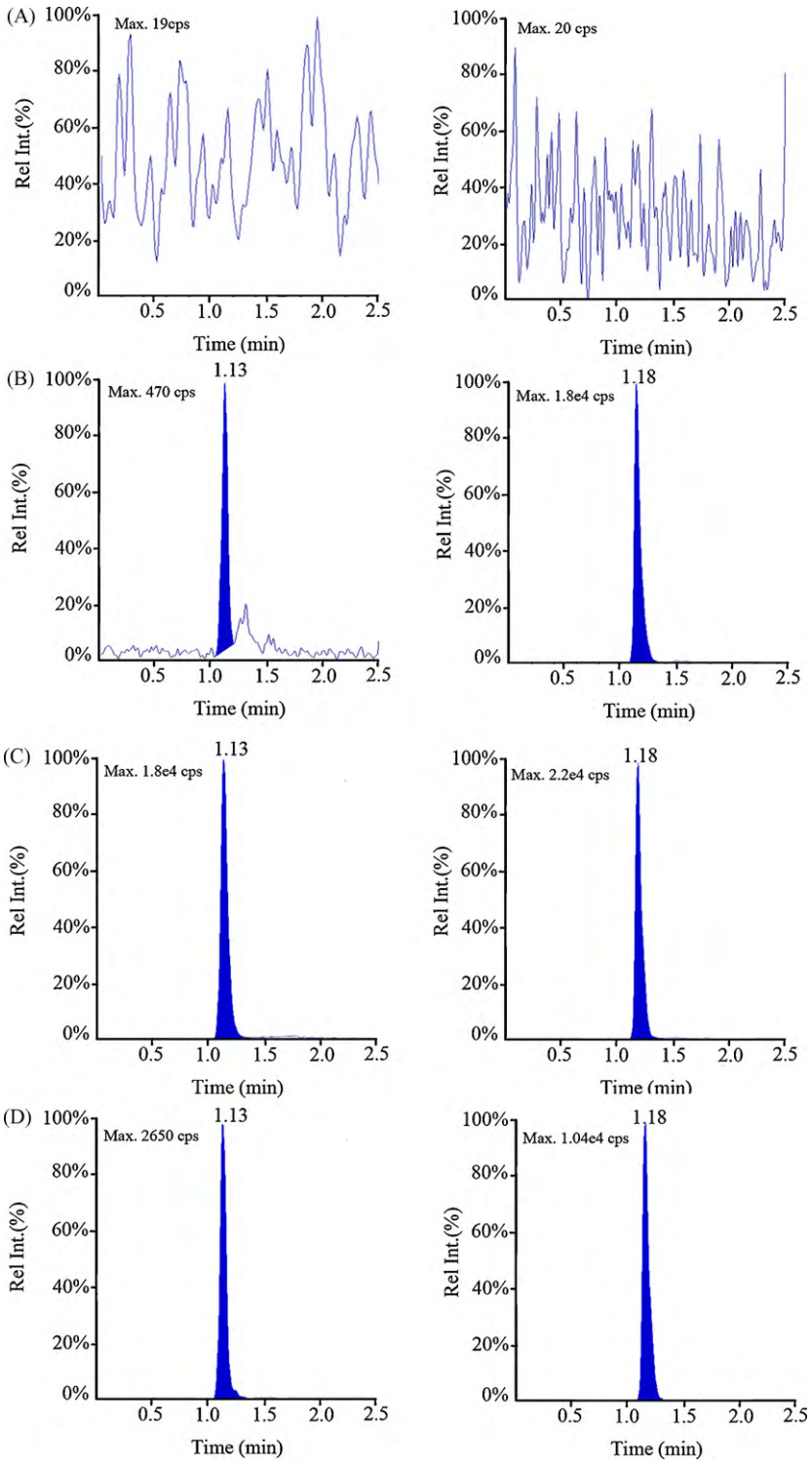

Fig. 2. Representative MRM chromatograms of CXB and I.S. in rat plasma. (A) Blank plasma sample; (B) blank plasma sample spiked with CXB at the LLOQ of 1.0 ng/mL and I.S. (100.0 ng/mL); (C) blank plasma sample spiked with CXB 50.0 ng/mL and I.S. (100.0 ng/mL); (D) plasma sample from a rat at 9 h after a single intravenous administration of 5.0 mg/kg CXB.

