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Quantitative determination of helicid in rat biosamples by liquid chromatography electrospray ionization mass spectrometry

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ABSTRACT

A simple liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) method with highly improved sensitivities for the determination of helicid in rat bile, urine, feces and most tissues was developed. The tissues and feces were firstly homogenized mechanically using deionized water as the media. Bile, urine, tissues and feces homogenates were extracted by liquid–liquid extraction with n-butyl alcohol for sample preparation. The subsequent analysis procedures were performed on a Shimadzu LCMS2010A system (electrospray ionization single quadrupole mass analyzer). A Luna C_{18} column (150 mm × 2.00 mm, 5 μm) was used as the analytical column, while a mixture of acetonitrile and ammonium chloride water solution was used as the mobile phase. The proportions of mobile phase were changed timely according to gradient programs. Chlorinated adducts of molecular ions $[M+Cl]^-$ at *m/z* 319.00 and 363.05 were used to quantify helicid and bergeninum (internal standard), respectively. The method was validated to be accurate, precise and rugged with good linearity. The proposed method was successfully applied to the preclinical tissue distribution and excretion studies of helicid in rats.

1. Introduction

In China, *Helicid nilgirica* Bedd has been used for thousands of years for cure of headache and insomnia, and helicid is found to be one of the effective constituents present in the *H. nilgirica* Bedd [1–4]. It has drawn more and more attention from the scientists because of its well-documented sedation and analgesic effects and low side effects [5,6]. A neurotoxicological teratology study showed even a high intragastric administration dose of 350 mg/kg still does not affect the early development of nervous system, neurobehavioral function and brain histology of rats' offspring [7]. A serials investigation on pharmacology and pharmacodynamics of helicid and its analogues are under way [8–10]. Some studies found that some helicid analogues may be novel acetylcholinesterase inhibitors or tyrosinase inhibitors [11,12].

Not much work has been done on pharmacokinetics studies of helicid up to now. Liu et al. have reported a liquid chromatography tandem mass spectrometry (LC/MS/MS) assay for determination of helicidum (helicid) and its metabolites in dog plasma [13]. However, this method was based on an external standard, which was generally considered not very reliable in LC/MS/MS analysis. More recently Shen Lan et al. reported a HPLC method to investigate the pharmacokinetics of helicid in rats with a LLOQ of 43.8 μg/L in rat plasma. The results showed that at the administrated doses of 2.23, 4.46, 6.70 mg/kg, the pharmacokinetics of helicid in rats is based on linear dynamics [14]. Furthermore, we have already reported a LC–ESI–MS method for identification and quantification of helicid in rat plasma with a LLOQ of 1 μg/L, and successfully investigated the pharmacokinetics in rats after intragastric administration of helicid with a single dose 50 mg/kg [15].

However, few preclinical tissue distribution and excretion studies of helicid were reported till now, because current analysis methods could not fully meet the requirements. In order to meet the increasing requirements from in vivo pharmacokinetic researches involved in new drug development, we further developed a reliable, sensitive and simple LC–ESI–MS method for quantitative determination of helicid in rat biosamples and applied it to tissue distribution and excretion studies.

2. Experimental

2.1. Chemical reagents and animals

Helicid (Batch No. 040801) was kindly provided by Kun Ming Baker Norton Co. Ltd. Bergeninum (Batch No. 1532-200202) was purchased from the National Institute for the Control of Pharma-

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ceutical and Biological Products (Beijing, China). The purities of all chemicals were above 99.9%. HPLC grade acetonitrile was obtained from Fisher Scientific (Toronto, Canada). HPLC grade methanol was supplied by Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). n-Butanol, all other chemicals and solvents used were obtained from standard vendors, and were of the highest quality available. Rats of both sex (Certificate No. SCXK-2002-0011) were provided by the animal breeding Center of the China Pharmaceutical University. The study was approved by the Animal Ethics Committee of the China Pharmaceutical University.

2.2. Sample preparation

Tissue (including lung, kidney, brain, ovary, testicle, skin, heart, spleen, muscle, pancreas, fat, stomach, intestines, liver) harvested from sacrificed dosed rats were rinsed with ice-cold 0.9% NaCl saline immediately, and then gently blotted with absorbent paper. The tested organs were firstly crushed to pieces in ice bath by an Ultra-Turrax T25 apparatus (IKA-Labortechnik, Germany). Then carefully weighed 0.3 g tissues were added with 1 mL deionized water and crushed and mixed in ice bath by an Ultra-Turrax T25 apparatus again to facilitate homogenization. After 20 μL of IS working solution (25 μg/mL) was added, the tissue samples were extracted with 4 mL n-butanol for 3 min and centrifuged at 3500 × *g* for 10 min. 3.0 mL supernatants were transferred out for evaporation at 45 °C in the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was reconstituted in 400 μL deionized water, and centrifuged at 23,000 rpm at 4 °C for 10 min. The supernatant (80 μL) was pipetted to an autosampler vial, and 10 μL was injected onto column for analysis.

