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Short communication

# Simple, sensitive and rapid HPLC–MS/MS method for the determination of cepharanthine in human plasma

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ABSTRACT

A rapid, sensitive and specific method for the determination of cepharanthine in human plasma using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) was described. Cepharanthine and the internal standard (I.S.), telmisartan, were extracted from human plasma by methanol to precipitate the protein. A centrifuged upper layer was then evaporated and reconstituted with 100 μL methanol. Chromatographic separation was performed on an AGILENT XDB-$C_8$ column (150 mm × 2.1 mm, 5.0 μm, Agilent, USA) using a gradient mobile phase with 1 mmol/L ammonium acetate in water with 0.05% formic acid and methanol. Detection and quantitation was performed by MS/MS using electrospray ionization (ESI) and multiple reaction monitoring (MRM) in the positive ion mode. The most intense $[M+H]^+$ MRM transition of cepharanthine at $m/z$ 607.3 → 365.3 was used for quantitation and the transition at $m/z$ 515.5 → 276.4 was used to monitor telmisartan. The calibration curve was linear within the concentration range of 0.5–200.0 ng/mL ($r$ = 0.9994). The limit of quantification (LOQ) was 0.5 ng/mL. The extraction recovery was above 81.1%. The accuracy was higher than 92.3%. The intra- and inter-day precisions were less than 9.66%. The method was accurate, sensitive and simple and was successfully applied to a pharmacokinetic study after single intravenous administration of 50 mg cepharanthine in 12 healthy Chinese volunteers.



## 1. Introduction

Cepharanthine is a biscoclaurine (bisbenzylisoquinoline) alkaloid extracted from the roots of *Stephania cepharantha hayata*. Its structure is shown in Fig. 1. Cepharanthine displays a wide range of pharmacological activities and is widely used, primarily in Japan to treat variety of diseases. It has been used for the treatment of patients with radiation-induced leukopenia or thrombocytopenia, nasal allergy, alopecia areata, exudative otitis media, venomous snakebites, malaria and septic shock [1–3]. In recent years, it was reported that cepharanthine is a membrane-stabilizing, multidrug resistance-reversing [4], anti-inflammatory, anti-HIV-1, free radical scavenging, immunomodulatory agent; and also possesses anti-tumor, apoptosis-inducing effects [5–7].

Yasuda et al. [8,9] reported a sensitive HPLC method to do the pharmacokinetic studies of cepharanthine following oral or intravenous doses in healthy subjects with the limit of determination 0.2 ng/mL. However, the solid-phase extraction (SPE) procedure was used for sample preparation with 1.0 mL plasma and 50 μL extracted sample solution was injected into the HPLC system for analysis in this method, where the SPE procedure was limited by its tedious work of sample preparation and high cost and large plasma volume was restricted in clinical situations. In addition, the method had no internal standard (I.S.) as a control, and it had a narrow linear range (the linear range was 0–5.0 ng/mL in human plasma for oral administration and 0–50.0 ng/mL for intravenous administration and if the concentration of cepharanthine is higher than 50.0 ng/mL, the linear range is inappropriate). Recently an article titled "Absorption of papaverine, laudanosine and cepharanthine across human intestine by using human Caco-2 cells monolayers model" [10] developed a HPLC method to determine cepharanthine in Hank's balanced salt solution (HBSS), Nevertheless, they did not use the method to determine cepharanthine in biological samples, and the method had no I.S. as a control and lacked sensitivity (the LOQ was $1.00 \times 10^{-5}$ mol/L, i.e. 6.06 μg/mL in the HBSS solution and if the concentration of cepharanthine is below that level it cannot be quantitated). Thus the development of an improved analytical method for the determination of cepharanthine in biological samples is necessary.

