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Quantitative determination of helicid in rat plasma by liquid chromatography–electrospray ionization mass spectrometry and its application to preliminary pharmacokinetic studies

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Abstract

A sensitive and selective liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method was developed for the identification and quantification of helicid in rat plasma. The method was based on simple liquid–liquid extraction (LLE). A Kromasil C18 column (150 mm × 2.00 mm, 3.5 μm) was used as the analytical column, while a mixture of acetonitrile and 500 μM ammonium chloride was used as the mobile phase. MS detection was performed using a single quadrupole mass spectrometer in a negative selected ion monitoring (SIM) mode. The deprotonated molecules $[M + Cl]^-$ at *m/z* 319.00 and 363.05 were used to quantify helicid and bergeninum (internal standard, I.S.), respectively. The lower limit of quantification of helicid was 1 ng/ml. The method was linear in the concentration range of 1–1000 ng/ml. The intra-day and inter-day precisions (R.S.D.%) were within 10.0% for the analyte. Helicid proved to be stable during all sample storage, preparation and analytical periods. The method was successfully applied to a pharmacokinetic study in rats after intragastric administration of helicid with a dose of 50 mg/kg. Only 50 μl of rat plasma at each sampling time was needed for analysis. The proposed method enables unambiguous identification and quantification for the preliminary pharmacokinetic studies of helicid.

Keywords: Helicid; LC–ESI-MS; Pharmacokinetics

1. Introduction

Traditional Chinese herbal medicines have been used to treat human diseases in China for centuries. They draw more and more attention because of their low toxicity and good therapeutic performance. The *Helicid nilgirica* Bedd has been used for thousands of years for curing headache and insomnia. Helicid (molecular structure shown in Fig. 1a) is found to be one of the main constituents present in the *H. nilgirica* Bedd [1]. In recent years, helicid has drawn more and more attention from scientists. Many pharmacodynamic studies show its well-documented sedation and analgesic effects and low side effects [2,3]. The effects of helicid on sedation and odynolysis are closely associated with its neuroprotective activity. Previous researches proved that helicid plays a neuroprotective role by affecting the excitotoxicity, nitric monoxide (NO) system, neuroglia, biomembrane, oxidative neurotoxicity, apoptosis et al. [4–6]. Recent researches also suggest that anti-inflammatory function inhibiting the release of excitatory amino acid glutamate and resisting glutamate toxicity may be the new targets of neuroprotective activity mechanism of helicid [7]. Neurotoxicological teratology study on the offspring of rats revealed that even a high intragastric administration dose of 350 mg/kg does not affect the early development of nervous system, neurobehavioral function, and brain histology of offspring [8]. In a clinical test, helicid showed good therapeutic effects in curing headache and dizziness [9]. Because of its satisfying effect–price ratio and low side effects, helicid is widely used throughout China though many synthetic drugs for curing neurosis have been developed and marketed successfully.

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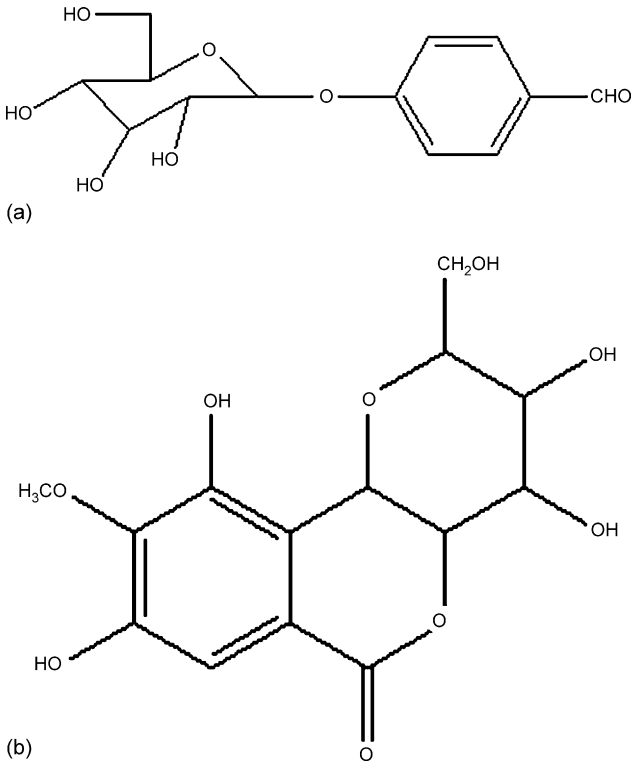

Fig. 1. Chemical structures of helicid (a) (mw = 284.1) and bergeninum (b) (internal standard, I.S., mw = 328.27).