Feces were firstly homogenized mechanically using water as media (0.3 g:1 mL, w/v). 0.5 mL bile, urine and feces homogenize were added 20 μL of IS working solution (25 μg/mL), then extracted with 3 mL n-butanol for 3 min and centrifuged at 3500 × g for 10 min. 2.5 mL supernatants were transferred out for evaporation at 45 °C. The rest of the operations were the same as those of the tissue homogenate.

2.3. Preparation of standards and quality control samples

The standard stock solutions of helicid (10 mg/mL) was prepared in deionized water, while bergeninum (1 mg/mL) in methanol. Appropriate serial dilutions of the stock solution were made in deionized water for spiking blank biometrics. Internal standard working solution was prepared by diluting internal standard stock solution with methanol. All solutions were stored at 4 °C.

Aliquots (10 μL) of the appropriately diluted stock solutions of helicid were added to blank fluids made according to Section 2.2 to yield calibration standards. Calibration standards of helicid were spiked at 1, 2.5, 5, 10, 25, 50 ng/mL in lung, kidney, brain, ovary, testicle, skin homogenates; 1, 2.5, 5, 10, 25, 50, 100 ng/mL in heart, spleen, and muscle homogenates; 1, 2.5, 5, 10, 25, 50, 100, 500 ng/mL in pancreas, fat homogenates; 5, 10, 25, 50, 100, 500 ng/mL in stomach and intestines homogenates; and 25, 50, 100, 500, 1000, 2500, 5000 ng/mL in liver homogenates; 10, 25, 50, 100, 250, 500, 1000 ng/mL in bile; 25, 50, 100, 250, 500, 1000 ng/mL in urine; 25, 50, 100, 250, 500, 1000, 2500 ng/mL in feces homogenate. Matrix-based calibration curves were obtained with those helicid-spiked samples, using for method validation. Calibration curves of helicid in all matrices were constructed at five replicates by plotting the mean peak-area ratios of target/IS versus nominal concentrations. Quality control (QC) samples were prepared in the same way as calibration standards with blank biometrics, and were stored in polypropylene tubes at −80 °C until analysis.

Table 1a
LC program for chromatographic separation for tissues.

Time (min)	Solvent A (%)	Solvent B (%)	Valve
0.03	88	12	1
1.5	88	12	–
1.8	65	35	–
2	65	35	0
2.5	65	35	–
3	30	70	–
5	30	70	–
5.1	88	12	–
6	88	12	1
10.05	Pump stop		

2.4. Instrument and analytical conditions

All the analytical procedures were performed on a Shimadzu (Kyoto, Japan) 2010A LC–ESI–MS system with a Shimadzu LCMS solution Workstation (ver. 2.02) for data acquisition. Liquid chromatographic separations were achieved using a Luna C_{18} column (150 mm × 2.00 mm, 5 μm). The column and autosampler tray temperatures were set at 40 and 4 °C, respectively. The mobile phase was made up of acetonitrile (solvent B) and water containing ammonium chloride (solvent A, 26.75 mg:1 L, w/v) at a flow rate of 0.2 mL/min from separate pumps, according to the elution programs listed in Table 1a for tissue samples, Table 1b for bile, urine and feces samples. All samples were ionized by negative ion electrospray ionization (ESI) probe in the negative mode under the following source conditions: gas flow: 4.5 L/min; curve dissolution line (CDL) voltage was fixed as in tuning, CDL temperature: 250 °C; block temperature: 200 °C. Qarray dc voltage and rf voltage were set at 0 and 150 V. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. Nitrogen gas (99.995%, from Gas Supplier Center of Nanjing University, China.) was used as the nebulizing gas (1.5 L/min) and sheath gas (2.0 L/min) source, respectively. The chlorinated molecular ion adduct $[M+Cl]^-$ of helicid at *m*/*z* 319.00 and bergeninum at *m*/*z* 363.05 were monitored.

2.5. Assay validation

The method was validated according to FDA guidelines on specificity, sensitivity, precision, recovery and stability [16].

2.5.1. Sensitivity and specificity

The analyte response at the LLOQ should be at least 10 times the response compared to blank, and were used as the lowest standard on the calibration curves. To evaluate the assay specificity, six samples of each blank matrix obtained from six different animals were tested to demonstrate that there were no interfering components.

Table 1b
LC program for chromatographic separation of bile, urine and feces.

Time (min)	Solvent A (%)	Solvent B (%)	Valve
0.03	88	12	1
2	88	12	0
2.2	40	60	–
5	40	60	–
5.2	88	12	–
6	88	12	1
8	Pump stop		

Solvent A (%) represented the proportion of aqueous phase; Solvent B (%) represented the proportion of organic phase. Valve was the command of flow channel selection value. When its value was 1, liquid switched to waste; when it was 0, liquid was loaded to MS analyzer.