The typical retention times for CXB and I.S. were 1.13 and 1.18 min, respectively.

3.4.2. Linearity and LLOQ

The peak area ratios of CXB to the I.S. versus the nominal concentrations displayed a good linear relationship over the concentration ranges of 1.0–500.0 ng/mL in plasma. The typical regression equation was $y = 0.0263x + 0.00984$ ($r = 0.9957$), y representing the peak area ratio of CXB to the I.S. and x representing the concentration of CXB in plasma. The LLOQ (1.0 ng/mL) was defined as the lowest concentration on the calibration curves.

3.4.3. Precision and accuracy

The method showed good precision and accuracy. The intra- and inter-batch precision and accuracy of the assay were investigated by analyzing LLOQ and QC samples. All the values are summa-

Table 2
Precision and accuracy for the analysis of CXB in rat plasma.

Nominal concentration (ng/mL)	Intra-batch (n = 6)		Inter-batch (n = 3)	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
1.0	7.92	99.5	1.30	100.8
2.0	7.86	98.7	5.68	96.0
50.0	5.26	89.4	7.11	96.5
500.0	4.52	90.4	2.88	89.4

Table 3
Absolute matrix effect of CXB in rat plasma (n = 3).

Nominal concentration (ng/mL)	Matrix effect	
	Recovery (%)	RSD (%)
2.0	102.9	4.87
50.0	107.0	4.20
500.0	95.9	4.44

rized in Table 2. The intra- and inter-batch RSDs were below 7.92% and 7.11%, respectively. The intra- and inter-batch accuracy ranged from 89.4 to 99.5% and from 89.4 to 100.8%, respectively. Therefore, the method was proved to be both precise and accurate.

3.4.4. Extraction recovery

The extraction recoveries of CXB were 91.9, 80.1 and 95.4% for QC samples at the concentrations of 2, 50 and 500 ng/mL (n = 3), respectively. The extraction recovery of the I.S. was also evaluated and the mean value was 79.5% (n = 9). The RSDs of these values were less than 7.11%.

3.4.5. Matrix effect

The matrix effect of CXB was evaluated by analyzing QC samples in three different lots of plasma at three concentration levels. The results are summarized in Table 3. The mean matrix effect values of CXB ranged from 95.9 to 107.0%. The matrix effect of the I.S. was also evaluated and the mean value was 93.2% (n = 9). The RSDs of these values were less than 4.87%. The results indicated that the extracts had little or no detectable co-eluting compounds that could influence the ionization of CXB and I.S.

3.4.6. Stability

The working solutions at the concentrations of 20, 500 and 5000 ng/mL for CXB and 1000 ng/mL for I.S. in methanol–water (50:50, v/v) were stable for 7 days at 4 °C. In rat plasma, CXB was found to be stable for at least 7 days at −20 °C, for at least 2 h at room temperature (about 20 °C), as well as during three freeze–thaw cycles. In the reconstitution solution, CXB was stable under the autosampler conditions (4 °C) for at least 12 h. Stability data are summarized in Table 4.

3.4.7. Dilution integrity

The mean back-calculated concentrations for 1/10 dilution samples of 5 × ULOQ and ULOQ were 104.5% and 103.6%, respectively. The RSDs were 7.2% and 11.0%, respectively.