As we all know, developing and validating satisfactory bioanalytical method is a preliminary step for further pharmacokinetic studies. However, no bioanalytical method has been developed for the determination of helicid concentration in vivo.

In this report, we describe a simple and sensitive HPLC–ESI-MS method to determine the concentration of helicid in rat plasma. This method was fully validated for its specificity, accuracy, precision, and sensitivity, and was successfully applied to the preliminary pharmacokinetic study of helicid in rats. Meanwhile, a series of studies are underway, especially for the pharmacokinetic–pharmacodynamic (PK–PD) correlation studies of helicid in clinical practice.

2. Experimental

2.1. Chemicals and reagents

Helicid was kindly provided by Kun Ming Baker Norton Co. Ltd., and bergeninum (internal standard, I.S.) [10] was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all chemicals were above 99.9%. The chemical structures are shown in Fig. 1. 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 of analytical grade.

2.2. Instrument and analytical conditions

A Shimadzu 2010A liquid chromatography–mass spectrometry (LC–MS) system with electrospray ionization (ESI) interface and Shimadzu LCMS solution workstation software (version 2.02) for the data processing were utilized to perform the analytical procedures. The system consisted of two Shimadzu LC-10ADvp pumps, a Shimadzu SIL-HTc autosampler, a Shimadzu CTO-10Avp column oven and a Shimadzu DGU-14AM online degasser. A Q-array-Octapole-Quadrupole mass analyzer was used as the detector. The LC process was carried out on a Kromasil C_{18} column (150 mm × 2.00 mm, 3.5 μm). The column and autosampler tray temperatures were set at 40 and 4 °C, respectively. A mobile phase composed of acetonitrile-500 μM ammonium chloride (12:88, v/v) at a flow rate of 0.2 ml/min was used to obtain the baseline separation of all analytes. Mass spectrometer was operated in negative ion mode. Under these conditions, helicid was eluted at a retention time of 4.07 min and bergeninum (I.S.) at 4.81 min.

2.3. Mass spectrometric conditions

All measurements were carried out using the negative ESI. Mass spectrometer conditions were optimized to obtain maximum sensitivity. The curved desolvation line (CDL) temperature was 230 °C, and the block temperature was 200 °C. A detector voltage of 1.5 kV and a probe voltage of 4.5 kV were fixed as done in tuning method. 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. Q-array 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 selected ion monitoring (SIM) mode and 1 s in scan mode. Quantification of helicid was carried out by monitoring the $[M + Cl]^-$ ions at *m/z* 319.00 and 363.05 (helicid and bergeninum, respectively).

2.4. Preparation of stock solutions

The standard stock solutions of 10 mg/ml of helicid and bergeninum were prepared in deionized water and methanol, respectively. A series of standard working solutions were obtained by further dilution of the standard stock solutions with the deionized water. Internal standard working solution (5 μg/ml) was prepared by diluting internal standard stock solution (10 mg/ml) with methanol. All solutions were stored at 4 °C. Appropriate amounts of working solution were diluted with drug-free plasma to span a calibration standard range of 1–1000 ng/ml.

2.5. Preparation of samples and quality control samples

Each collected blood sample was immediately centrifuged at 4000 rpm for 5 min, and plasma was transferred into a clean Eppendorf tube. All plasma samples were stored at −20 °C until analysis. An aliquot (50 μl) of the plasma, spiked with internal standard working solution (10 μl), was vortex-mixed for 30 s and extracted with *n*-butanol (1000 μl) using a vortex

mixer (Scientific Industries Inc., USA) for 3 min. Then the tubes were centrifuged at 20,000 rpm at 4 °C for 10 min (Micromax RF, Thermo Electron Corporation, USA). The upper organic phase (800 μl) of each tube was transferred into a clean tube and evaporated to dryness in the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was immediately reconstituted in 200 μl water and centrifuged at 20,000 rpm at 4 °C for 10 min. The supernatant (80 μl) was pipetted to an autosampler vial, and 10 μl of it was injected onto column for analysis.

Quality control (QC) samples (1, 100, and 1000 ng/ml) were made by spiking blank rat plasma with appropriate standard solutions to the required plasma concentrations, followed by the same operation listed above.

2.6. Method validation

2.6.1. Selectivity

Selectivity was ascertained by analyzing six blank rat plasma samples without adding I.S. to determine the interference with the analyte.

