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LC–MS/MS determination of helicid in human plasma and its application in pharmacokinetic studies

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ABSTRACT

Helicid is a traditional Chinese medicine used to treat headache and insomnia with definite effects. To facilitate pharmacokinetic studies of helicid in man, a sensitive and specific LC–MS/MS method for the quantitative detection of helicid in human plasma was developed and validated. The method involved the addition of bergeninum as the internal standard (IS), protein precipitation, HPLC separation, and quantification by MS/MS system using negative electrospray ionization in the multiple reaction monitoring mode (MRM). The precursor → product ion transitions were monitored at m/z 282.8 → 120.9 for helicid and m/z 326.9 → 192.2 for the IS, respectively. The lower limit of quantification (LLOQ) was 0.2 μg/L. The calibration curves for helicid was linear over a concentration range of 0.2–20 μg/L. The intra- and inter-batch analyses of QC samples at 0.4, 2, 20 μg/L indicated good precision (%R.S.D. between 2.69 and 5.47%) and accuracy (between 96.15 and 105.05%). The helicid was stable in human plasma stored at room temperature for at least 24 h, 4 °C for at least 24 h, −20 °C for at least 1 month, and for routine three freeze–thaw cycles. This accurate and specific assay provides a useful method for evaluating the pharmacokinetic profile of helicid in humans.

1. Introduction

Helicia nilagirica Bedd, as a traditional Chinese herb, has been used therapeutically throughout southwest China because of its experience-based safety and clinical effectiveness for a long history. Helicid, the main constituent of Shenshuaiguo tablet, extracted from *Helicia nilagirica Bedd*, is a medicine sold in market to treat neurasthenia, neurasthenia syndrome, and vascular headache. The clinical practice showed that helicid has significant effects while no side effects and toxicities in patients have been observed so far [1–5].

Researches on its pharmacological effect mechanism were carried out by scientists all over the world [6–9]. Our group evaluated the pharmacokinetics behavior of helicid in different species based on developed quantitative methods [10–12]. We reported an 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 intra-gastric administration of helicid with a single dose 50 mg/kg. Then, this LC–ESI-MS method was further developed for quantitative determination of helicid in rat biosamples to study its tissue distribution and renal excretion.

In spite of its frequent clinical use, there were few reports on pharmacokinetic of helicid in human. The preliminary experiment showed that the C_{max} of helicid was about 12 μg/L in human plasma, thus all the previously reported methods could not meet the quantization requirements. So, a sensitive and convenient method for determination of helicid in human plasma was carried out in order to assess the pharmaceutics parameters of helicid in human subjects.

2. Materials and methods

2.1. Chemicals and materials

Helicid (Batch No. 040801, Fig. 1A) was kindly provided by Kunming Baker Norton Co., Ltd. Bergeninum (Batch No. 1532-200202, internal standard, Fig. 1B) 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%. HPLC grade acetonitrile was obtained from Fisher Scientific (Toronto, Canada). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Ammonia solution, all other chemicals and solvents used were obtained from standard vendors, and were of the highest quality available.