In our study, we designed a simple, rapid, accurate, sensitive and highly selective HPLC–MS/MS method for the determination of cepharanthine in human plasma samples for the first time. This method was successfully applied to a pharmacokinetic study in 12

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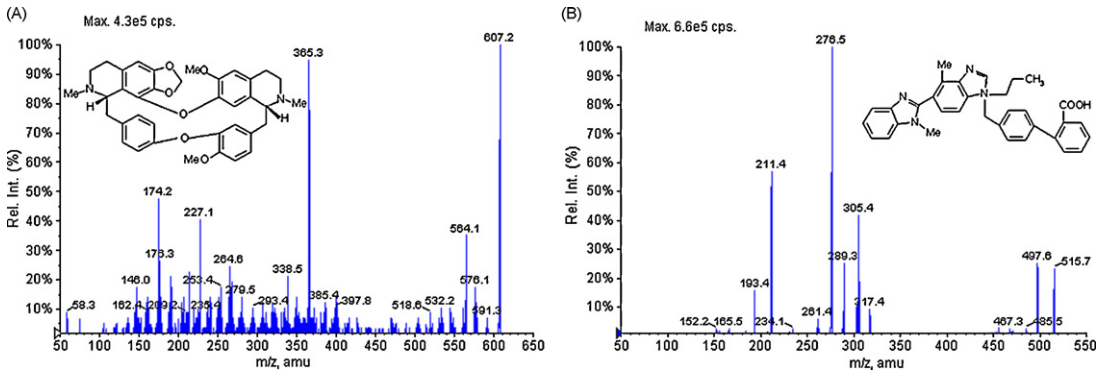


**Fig. 1.** Full-scan product ion scans of $[M+H]^+$ ions and structures for (A) cepharanthine and (B) telmisartan (I.S.).

healthy Chinese volunteers following single intravenous administration of 50 mg cepharanthine.

## 2. Experimental

### 2.1. Chemicals and reagent

Cepharanthine hydrochloride (>99.68%) was supplied by Henan Institute of Medical Sciences (Zhenzhou, PR China), and telmisartan (>99.5%, I.S.) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The primary stock solutions were prepared separately in methanol (100.0 μg/mL for cepharanthine and 100.0 μg/mL for I.S.), working solutions of cepharanthine and I.S. (1.0 ng/mL) were obtained by diluting the stock solutions with methanol. All the standard solutions were stored at −20 °C and the volumetric flask's bouche was wrapped by Parafilm membrane to prevent methanol evaporation when not in use. Methanol, ammonium acetate and formic acid (HPLC grade) were purchased from Dima Technology Inc. (Richmond Hill, Canada). All other chemicals were of analytical grade and used as received. Blank (drug free) human plasma was obtained from the healthy subjects. Deionized water was generated from Milli-Q water purifying system purchased from Millipore Corporation (Bedford, MA, USA), and was used throughout the study.

### 2.2. Calibration standards and control samples

Routine daily calibration curves were prepared in drug free plasma. Appropriate volumes of working solutions and drug free human plasma were added to each test tube. Final concentrations were 0.5, 1.5, 5.0, 10.0, 25.0, 100.0 and 200.0 ng/mL. Similarly, quality control samples that were run in each assay at concentrations of 1.5, 25.0 and 200.0 ng/mL were also prepared.

### 2.3. HPLC–MS/MS conditions and quantifications

High performance liquid chromatography was performed using a Shimadzu HPLC system consisting of a LC-20AD binary pump and a DGU-20A3 degasser and a SIL-20A autosampler and a CTO-10AS$_{VP}$ column oven (Shimadzu Corporation, Kyoto, Japan). The analytical column was an AGILENT XDB-$C_8$ column (150 mm × 2.1 mm i.d., 5.0 μm, Agilent, USA) maintained at 20 °C, which was the same with the ambient temperature of the laboratory. The mobile phases for HPLC were (A) 1 mmol/L ammonium acetate in water with 0.05% formic acid and (B) methanol. The cepharanthine and I.S. were separated with a gradient mobile phase at a flow rate of 0.5 mL/min with a run time of 8.5 min. The following gradient was used: 0–1.5 min, 95% A; 1.5–1.6 min, 95–4.75% A; 1.6–4.7 min, 4.75% A; 4.7–4.8 min, 4.75–95% A; followed by a 3.7 min post-run interval at 95% A until the next sample was injected. Detection was carried out on an API 3200 MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray interface (ESI) and operated in the positive ionization mode. Multiple reaction monitoring (MRM) at unit resolution was employed to monitor the transitions of the protonated molecular ions ($[M+H]^+$) of cepharanthine at $m/z$ 607.3 → 365.3 (quantifier) and of I.S. at $m/z$ 515.5 → 276.4 (quantifier). The most intense product ions of cepharanthine at $m/z$ 365.3 and I.S. at $m/z$ 276.4 were selected and used as the third quadrupole (Q3) ion to be monitored. Optimized MS parameters were: Curtain gas, gas 1 and gas 2 (nitrogen) 15, 45 and 80 psi, respectively; dwell time 150 ms; ion spray voltage 5500 V; ion source temperature 550 °C; declustering potential (DP) 75 V; collision energy (CE) 54 V for cepharanthine and 60 V for I.S. Data acquisition and analysis were performed by using the analyst software version 1.4.2. (Applied Biosystems).