2.6.2. Linearity of calibration curves and lower limit of quantification

Five calibration curves containing helicid at the concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/ml were constructed by plotting the peak–area ratios of target/I.S. to the spiked concentrations. Calibration curves were analyzed by weighted linear regression analysis.

The lower limit of quantification (LLOQ) was evaluated by analyzing the six replicates of plasma sample spiked with the analyte at a final concentration of 1 ng/ml at which the signal-to-noise ratio (S/N) was preliminary found to be larger than 10.

2.6.3. Recovery and matrix effect

To determine recovery and matrix effect, extracted samples, unextracted samples (pure sample freshly prepared in deionized water) and a set of post-extracted spiked samples (at three concentrations: 1, 100, and 1000 ng/ml, $n = 6$) were analyzed in the same assay run. The recovery was determined by measuring an extracted sample against a post-extracted sample of the peak–area ratio (analyte/I.S.). The matrix effect was measured by comparing the peak response of the post-extracted spiked sample with that of the unextracted sample containing equivalent amounts of the analyte of interest [11].

2.6.4. Precision and accuracy

Intra-day accuracy and precision were evaluated by analyses of QC samples (each, $n = 6$) at different times on the same day. Inter-day accuracy and precision were determined by repeated analyses of QC samples over 6 consecutive days ($n = 1$, series per day). The concentration of each sample was determined using newly prepared calibration standards.

2.6.5. Stability

The effects of three freeze-thaw cycles on the compound stability at room temperature in plasma for 24 h were evaluated by repeated analyses ($n = 6$) of QC samples. Long-term stability in plasma was also tested by assaying frozen QC samples after storage at −20 °C for 6 months. The post-preparative stability was tested by reanalyzing QC samples kept under the autosampler condition (4 °C), over the anticipated run time of 24 h. Stability was expressed as a percentage of nominal concentration.

2.7. Pharmacokinetic study

Six Sprague–Dawley rats (190–210 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) and were housed with unlimited access to food and water except for fasting 12 h before the experiment. The animals were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and 60% relative humidity. After intragastric administration of helicid (50 mg/kg), 150 μl of blood sample via the orbital sinus were collected at 0.08, 0.17, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h. Blood samples were centrifuged immediately to separate plasma and were stored at −20 °C until analysis.

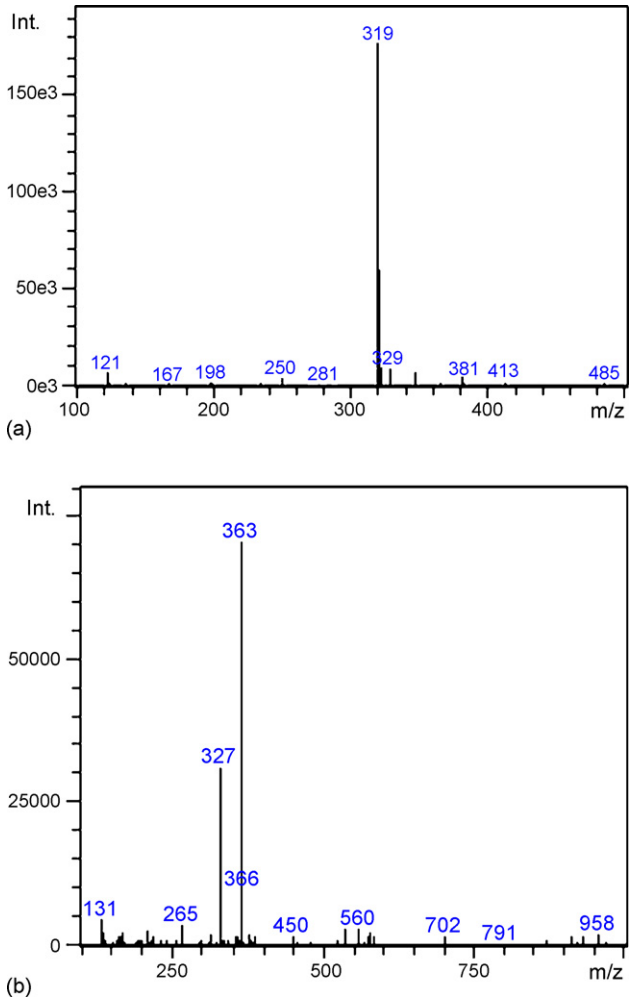

Fig. 2. Negative ion electrospray mass spectrum obtained in scan mode from standard samples of helicid (a, 2 μg/ml) and bergeninum (b, 250 ng/ml), respectively, with abundance of $[M + Cl]^-$.