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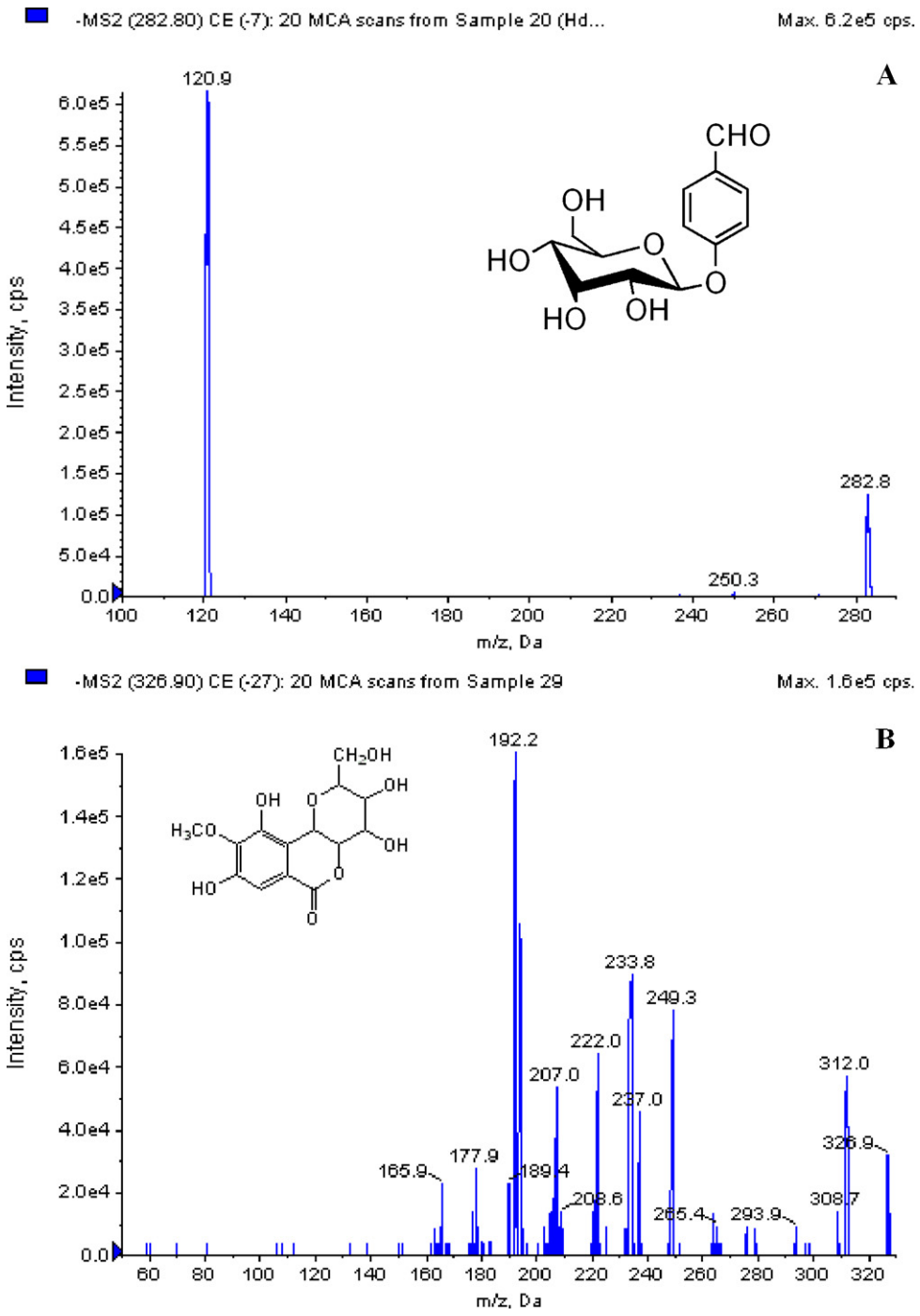

Fig. 1. Chemical structures and product ion spectra of $[M-H]^-$ of helcid (A) and burgenium (B).

2.2. Drug administration and plasma sample collection

In a single-dose pharmacokinetic study, six healthy volunteers, each received an oral dose 100 mg of helicid (provided by Kunming Baker Norton Co., Ltd., Batch No. 070517, 50 mg/tablet) Venous blood samples (2.5 mL aliquots) were collected into heparinized tubes via an indwelling catheter pre-dose and at the following times: 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 12 and 24 h post-dose. The study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University and the volunteers were provided with informed written consent. All study procedures were strictly acted in obedience to the Declaration of Helsinki. 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.

2.3. LC–MS/MS conditions

The LC–MS/MS system consisted of a Shimadzu (Kyoto, Japan) UPLC and an API4000 mass spectrometer (Applied Biosystems, Foster City, CA). A Luna C_{18} column (150 mm × 2.00 mm, 5 μm) was used as the analytical column. A mobile phase composed of acetonitrile–0.1% ammonia solution (20:80, v/v) at a flow rate of 0.3 mL/min was used to obtain the baseline separation of all analytes. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 282.8 → 120.9 for