### 2.4. Sample preparation

To a 50 μL aliquot of human plasma in a 1.5 mL Eppendorf tube, 100 μL I.S. working solution and 1 mL methanol were added. The mixture was vortex-mixed for 30 s and centrifuged at 17,968 × $g$ under 4 °C for 10 min. The centrifuged upper layer was carefully transferred in a 10 mL glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was then reconstituted with 100 μL methanol, and vortex-mixing for 30 s, 10 μL solution was injected into the HPLC–MS/MS system.

### 2.5. Pharmacokinetic study

The method described above was applied to the pharmacokinetic study in which plasma concentrations of cepharanthine in 12 healthy Chinese volunteers were determined up to 192 h after single intravenous administration of 50 mg cepharanthine over a period of 60 min. Blood samples were drawn before drug intake and at different time points (0.33, 0.66, 1, 2, 3, 5, 8, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h) after dosing of cepharanthine. The clinical pharmacokinetic study was approved by the Ethics Committee of Affiliated Hospital of Academy of Military Medical Sciences. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

## 3. Results and discussion

### 3.1. HPLC–MS/MS

The HPLC–MS/MS with ESI positive ionization and the MRM mode provided a highly selective method for the determination of cepharanthine and the I.S. The ESI positive ion mode was chosen

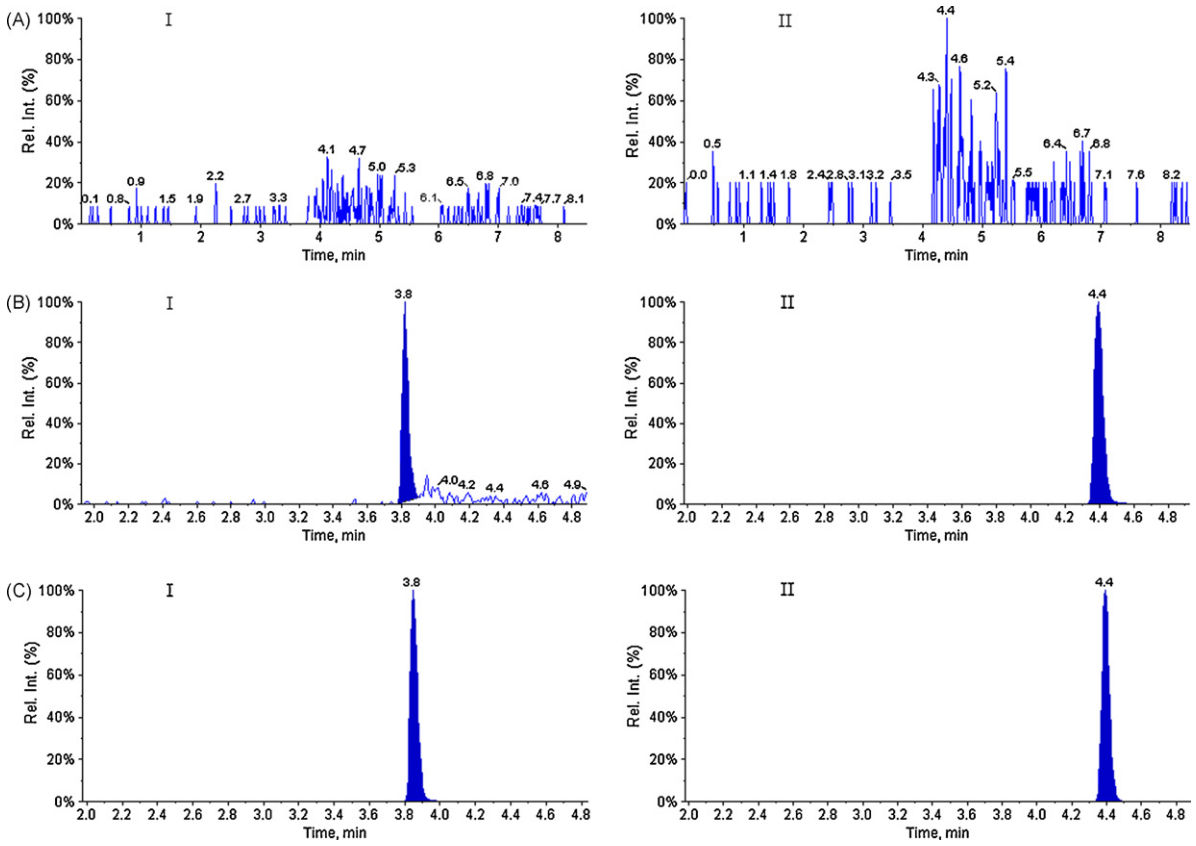


**Fig. 2.** MRM chromatograms of cepharanthine (I) and I.S. (II) obtained from human plasma samples: (A) blank plasma; (B) blank plasma spiked with standard solution (LOQ); (C) plasma sample from a healthy subject 1 h after single intravenous administration of 50 mg cepharanthine with concentration of 82.7 ng/mL.

for ion product since there are two hetero-*N* atoms in the structure of cepharanthine. The ion source temperature was set at 550 °C which was to make sure the gradient mobile phase thoroughly atomized and enhance the sensitivity as well. From full-scan mass spectrum via the first quadrupole (Q1) mass filter, the $[M+H]^+$, at *m*/*z* 607.3 for cepharanthine and *m*/*z* 515.5 for I.S. were chosen as the precursor ion. By introducing those $[M+H]^+$ ions into the second quadrupole (Q2) cell with the optimum CE, the MS/MS fragmentation was achieved. After the dissociation via the Q3 mass filter, the most abundant ions in the product ion mass spectrum at *m*/*z* 365.3 for cepharanthine and *m*/*z* 276.4 for I.S. were monitored for quantification. Full-scan product ion mass spectrum of cepharanthine and I.S. are shown in Fig. 1. As the $[M+H]^+$ MRM transition at *m*/*z* 607.3 → 365.3 for cepharanthine and *m*/*z* 515.5 → 276.4 for I.S. were the most intense ones, thus were used as the quantifier.

Various mobile phase combinations of ammonium acetate (1 mmol/L) in water and methanol or acetonitrile were investigated to optimize sensitivity, speed and peak shape. The results (data not shown) demonstrated that methanol gave a better response than acetonitrile and ammonium acetate (1 mmol/L) in water containing 0.05% formic acid improved the efficiency of ionization and peak shapes, at the same time, increase the sensitivity. Thus, HPLC separation was achieved in a total runtime of 8.5 min on an AGILENT XDB-$C_8$ column with ammonium acetate (1 mmol/L) in water with 0.05% formic acid–methanol gradient (Fig. 2C). Because of the highly selective detection method, there were no interfering peaks present in six different batches of blank samples investigated from six separate healthy volunteers. An example of a blank plasma sample is shown in Fig. 2A, the lowest quality control sample (0.5 ng/mL) in Fig. 2B. In this study, we utilized a relatively simple method of protein precipitation to prepare the blood sample, which is a small solvent consumed procedure. The time of protein precipitation and centrifugation was also short. As demonstrated in this assay, this method is sensitive, specific, allowing for analyzing samples in batches and perfectly suitable for a high-throughput routine such as pharmacokinetic studies.