3. Results and discussion

3.1. Chromatography and mass spectrometry conditions

Under the current chromatographic conditions, all analytes were rapidly eluted within 6.0 min. Although higher acetonitrile ratios had been tried for decreasing the analysis time, the analytes could not be totally separated from the endogenous plasma components. Addition of 500 μM ammonium chloride to the mobile phase was found to be an important factor for acquiring the high sensitivity. When the ion adduct $[M + Cl]^-$ was selected for determination, the addition of ammonium chloride caused a

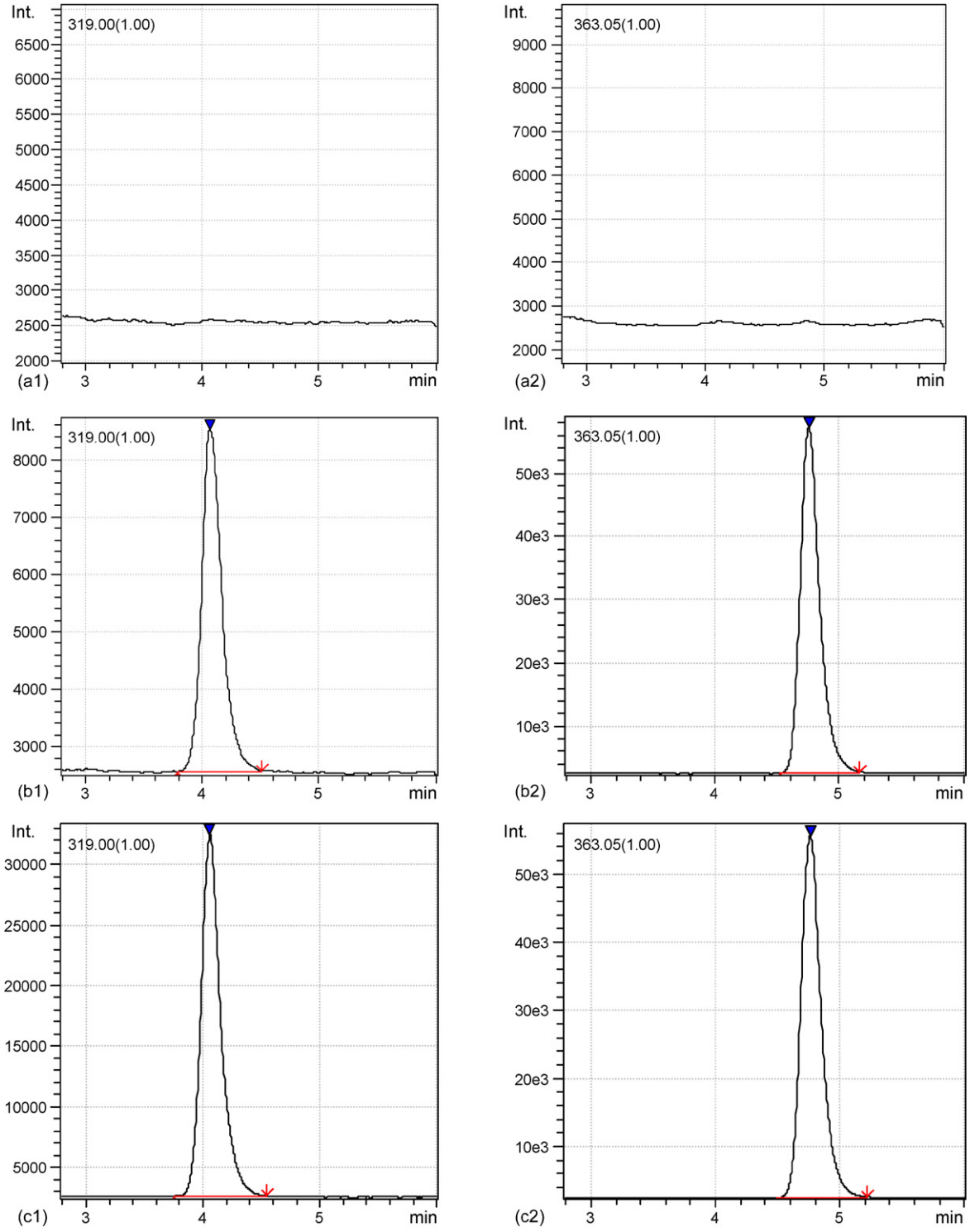

Fig. 3. Mass chromatograms of analytes under SIM mode from rat plasma: (a) blank rat plasma, (b) blank rat plasma spiked with helicid (100 ng/ml) and internal standard (250 ng/ml), respectively, (c) plasma sample 1 h after oral administration of helicid (50 mg/kg); (1), (2) represent the monitored ions of helicid and bergeninum separately in the SIM mode.

significant sensitivity increase according to our previous experiment [12]. Five hundred micro molar ammonium chloride was the concentration after optimization using a series of concentration.