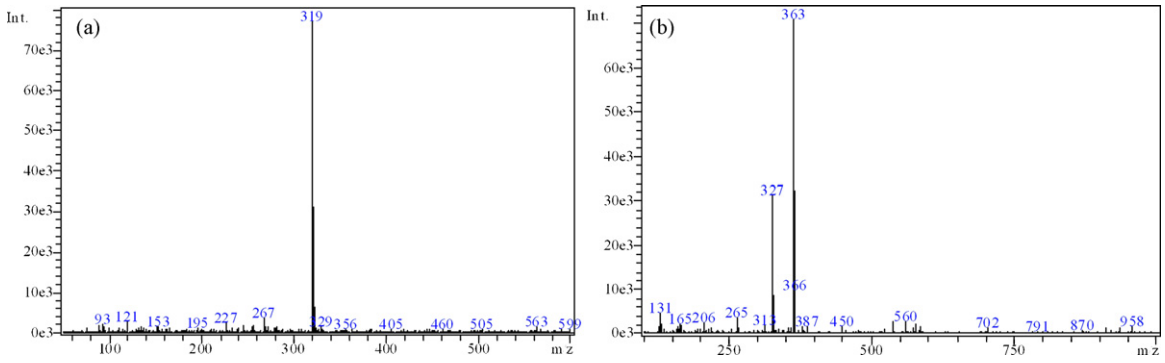

Fig. 1. Negative ion electrospray mass spectrum obtained in scan mode from standard samples of helicid (a, 1 μg/mL) and bergenin (b, 250 ng/mL) respectively, with abundance of $[M+Cl]^-$.

2.5.2. Accuracy and precision

Intra-day precision was tested by analysis of the QC samples of bile, urine and feces at three concentrations (each $n=6$) in the same day. Inter-day precision (each $n=6$) was determined by repeated analysis of the same samples over 5 consecutive days. Precision was determined by coefficient of variation (%CV) of peak-areas, which determined at each concentration level should not exceed 15%. The deviation of the mean from the true value serves as the measure of accuracy. The mean value of QC samples at each concentration should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%.