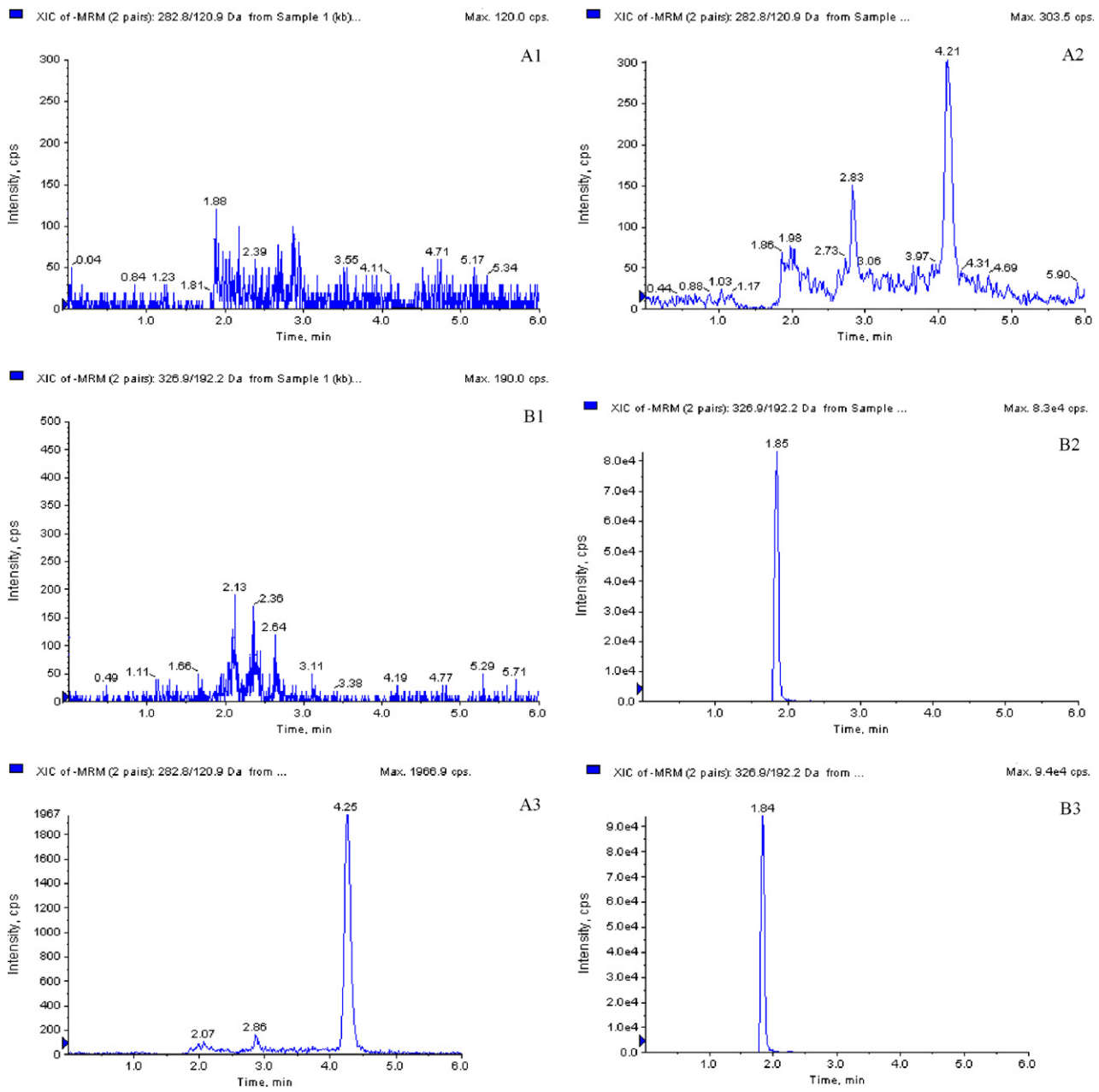

Fig. 2. Representative MRM chromatograms for helicid (A) and burgenium (B, I.S.) from (1) a blank plasma sample; (2) a blank plasma sample spiked with helicid at the LLOQ of 0.2 μg/L and I.S. standards; (3) a plasma sample from a volunteer 0.5 h after oral administration of helicid (100 mg).

helicid, m/z 326.9 → 192.2 for I.S., respectively. The electrospray ionization (ESI) source was set in negative ionization mode. The optimal MS parameters were as follows: the turbo-gas temperature was set at 400 °C and the ion spray needle voltage was adjusted to −4500 V; GS1, GS2, curtain gas and collision gas, were set at 35, 45, 25 and 4 psi, respectively; interface heater (ihe) on. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3, with a dwell time of 100 ms per MRM channel. The collision energy was set at −7 and −27 for helicid and IS, respectively. Data acquisition was performed with analyst software (Version1.5).

2.4. Preparation of calibration and quality control (QC) samples for LC–MS/MS analysis

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 plasma. Internal standard working solution was prepared by diluting internal standard stock solution with methanol. All solutions were stored at 4 °C.

Calibration standards ranging from 0.2 to 20 μg/L at seven concentration levels were prepared by spiking helicid standards into human plasma blanks. The standards were prepared using the sample preparation procedure given below. QC samples at concentration levels of 0.4, 2, 20 μg/L were prepared by spiking helicid standards into human plasma blanks.