### 3.2. Matrix effects

The matrix effect (ME, i.e. potential ion suppression or enhancement effects of co-eluting and undetected matrix components in plasma) was investigated. It was evaluated by comparing the peak area of cepharanthine and I.S. spiked in post-extracted blank plasma samples to that of cepharanthine and I.S. spiked in mobile phase at equivalent concentration. The matrix effect of the assay was evaluated at three quality control concentrations (1.5, 25.0 and 200.0 ng/mL) of cepharanthine and the I.S. concentration level of 1.0 ng/mL. Six samples at each concentration level of the analytes were analyzed. The blank plasmas used in this study were from six different batches of healthy human blank plasma. If the ME <85% or >115%, an endogenous matrix effect is implied. In the present study, the ME of nominal quality control samples at concentrations of 1.5, 25.0 and 200.0 ng/mL were 102.7 ± 4.8, 87.5 ± 2.8 and 88.0 ± 1.7%, respectively. The ME of I.S. was 92.7 ± 3.8%. The results obtained were well within the acceptable limit, it indicated that the analysis of cepharanthine was not interfered with by endogenous substances in plasma.

### 3.3. Validation of the method

The extraction recoveries of analyte were determined at three different concentration levels by comparing the peak areas of analyte obtained from the quality control samples ($n = 5$) after extraction to those obtained from the reference samples which

**Table 1**
Extraction recoveries, intra- and inter-day accuracy and precision.

| Concentration (ng/mL) | Extraction recoveries ($n = 5$) | | Accuracy (%, $n = 6$) | | Intra-day precision ($n = 6$) | | Inter-day precision ($n = 6$) | |
|---|---|---|---|---|---|---|---|---|
| | Recoveries (%) | RSD (%) | Intra-day | Inter-day | Mean | RSD (%) | Mean | RSD (%) |
| 1.5 | 84.04 | 1.38 | 97.08 | 102.58 | 1.46 | 4.31 | 1.54 | 9.66 |
| 25.0 | 81.13 | 0.45 | 99.60 | 100.07 | 24.83 | 2.62 | 25.00 | 5.90 |
| 200.0 | 85.30 | 4.24 | 96.23 | 97.03 | 192.33 | 3.11 | 194.00 | 5.05 |

*Note*: R.S.D., relative standard deviations; extraction recovery (%) = [mean peak area (extraction samples)/mean peak area (reference samples)] × 100%.

**Table 2**
Stability test of cepharanthine ($n = 5$).

| Concentration (ng/mL) | Room temperature | | −40 °C for 3 months | | Freeze/thaw | | Autosampler | |
|---|---|---|---|---|---|---|---|---|
| | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) |
| 1.5 | 1.51 | 11.38 | 1.61 | 10.93 | 1.52 | 11.84 | 1.45 | 4.97 |
| 25.0 | 21.80 | 2.00 | 27.57 | 3.04 | 25.40 | 2.36 | 24.07 | 1.27 |
| 200.0 | 192.00 | 7.86 | 205.67 | 1.56 | 198.67 | 2.32 | 185.00 | 3.38 |

*Note*: R.S.D., relative standard deviations.

were prepared by adding the analyte to the post-extracted blank plasma samples at the same concentrations.

The intra-assay precision and accuracy was assessed by measuring the concentration of cepharanthine in six aliquots of three different quality control samples extracted and analyzed on a single day. Inter-assay precision and accuracy was determined from the results of three different quality control samples which were extracted and analyzed six fold on three consecutive days. All the results are summarized in Table 1.

The calibration curve of cepharanthine was linear over the concentration range of 0.5–200.0 ng/mL. The equation was $Y = 0.0157X + 0.00639$ ($r = 0.9994$). The LOQ validated was 0.5 ng/mL (S/N = 10) and the assay only requires 50 μL of plasma. These features are important for clinical situations when blood volume is restricted and yet high assay sensitivity is required.

### 3.4. Stability

The stability of quality control plasma samples (1.5, 25.0 and 200.0 ng/mL) were found stable in plasma when placed in the short-term (4 h) room temperature, three freeze/thaw (−40 °C) cycles and stored at −40 °C for 3 months. In addition, the stability of cepharanthine in processed quality control samples (i.e. ready for HPLC–MS/MS analysis) was also found stable when placed in the autosampler at the ambient temperature (20 °C) for 5 h (Table 2).