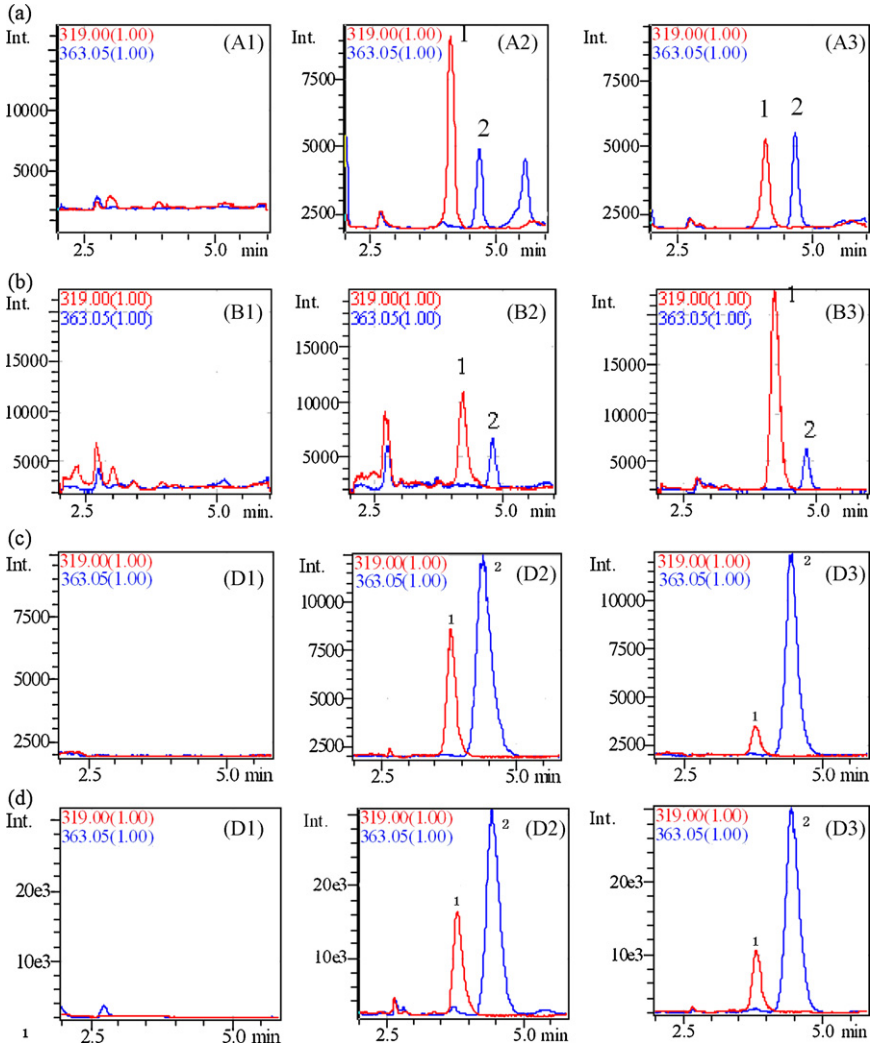

Fig. 2. Mass chromatograms of analytes under SIM mode: A and B represent bile and feces samples; C and D were samples from typical tissues (heart and stomach); 1–3 represent the blank matrices, blank matrices spiked with the standards of helicid and internal standard of known concentrations, and the corresponding biosamples collected after drug administration. A3 bile sample from a rat 6–8 h after oral administration of 50 mg/kg helicid; B3 represent feces sample obtained 4–8 h after oral administration of 50 mg/kg helicid; C3 heart sample and D3 stomach sample obtained from rats at 1.5 h after oral administration of 50 mg/kg helicid. 1 helicid peak, 2 IS peak.

Table 2
Linearity of the calibration curves for helicid determination in rat biosamples.

Tissue	Concentration (ng/0.3 g)												Equation		
	1	2.5	5	10	25	50	100	500	1000	2500	5000		a	b	r^2
Lung	0.0057	0.012	0.0365	0.0635	0.1776	0.3501	–	–	–	–	–		−0.0027	0.0071	0.9993
Kidney	0.0253	0.0282	0.0825	0.1496	0.3687	0.7328	–	–	–	–	–		0.004	0.0146	0.9994
Brain	0.0264	0.0526	0.1654	0.4344	1.0571	2.0987	–	–	–	–	–		−0.0269	0.0427	0.9991
Skin	0.0186	0.0345	0.07	0.1195	0.2714	0.5574	–	–	–	–	–		0.0088	0.0109	0.9992
Ovary	0.0173	0.0366	0.0662	0.1515	0.3562	0.7226	–	–	–	–	–		0.0005	0.0144	0.9997
Testicle	0.015	0.036	0.0845	0.1892	0.4635	0.9144	–	–	–	–	–		−0.0036	0.0184	0.9997
Spleen	0.0136	0.0295	0.0723	0.1413	0.3116	0.5985	1.2412	–	–	–	–		0.0045	0.0123	0.9994
Heart	0.0092	0.0151	0.0499	0.085	0.1778	0.3401	0.6919	–	–	–	–		0.007	0.0068	0.9991
Muscle	0.0156	0.0352	0.0687	0.099	0.2409	0.5109	1.0155	–	–	–	–		0.0053	0.0101	0.9994
Pancreas	0.0187	0.0422	0.0739	0.1743	0.4108	0.6781	1.3508	6.3901	–	–	–		0.0396	0.0127	0.9998
Fat	0.0209	0.0423	0.066	0.1238	0.3357	0.6471	1.0298	5.7819	–	–	–		0.0079	0.0115	0.9992
Stomach	–	–	0.0945	0.1218	0.173	0.2999	0.458	2.9667	5.9527	–	–		0.0061	0.0059	0.9991
Intestines	–	–	0.0648	0.0857	0.1783	0.2807	0.4808	2.3621	4.7989	–	–		0.0047	0.0047	0.9998
Liver	–	–	–	–	0.0119	0.038	0.2575	0.5555	1.0956	5.9514	11.961		−0.0519	0.0024	0.9999
Concentrations (ng/mL)	10	25	50	100	250	500	1000	2500							
Bile	0.1051	0.1981	0.4488	0.7483	1.9045	3.6225	7.1843	–					0.0566	0.0071	0.9998
Urine	–	0.0813	0.2088	0.3512	0.9838	1.9636	3.8565	–					−0.0022	0.0039	0.9997
Feces	–	0.0486	0.0923	0.2206	0.4508	0.9582	1.8951	3.7691					0.0275	0.0037	0.9997

2.5.3. Recovery and ionization

The recovery value was calculated by comparison of the peak-area of the analyte extracted from each matrix with that of the same amount of the compound dissolved in water. The resulting peak-areas of the two above were compared to provide the recovery. Recovery of the analyte need not be 100%, but the extent of recovery of the analyte and of the internal standard should be consistent, precise, and reproducible. Furthermore, the co-eluting matrix effect was investigated by comparing the peak-area for a known amount of helicid added to the n-butanol extraction of blank matrix collected from six Sprague–Dawley rats, with the peak-area of the same amount of the test compound in the deionized water.

2.5.4. Stability

The tissue samples were extracted and analyzed after we harvested them. So we only investigated the post-preparative stability of tissue samples by re-analyzing six duplicates of QC samples kept under the autosampler conditions (4 °C) at intervals within every routine analysis (24 h). QC samples were more than 5% of the assayed samples. The resulting peak-area was compared with that of the samples determined at once after prepared.