The total negative ion spectra, scanned from *m/z* 100–500 of helicid and 100–1000 of bergeninum (I.S.), are shown in Fig. 2. The most intense ions observed were $[M+Cl]^-$ (*m/z* 319.00) for helicid and $[M+Cl]^-$ (*m/z* 363.05) for bergeninum. Thus, these ions were chosen for monitoring in the SIM mode.

3.2. Liquid–liquid extraction

One of the most versatile techniques for the extraction and enrichment of analytes from liquid samples is liquid–liquid extraction (LLE). Compared with the more recent and popular technique of solid-phase extraction (SPE), the separation of LLE is through the use of specific reagents. This results in extracts with less potential for interfering compounds. Furthermore, liquid–liquid extraction is easy and economical.

The low molecular weight and high polarity of helicid lead to poor solubility in organic solvent. We tried several organic solvents for the extraction of helicid and found *n*-butanol to be a suitable solvent. Compared with the other organic solvents, it has a relatively higher polarity. The procedure fits for the "rule of similarity".

3.3. Method validation

3.3.1. Selectivity

Under the current optimized HPLC and MS conditions, helicid and the internal standard were baseline separated chromatographically with the retention times of 4.07 ± 0.03 and 4.81 ± 0.05 min, respectively. Due to high selectivity of the selected reaction mode, no interference was observed in the retention time of helicid and I.S. The chromatograms, shown in Fig. 3, support the high selectivity of this method.

3.3.2. Linearity of calibration curves and lower limits of quantification

The peak-area ratio of helicid to I.S. in rat plasma varied linearly with concentration over the range tested (1–1000 ng/ml). The calibration curves were selected based on the analysis of the data by linear regression with and without weighting factors ($1/x$, $1/x^2$). The residuals improved by weighted ($1/x$) least-squares regression. Best fit for the calibration curve could be achieved by a linear equation of $y = 0.0012x + 0.0009$, with $1/x$ weighting factor (where, y is the peak–area ratio and x the concentration). The correlation coefficient (r^2, mean value, $n = 5$) for helicid was 0.9994.

The lower limit of quantification for helicid in plasma, defined at a signal-to-noise ratio of 10, was 1 ng/ml. The precision of six replicates was 3.92%, much less than 20%. The results in detail are shown in Table 2.

Table 1
Recovery of helicid from spiked rat plasma ($n = 6$)

Compound	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Recovery (%)
Helicid	1	0.91 ± 0.48	91.35 ± 4.79
	100	99.57 ± 4.03	99.57 ± 4.03
	1000	985.15 ± 7.49	98.52 ± 0.75

3.3.3. Recovery and matrix effect

The extraction recoveries of helicid from spiked rat plasma were determined by samples at the following concentrations: 1, 100, and 1000 ng/ml. A single-step liquid–liquid extraction with *n*-butanol proved to be simple, rapid and successful with an average recovery rate greater than 90% for the analyte under all tested concentrations. The results in detail are shown in Table 1. The extraction recovery of the internal standard was determined to be 93.8% at the spiked concentration (5 μg/ml). The possibility that a matrix effect, caused by ionization competition, would occur between the analyte and the endogenous co-eluents was evaluated at the three concentrations mentioned above ($n = 6$). The matrix effects of helicid and I.S. were calculated using the following formula: matrix effect% = $[(P - T)/T] \times 100\%$ (P represents the peak responses of the post-extracted spiked samples, and T peak responses of the pure standards prepared in deionized water). For helicid and I.S., the matrix effects were −5.78 and −7.18%, respectively. These results suggested that negligible matrix effect occurred in this method.