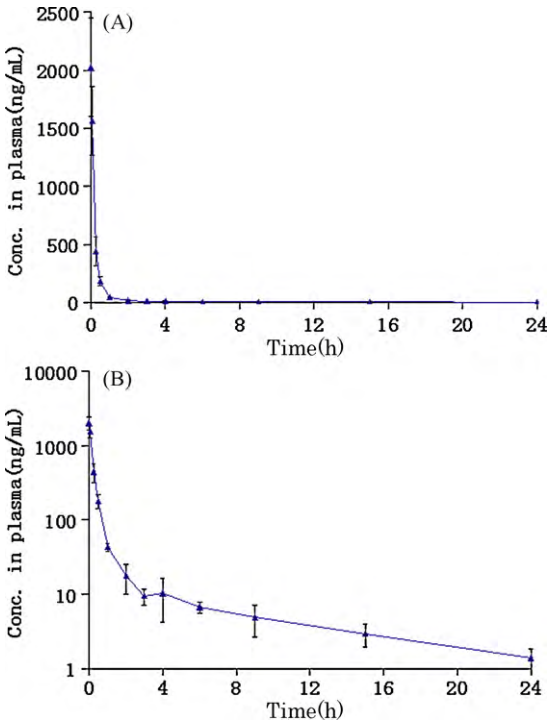

Fig. 3. Mean plasma concentration–time profiles of CXB following a single intravenous administration of 5.0 mg/kg to six male SD rats, each point and bar represents the mean ± S.D. (n = 6). (A) A standard scale for concentration; (B) a logarithm scale for concentration.

Table 5
The main pharmacokinetic parameters of CXB after a single intravenous administration of 5.0 mg/kg CXB to six male rats.

Parameter	Value
Vc (L/kg)	2.60 ± 1.88
$t_{1/2}$ pi (h)	0.109 ± 0.058
$t_{1/2}$ α (h)	0.503 ± 0.173
$t_{1/2}$ β (h)	8.17 ± 0.99
k_{12}(1/h)	1.38 ± 1.36
k_{21}(1/h)	2.24 ± 1.77
k_{13}(1/h)	1.17 ± 0.178
k_{31}(1/h)	0.113 ± 0.021
k_{10} (1/h)	4.53 ± 1.65
CL_s (L/kg/h)	9.52 ± 1.73
AUC_{0-t} (ng h/mL)	566.0 ± 76.4
$AUC_{0-\infty}$ (ng h/mL)	583.1 ± 79.7

Vc: Apparent volume of distribution; $t_{1/2}$ pi: Rapid-distribution half-life; $t_{1/2}$ α: Slow-distribution half-life; $t_{1/2}$ β: Elimination half-life; k: Rate constant; CL_s: Clearance; AUC: Area under concentration–time curve.

3.5. Application to pharmacokinetic study

The method was successfully applied to the determination of CXB in plasma obtained from six rats following a single intravenous administration of 5.0 mg/kg CXB. Plasma concentrations of CXB in rat were detectable for at least 24 h after intravenous administration. The pharmacokinetic profiles of CXB are shown in Fig. 3. After

Table 4
Stability of CXB in rat plasma (n = 3).

Analyte concentration (ng/mL)	Three freeze–thaw cycles stability		Long-term stability		Short-term stability		Auto sampler stability	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
2	96.5	12.8	107.7	4.39	95.0	8.63	97.3	8.97
50	103.5	6.36	104.0	6.72	100.9	8.15	98.5	12.0
500	103.6	14.3	93.3	12.3	100.0	5.41	91.6	9.38

intravenous administration, a rapid distribution was followed by a slow elimination. The main pharmacokinetic parameters of CXB in rats after intravenous administration are presented in Table 5, which are reported for the first time.

4. Conclusions

An HPLC–ESI-MS/MS method for the quantification of CXB in rat plasma was developed and validated. The method was sensitive with an LLOQ at 1.0 ng/mL for CXB using 100 μL rat plasma. The total run time was only 2.5 min per sample and the plasma sample pretreatment was a one-step LLE procedure. The results indicate that it is suitable for routine analysis of large batches of biological samples. The method has the advantages of simplicity, specificity and sensitivity, which could be a reference to the study of the analogs of CXB in biological samples.

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