As to bile, urine and feces samples, post-preparative, short-term stability, long-term stability, and freeze–thaw stability were investigated. To evaluate the post-preparative stability, six duplicates of QC samples at three concentration levels (low, medium, high) of bile, urine and feces were re-analyzed, after stored at 4 °C for 24 h. Short-term stability was determined by evaluating QC samples stored at room temperature (25 °C) for 24 h. Long-term stability and freeze–thaw stability were together tested by analyzing the QC samples, which were stored at −80 °C for a month and then experienced freeze–thaw three cycles.

Stock solution stability was checked by evaluating the working solutions of helicid and IS maintained at 25 °C for 24 h. Concentrations of helicid standard prepared in deionized water, which were determined by LC–ESI–MS analysis, were compared before and after an evaporation at 45 °C for 1 h to estimate the possibility instability caused by evaporation in the sample preparation.

2.6. Method application

Twenty-four rats were divided into four groups, half male and half female, oral administration helicid 50 mg/kg and killed by exsanguination from the abdominal aorta under isoflurane anaesthesia at 0 min, 15 min, 1.5 h, and 4 h after blood sampling. Tissues (including lung, kidney, brain, ovary, testicle, skin, heart, spleen, muscle, pancreas, fat, stomach, intestines, and liver) were harvested and homogenized by the proposed preparation methods for analysis. For bile collection, six rats of both sex received a dose of 50 mg/kg via oral gavage, and bile samples from 0, 0–2, 2–4, 4–6, 6–8, 8–12 h were obtained through bile duct cannulas. Another six rats received a dose of 50 mg/kg orally, and then were housed in individual metabolism cages designed for separation and collection of urine and feces at 0, 0–4, 4–8, 8–12, 12–24, 24–36 h. All these samples were kept immediately in an ice bath at the end of each collection interval and stored at −80 °C until detection (within 1 week).

3. Results and discussion

3.1. Chromatography and mass spectrometry conditions

Addition of 26.75 mg ammonium chloride to the 1 l mobile phase was found to be an important factor for acquiring the high sensitivity, based on our previously research [15] and other fundamental

Table 3
Precision and accuracy of the method for the analysis of helicid ($n = 6$).

Biomatrices	Spiked concentration (ng/mL)	Intra-day			Inter-day		
		Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
Bile	25	25.14 ± 0.24	0.95	100.56	24.11 ± 0.31	1.29	96.44
	250	249.13 ± 2.14	0.86	99.65	247.21 ± 0.67	0.27	98.88
	1000	1006.31 ± 6.79	0.67	100.63	979.98 ± 4.36	0.44	98.00
Urine	25	24.96 ± 1.75	7.01	99.84	25.03 ± 0.11	0.44	100.12
	250	241.77 ± 3.16	1.31	96.71	246.77 ± 1.85	0.75	98.71
	1000	996.33 ± 9.19	0.92	99.63	995.68 ± 6.68	0.67	99.57
Feces	25	24.35 ± 1.55	6.37	97.40	24.16 ± 2.07	8.57	96.64
	250	234.33 ± 12.11	5.17	93.73	229.15 ± 11.46	5.00	91.66
	1000	977.28 ± 16.16	1.65	97.73	968.56 ± 14.23	1.47	96.86

CV (%) = measured concentration/SD × 100%.
Accuracy (%) = measured concentration/spiked concentration × 100%.

research from our laboratory [17–19]. Fig. 1 shows that the chlorinated molecular ion adducts $[M+Cl]^-$ m/z 319.00 for helicid and m/z 363.05 for bergeninum were truly predominant in negative mode. Thus, these ions were chosen for monitoring in the SIM mode during subsequent quantification.

3.2. HPLC gradient programme

We further developed the previous helicid plasma detection method [15] to the research of helicid in rat bile, urine, feces and most tissues, through choosing gradient programme. The HPLC gradient programme, which we described in Table 1, can effectively avoid the endogenous interference in biosamples and acquire good chromatographic peak shape. Representative chromatograms for helicid and IS in actual rat biosamples are presented in Fig. 2.

3.3. Liquid–liquid extraction

The easy and economical liquid–liquid extraction was also used in our present work. n-Butanol found to be a suitable solvent to extract helicid from rat bile, urine, feces and most tissues samples through one-step liquid–liquid extraction (LLE). Compared with the other organic solvents, helicid has a relative higher polarity. The procedure fits for the "rule of similarity".

3.4. Method validation

3.4.1. Specificity

Under the current optimized LC/ESI/MS conditions, helicid and IS in tissue samples were eluted at retention times of 3.82 ± 0.09, 4.45 ± 0.11 min, meanwhile, 4.22 ± 0.13, 4.75 ± 0.15 min in bile, urine and feces samples, separately (Fig. 2). No interferences of the analytes were observed in all matrices.