2.5. Sample preparation for LC–MS/MS analysis

An aliquot (100 μL) of the plasma, spiked with internal standard working solution (5 μg/mL, 20 μL), was vortex-mixed for 3 min and then deproteinized with 300 μL of acetonitrile. The precipitate

Table 1
Precision and accuracy of the method for the analysis of helicid ($n = 6$).

Compound	Spiked concentration (μg/L)	Intra-batch			Inter-batch		
		Measured concentration (mean ± SD, μg/L)	CV (%)	Accuracy[a] (%)	Measured concentration (mean ± SD, μg/L)	CV (%)	Accuracy[a] (%)
Helicid	0.4	0.42 ± 0.01	2.69	104.73	0.38 ± 0.02	4.86	96.15
	2	2.10 ± 0.08	3.80	105.05	2.09 ± 0.10	4.83	104.56
	20	19.59 ± 0.91	4.65	97.95	20.14 ± 1.10	5.47	100.70

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

was removed by centrifugation at 15,000 rpm at 4 °C for 10 min (Micromax RF, Thermo Electron Corporation, USA). The supernatant (80 μL) was pipetted to an autosampler vial, and 10 μL was injected into column for analysis.

2.6. Method validation

The method was validated according to FDA guidelines on specificity, sensitivity, precision, recovery and stability. To evaluate the assay specificity, six blank plasma samples obtained from six volunteers were tested to demonstrate that there were no interfering components. The linearity of the method was determined by plotting the peak area ratios of the analyte to the IS against the concentrations of helicid in human plasma in duplicate on three consecutive validation days. The lower limit of quantification (LLOQ) was determined in six replicates on three consecutive validation days. The precision and accuracy of the method were assessed by the determination of QC samples at three concentration levels (0.4, 2, and 20 μg/L) in six replicates on three validation days. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. The recovery of helicid was determined by comparing the mean peak areas of the regularly pretreated QC samples at three concentration levels (six samples each) to those of spike-after-extraction samples. To evaluate the matrix effect (ME), six different lots of blank plasma were extracted and then spiked with QC samples and the IS. The corresponding peak area of the analyte in plasma spiked post-extraction (A) were then compared with those of the solution standards in the mobile phase (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the matrix factor (MF). The stabilities of helicid in human plasma were evaluated by analyzing replicates ($n = 6$) of plasma samples at three levels, which were exposed to different conditions (time and temperature).

2.7. Pharmacokinetic analysis

Plasma concentrations equal to or above LLOQ were used for pharmacokinetics analysis using a moment method (DAS software Ver2.0, Chinese Mathematical Pharmacology).

3. Results

3.1. Separation, selectivity, and sensitivity

Fig. 1 shows that the molecular ion adducts m/z 282.8 → 120.9 for helicid and m/z 326.9 → 192.2 for bergeninum (I.S.) were truly predominant compared with other adducts in negative mode. Analysis of blank plasma showed no interference in the final extract, although there are several endogenous peaks that exhibited the same m/z transitions at different retention times. Typical chromatograms of blank plasma extracts are presented in Fig. 2(1). The results indicate that the method provides adequate separation and selectivity through HPLC separation and MS/MS detection. The method provides acceptable sensitivity for the compound of interest. LLOQ sample has a signal-to-noise ratio greater than 10 in the validation. Typical chromatograms for the LLOQ sample and volunteer plasma sample are presented in Fig. 2(2 and 3), respectively.

3.2. Recovery

The mean extraction recoveries of helicid from human plasma were 98.73 ± 1.24, 93.62 ± 1.53, 95.76 ± 7.77 ($n = 6$) at the three concentrations (0.4, 2, and 20 μg/L). The recovery of IS was done the same way; and it was 96.21% ($n = 6$).

3.3. Standard curve linearity

Best fit for the calibration curve could be achieved by a linear equation of $y = 0.0107x - 0.0006$, with $1/x^2$ weighting factor (where, y is the peak-area ratio and x the concentration). The square of the correlation coefficient (r^2, mean value, $n = 6$) for helicid was 0.9993.

3.4. Accuracy and precision

Accuracy and precision of the method were determined by analyzing six QC replicates at 0.4, 2, and 20 μg/L for helicid, in each validation run. Table 1 summarized the accuracy and precision on each of three assays for helicid with good precision (%R.S.D. between 2.69 and 5.47%) and accuracy (between 96.15 and 105.05%), respectively.

3.5. Stability

The helicid in human plasma was found to be stable at room temperature for 24 h, at 4 °C for 24 h, at the −20 °C for 1 month, at three freeze and thaw cycles (Table 2). The results from the tests demonstrated a good stability of helicid over all steps of the determination.