3.3.4. Precision and accuracy

QC samples at the three concentrations were analyzed in six replicates for determining the precision and accuracy of this method. The intra-day accuracy for helicid ranged from 102.00 to 109.23% at the tested concentrations with the precision (R.S.D.) between 2.50 and 4.97%. The inter-day accuracy for helicid ranged from 102.00 to 111.63% at three different

Table 2
Precision and accuracy of the method for the analysis of helicid ($n = 6$)

Compound	Spiked concentration (ng/ml)	Intra-day			Inter-day		
		Measured concentration (mean ± S.D., ng/ml)	R.S.D. (%)	Accuracy (%)	Measured concentration (mean ± S.D., ng/ml)	R.S.D. (%)	Accuracy (%)
Helicid	1	1.02 ± 0.04	3.92	102.00	1.02 ± 0.05	4.90	102.00
	100	104.07 ± 2.60	2.50	104.07	107.38 ± 2.32	2.16	107.38
	1000	1092.34 ± 54.29	4.97	109.23	1116.34 ± 12.06	1.08	111.63

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

Table 3
Stability of helicid in rat plasma ($n = 6$)

Compound	Spiked concentration (ng/ml)	Remaining (%)			
		Freeze-thaw (three cycles)	Room temperature (for 24 h)	Stored at −20 °C (for 6 months)	Stored at −4 °C (for 24 h)
Helicid	1	100.00 ± 0.11	107.00 ± 0.23	96.07 ± 0.22	99.71 ± 0.06
	100	97.37 ± 3.83	98.54 ± 3.68	95.21 ± 3.54	97.61 ± 2.94
	1000	98.04 ± 1.04	98.89 ± 5.91	97.36 ± 3.79	97.86 ± 4.66

concentrations with the precision between 1.08 and 4.90%. The results are shown in Table 2. These results indicate that this method has satisfactory precision, accuracy and reproducibility.

3.3.5. Stability

The stability of helicid during the sample storing and processing procedures was fully evaluated by analyzing QC samples. The concentration variations found after three cycles of freezing at −20 °C and thawing at 37 °C were within ±4% of nominal concentrations, indicating no significant loss of helicid during three freeze-thaw cycles. When processed samples were stored at −20 °C, helicid showed very good stability at the concentrations studied; the responses varied not more than ±6% within 6 months of storage. After storage at room temperature for 24 h, helicid concentrations in plasma deviated from the concentrations in unstored plasma by less than ±7%. When processed samples were stored at 4 °C in the autosampler, helicid showed a very good post-preparative stability; at the concentrations studied, the responses varied not more than 5% within 24 h of storage. The results in detail are shown in Table 3.

3.4. Pharmacokinetic study

This simple, precise and accurate LC/MS method yields satisfactory results for determination of helicid in rat plasma and was successfully applied to a pharmacokinetic study after intragastric administration of 50 mg/kg helicid to six rats. The mean plasma concentration–time profile of helicid is illustrated in Fig. 4.

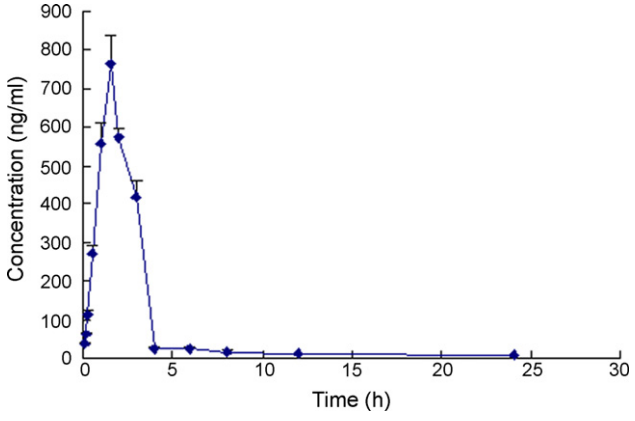

Fig. 4. Mean plasma concentration vs. time profiles in six rats after intragastric administration of single 50 mg/kg dose of helicid.

Only 50 μl of rat plasma was needed in this method due to the high sensitivity, and the method allows serial blood sampling in small laboratory animals. Not more than 150 μl of blood was taken from the rats in our trial at each sampling time, resulting in negligible total blood loss during pharmacokinetic experiments. The low blood loss is better for the animals and gives better confidence to the data.

4. Conclusion

By using detection of deprotonated molecules $[M + Cl]^-$, the LC–ESI-MS method described in the article achieved good sensitivity and selectivity for the quantification of helicid in rat plasma. No interference caused by endogenous compounds was observed. This simple and rapid assay proved to be well suitable for pharmacokinetic study of helicid in rat plasma.

In addition, this method has now been extended to the determination of helicid concentration in other biological samples including cell and tissue homogenates, cerebrospinal fluid, urine and feces. Furthermore, this method has been used for a series of clinical PK–PD correlation studies.

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