3.4.2. Calibration curves and sensitivity

The method showed good linear response over the selected concentration range in all biosamples. The concentration of plasma we detected by the method we already reported before [15]; meanwhile, the other biosamples are detected by the method presented in this paper. The mean regression equations and their correlation coefficients (r^2) for the curves were shown in Table 2.

Additional evaluation of LLOQ, the lowest standards in the calibration curves of plasma, bile, urine, and feces, whose signal-to-noise ratio (S/N) were larger than 10, were 1, 10, 25, and 25 ng/mL, respectively. Our method therefore exhibited a relatively good linearity and sensitivity compared to any other reported methods (with all correlation coefficients (r^2) above 0.99) [13,14].

3.4.3. Precision and accuracy

The results of accuracy and precision data in detail are shown in Table 3. The intra-batch precision and inter-batch precision values, expressed as R.S.D., were less than 15% at all concentration within the standard. The accuracy values were all within 85–115%. The presented results showed reliable precision and accuracy in rat bile, urine and feces.

3.4.4. Recovery and ionization

The extraction recoveries of helicid were determined by QC samples of all tested biofluids. The single step liquid–liquid extraction with n-butanol proved to be simple, rapid and successful with an average recovery rate within 85–115% for all the analytes under all tested concentrations. The results in detail are shown in Table 4. The extraction recovery of the internal stan-

Table 4
Recovery of helicid in rat biomatrices (mean ± SD, $n = 6$).

Biomatrices	Remaining (%)		
Concentration (ng/0.3 g)	2.5	10	50
Lung	100.40 ± 4.40	99.81 ± 7.71	100.22 ± 7.42
Kidney	98.80 ± 1.22	97.60 ± 6.87	95.74 ± 4.27
Brain	100.40 ± 1.22	95.51 ± 13.40	92.22 ± 5.38
Ovary	98.80 ± 5.41	96.13 ± 7.44	91.96 ± 4.98
Testicle	99.61 ± 6.47	96.64 ± 9.92	100.42 ± 3.42
Skin	97.65 ± 9.60	95.70 ± 9.73	97.06 ± 4.68
Concentration (ng/0.3 g)	2.5	10	100
Heart	100.81 ± 1.63	99.14 ± 6.51	105.11 ± 4.94
Spleen	100.00 ± 4.40	100.22 ± 9.12	98.79 ± 7.45
Muscle	98.84 ± 0.84	101.13 ± 7.71	108.13 ± 2.72
Concentration (ng/0.3 g)	2.5	50	500
Pancreas	98.45 ± 2.82	96.62 ± 0.95	98.66 ± 5.44
Fat	96.41 ± 12.84	93.69 ± 6.24	99.15 ± 6.95
Concentration (ng/0.3 g)	5	50	500
Stomach	97.59 ± 14.48	98.42 ± 4.14	96.53 ± 7.77
Intestines	99.63 ± 5.21	92.67 ± 4.94	97.51 ± 4.96
Concentration (ng/0.3 g)	25	100	2500
Liver	98.85 ± 5.63	103.47 ± 7.63	95.37 ± 4.25
Concentration (ng/mL)	25	250	1000
Bile	100.24 ± 1.21	98.80 ± 2.56	100.46 ± 0.79
Urine	100.04 ± 0.56	94.71 ± 1.16	98.7.6 ± 0.96
Feces	98.64 ± 0.57	92.79 ± 5.24	97.90 ± 0.68

Remaining (%) = measured concentration/spiked concentration × 100%.

Table 5
Stability of helicid in rat biomatrics ($n = 6$).

Biomatrices	Spiked concentration (ng/mL)	Remaining (%)		
		Short-term stability	Post-preparative stability	Long and freeze–thaw stability
Bile	25	100.02 ± 0.16	99.87 ± 0.21	99.01 ± 0.44
	250	100.11 ± 0.32	97.34 ± 0.22	97.14 ± 0.35
	1000	98.79 ± 0.27	98.77 ± 0.19	95.74 ± 0.26
Urine	25	100.08 ± 0.11	101.01 ± 0.14	98.16 ± 0.06
	250	100.16 ± 0.07	99.77 ± 0.24	98.15 ± 0.13
	1000	99.79 ± 0.21	98.11 ± 0.23	97.64 ± 0.06
Feces	25	99.54 ± 0.27	97.61 ± 0.13	95.27 ± 0.26
	250	100.01 ± 0.24	99.71 ± 0.22	98.66 ± 0.31
	1000	97.36 ± 1.14	97.25 ± 1.21	96.59 ± 1.91

Remaining (%) = measured concentration/spiked concentration × 100%.