3.6. Matrix effect

The matrix effect was estimated by spiking QC neat solutions into extracted blank plasma samples with the concentration the same as normal QC samples (0.4, 2, 20 μg/L, $n = 6$). Results were calculated by comparing the mean peak areas of helicid in these post-spiked samples with those in corresponding neat solutions. The matrix effects were 96.75% for 0.4 μg/L, 97.85% for 2 μg/L, 96.88% for 20 μg/L.

3.7. Pharmacokinetics studies

After administration of a single dose of 100 mg helicid, the C_{max} and T_{max} were 10.6 ± 3.11 μg/L and 1.08 ± 0.47 h, respectively. Plasma concentration declined with the $t_{1/2}$ of 5.27 ± 0.89 h. The AUC_{0-24} and $AUC_{0-\infty}$ values obtained were37.25 ± 5.33 and 40.34 ± 6.04 μg h/L, respectively. The mean plasma concentration–time curves for helicid are shown in Fig. 3.

Table 2
Stability of helicid in human plasma ($n = 6$).

Compound	Spiked concentration (μg/L)	Remaining (%)			
		Freeze–thaw (three cycles)	Room temperature (for 24 h)	Stored at −20 °C (for 1 month)	Stored at 4 °C (for 24 h)
Helicid	0.4	96.00 ± 0.13	94.31 ± 0.56	93.97 ± 0.72	95.71 ± 0.11
	2	95.97 ± 3.43	95. 45 ± 5.68	95.14 ± 2.55	96.11 ± 3.94
	20	94.91 ± 5.14	94.89 ± 4.96	93.36 ± 1.79	94.86 ± 3.15

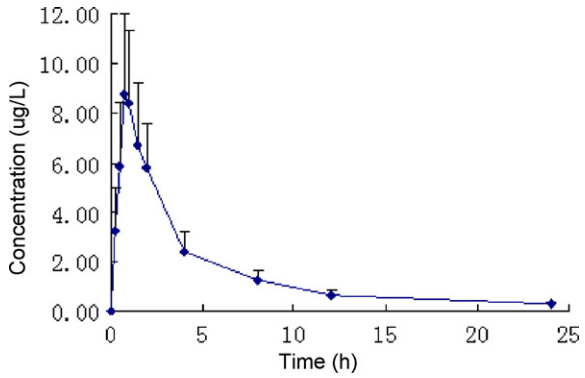

Fig. 3. Mean plasma concentration–time curve of helicid in six volunteers after a single oral dose (100 mg) of helicid.

Due to the excellent sensitivity of assay, with a LLOQ of 0.2 μg/L, plasma concentrations were determined up to 24 h.

4. Discussion

Recently, the introduction of an HPLC–ESI/MS/MS system (HPLC combined with electrospray ionization tandem mass spectrometry) has provided a good technique for developing a more sensitive and specific assaying method. 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]. More recently, Shen Lan et al. reported an HPLC method to investigate the pharmacokinetics of helicid in rats with a LLOQ of 43.8 μg/L in rat plasma. Previously, we reported an LC–MS/MS method for the determination 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 [14].

However, after a single oral dose of 100 mg of helicid, the peak of helicid concentrations in healthy subjects' plasma were not higher than 12 μg/L; thus all previously developed methods were not suitable for human plasma detection. Now, we developed the current LC–MS/MS method, with a LLOQ of 0.2 μg/L. This higher sensitive method can enable us to determine human plasma concentration up to 24 h.

There are some interesting results of our previous studies. The absolute bioavailability is 48.34% in rats, and 22.85% in dogs, respectively. Meanwhile, the plasma concentration was much lower in human; all the above results suggest the existence of species differences. We have investigated the plasma protein binding of helicid by equilibrium dialysis (to be published in another journal); however, there is no significant species difference between human and rats for the plasma protein binding of helicid. A series of studies are underway to uncover the reason for the species differences.

5. Conclusion

An LC–MS/MS assay for the measurement of helicid in human plasma has been established. The method is specific, sensitive, and accurate over a concentration range of 0.2–20 μg/L. This method demonstrated a relatively short analysis time and the good precision, selectivity, recovery and stability. The lowest standards in the calibration curves of plasma, whose signal-to-noise ratio (S/N) were larger than 10, were 0.2 μg/L, which is the lowest detection level reported so far. The intra-batch precision and inter-batch precision values, expressed as R.S.D., were less than 15% at all concentration tested. The single step protein-precipitation with acetonitrile proved to be simple, rapid and convenient. The developed method was fully validated and successfully applied to the human pharmacokinetics studies following a single oral dose of 100 mg helicid.

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