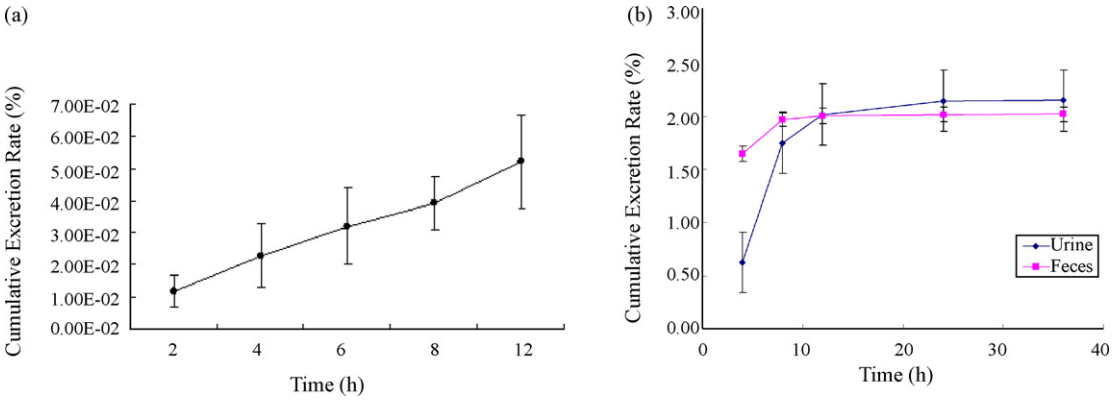

Fig. 3. Cumulative excretion rate of helicid in rat bile, urine and feces after oral administration at 50 mg/kg: (a) bile, (b) urine and feces.

dard was determined to be 89.3% at the spiked concentration (25 μg/mL). The possibility that matrix effects of helicid, caused by ionization competition would occur between the analyte and the endogenous co-eluents, were calculated using following formula: matrix effect% = $[(P - T)/T] \times 100\%$ (P, represents the peak responses of the post-extraction spiked samples, and T peak responses of the pure standards prepared in deionized water). The matrix effects of helicid in bile, urine, and feces were −5.61%, −7.97%and −7.78%, respectively. These results suggested negligible matrix effect occurred in this method.

3.4.5. Stability

Post-preparative stability data of tissue samples indicated there were no stability-related problems during the routine sample analysis of tissue samples in our studies, with RSD all far below 15% (detail data not shown). Moreover, post-preparative, short-term stability, long-term and freeze-thaw stability of bile, urine and feces samples were proved to be good enough. The results in detail are shown in Table 5. We considered the samples are stable, only if the average remaining within 85–115% for all the analytes under all tested concentrations.

The working solution of helicid and IS (in deionized water) proved to be stable for at least 24 h at 25 °C, the RSD of helicid and IS were 2.71% and 1.97%, respectively. The concentration of helicid standard exhibited no significant differences when compared before and after a 45 °C evaporation for 1 h with RSD lower than 5%, which showed the evaporation temperature was suitable.

3.4.6. Method application

This method was successfully applied to determine helicid in rat biofluids. Fig. 3 and Table 6 show the tissue distribution results in rats after an intragastric administration of helicid with a dose of 50 mg/kg. The tissue distributions were all lower than or nearly equal to the corresponding plasma concentrations except that in liver, stomach, and intestine extremely high distribution were observed. The high gastrointestinal distribution and long residence time could be the reason for the extensive pre-system metabolism, as reported before [13]. For the excretion studies, the curves of cumulative helicid excretion rate (cumulative amount of drug excreted/total drug amount that dosed × 100%) in bile, urine and feces are shown in Fig. 3. Only 0.0521 ± 0.0145% of helicid was excreted as parent from bile up to 12 h; while in urine and feces,

Table 6
Tissue concentrations of helicid at 5 min, 1.5 h and 4 h after oral administration at 50 mg/kg to rats (mean ± SD, $n = 6$).

Tissue	Time		
	15 min	1.5 h	4 h
Lung	3.22 ± 2.15	5.50 ± 6.20	8.15 ± 7.86
Kidney	13.25 ± 6.00	5.15 ± 5.47	1.39 ± 0.77
Brain	1.99 ± 1.67	1.66 ± 0.54	1.11 ± 0.16
Skin	12.69 ± 14.78	5.13 ± 3.71	2.84 ± 2.75
Ovary	4.00 ± 0.68	18.93 ± 9.75	6.58 ± 7.11
Testicle	1.06 ± 0.21	6.79 ± 0.87	6.53 ± 3.40
Spleen	14.86 ± 6.64	8.21 ± 4.50	2.32 ± 1.08
Heart	4.14 ± 2.67	20.66 ± 13.58	1.64 ± 0.93
Muscle	9.05 ± 11.84	4.17 ± 3.83	1.53 ± 1.26
Pancreas	19.87 ± 12.25	189.27 ± 204.78	23.49 ± 32.56
Fat	16.01 ± 12.06	6.39 ± 7.37	2.25 ± 2.82
Stomach	1086.19 ± 889.53	276.76 ± 331.42	36.93 ± 48.04
Intestines	3092.23 ± 1892.96	3056.56 ± 1951.85	144.92 ± 156.26
Liver	887.06 ± 839.53	74.02 ± 26.30	33.00 ± 6.83

the total helicid excretion as parent up to 36 h, were 2.02 ± 0.91% and 2.15 ± 1.13%, respectively.

4. Conclusion

By using detection of chlorinated molecules $[M+Cl]^-$, this LC–ESI–MS method described in the article achieved good sensitivity and linearity for the quantification of helicid in rat biofluids. No interference caused by endogenous compounds was observed. Single step liquid–liquid extraction with n-butanol was used for preparation of tissue, bile, urine and feces samples. This method demonstrated a relatively short analysis time and the acceptable sensitivity, precision, accuracy, selectivity, recovery and stability. The lowest standards in the calibration curves of plasma, bile, urine, and feces, whose signal-to-noise ratio (S/N) were larger than 10, were 1, 10, 25, and 25 ng/mL, respectively. All correlation coefficients (r^2) of all biosamples are above 0.99. The intra-batch precision and inter-batch precision values, expressed as R.S.D., were less than 15% at all concentration within the standard. The accuracy values were all within 85–115%. The single step liquid–liquid extraction with n-butanol proved to be simple, rapid and successful with an average recovery rate within 85–115% under all tested concentrations. And the stabilities are all above 90%. The proposed method was successfully applied to the tissue distribution and excretion studies. And to our knowledge, it is the first report of LC/MS method on the determination of helicid in tissues, bile, urine and feces samples. The results showed helicid distributed very little to most tissues and only no more than 5% of the total parent drug was excreted through the main physiological routes. These results indicated extensive metabolism instead of excretion was the reason for the elimination of helicid in rats. Which one on earth played the sedation and analgesic effects, helicid or its metabolites? A series of studies are under their way to uncover the mechanism of how helicid works.

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References

[1] G.Y. Liu, Y.M. Zhang, J.M. Xu, R.C. Lin, China J. Chin. Mater. Med. 30 (2005) 830.
[2] J.M. Sha, H.K. Mao, Chin. Pharm. Bull. 22 (1987) 27.
[3] G.Y. Liu, S.C. Ma, G.J. Zhen, G.J. Zhan, Chin. Traditional Herbal Drugs 36 (2005) 814.
[4] G.Y. Liu, S.C. Ma, G.J. Zhen, G.J. Zhan, China J. Chin. Mater. Med. 30 (2005) 830.
[5] X. Zhou, T.R. Zhao, G.H. Nan, J.Y. Li, Acta Pharmacol. Sin. 8 (1987) 393.
[6] J. Li, P. Liu, D.Q. Na, X.N. Wu, J. Health Toxicol. 15 (2001) 110.
[7] P. Liu, J. Li, D.Q. Na, Chin. Traditional Herbal Drugs 33 (2002) 238.
[8] X. Zhou, T.R. Zhao, G.H. Nan, J.Y. Li, Acta Pharmcol. Sin. 8 (1987) 393.
[9] H. Xie, G. Wang, L. Fan, L. Zhang, X. Dai, H. Zhou, Chin. J. Pharmacol. Ther. 13 (2008) 1416.
[10] J. Tong, R. Sun, S. Jiang, B. Yang, J. Rui, J. Li, H. Xie, Chin. J. Pharmacol. Ther. 13 (2008) 1277.
[11] W. Yi, R. Cao, H. Wen, Q. Yan, B. Zhou, Y. Wan, L. Ma, H. Song, Bioorg. Med. Chem. Lett. 18 (2008) 6490.
[12] H. Wen, C. Lin, L. Que, H. Ge, L. Ma, R. Cao, Y. Wan, W. Peng, Z. Wang, H. Song, Eur. J. Med. Chem. 43 (2008) 166.
[13] Qingfei Liu, Xiangdong Liu, Guoan Luo, J. Chromatogr. B 832 (2006) 185.
[14] Chen Z., Jiang X.H., Ren J., Liu T.M., Ma G., Wang L. 33 (2008) 2662.
[15] Y. Jia, G. Wang, H. Xie, X. Dai, Y. Wang, W. Wang, M. Xu, R. Wang, C. Yao, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 847 (2007) 72.
[16] Guidance for Industry Bioanalytical Method Validation, May, U.S. Department of Health and Human Services/Food and Drug Administration/Center for Drug Evaluation and Research (CDER)/Center for Veterinary Medicine (CVM), 2001, BP.
[17] Haitang Xie, Guangji Wang, et al., J. Chromatogr. B 818 (2005) 167.
[18] Y. Gu, G.J. Wang, J.G. Sun, Y.W. Jia, M.J. Xu, W. Wang, Biol. Pharm. Bull. 29 (2006) 951.
[19] C. Xia, G. Wang, J. Sun, H. Hao, Y. Xiong, S. Gu, L. Shang, C. Zheng, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 862 (2008) 72.