### 3.5. Pharmacokinetic application

The validated HPLC–MS/MS method has been successfully used to determine the concentrations of cepharanthine in human plasma samples. The mean plasma concentrations–time profile of cepharanthine after single intravenous administration of 50 mg cepharanthine is shown in Fig. 3. The concentrations of cepharanthine in human plasma decreased quickly in the first 2 h after administration and it was similar to previous study [9].

The pharmacokinetic parameters were estimated by the DAS version 2.1.1 software. Pharmacokinetic analysis of cepharanthine was performed by non-compartmental methods. The concentration maximum ($C_{max}$) and the time to it ($T_{max}$) were noted directly. The elimination rate constant ($K_e$) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) was calculated through $0.693/K_e$. The area under the plasma concentration–time curve of cepharanthine, from time zero to infinity ($AUC_{0-\infty}$) was determined by the linear trapezoidal rule to the last measurable plasma concentration ($C_t$) plus the additional area from time $t$ to infinity, calculated as $C_t/K_e$. The mean $C_{max}$ of cepharanthine was 135.9 ± 66.9 ng/mL occurred around 0.75 ± 0.21 h post-dosing. The $t_{1/2}$ was 131.9 ± 48.4 h and the mean area under the plasma concentration–time curve ($AUC_{0-192}$) was 566.6 ± 216.6 ng h/mL.

The mean $C_{max}$ after single intravenous administration with the same dosage was much lower (135.9 ± 66.9 ng/mL) in this study than previous reported (433.0 ± 25.0 ng/mL) in the literature [9], which could be explained by an infusion duration of 60 min instead of 5 min used in this study. However, Yasuda et al. reported the mean $C_{max}$ after dosing with cepharanthine 60 mg oral tablets was just 3.5 ± 0.3 ng/mL in human plasma and the absolute bioavailability of cepharanthine was 6–9% after oral dosing. The results suggested that cepharanthine could be extensively metabolized in liver [8,9]. In addition, the $t_{1/2}$ was quite longer in this study (131.9 ± 48.4 h) than previous reported (36.9 ± 3.6 h) in the literature [9]. Differences between the studies might be related to the duration of sampling times, for in our study the terminally sampling time was until 192 h while in the previous literature was until 48 h after dosing. Besides, plasma concentrations were always fluctuated in the range of 2.6–1.2 ng/mL from 24 to 192 h after dosing. These results might further certify that cepharanthine was rapidly distributed into organs and largely uptaken to tissues, binding strongly in the first 2 h after intravenous administration [9], and then gradually eliminated in vivo which contributed to the

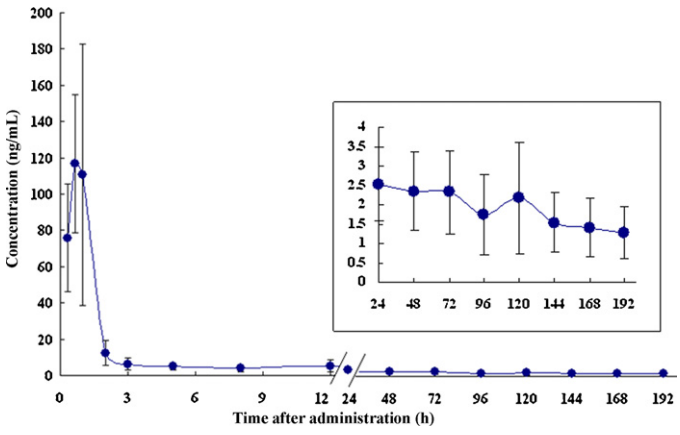


**Fig. 3.** Plasma concentrations–time profile of cepharanthine after single intravenous administration of 50 mg dose. Data are means ± S.D. for 12 healthy Chinese volunteers. The inset is for expanding the *Y*-axis between 24 h with 192 h after dosing.

long $t_{1/2}$. Thus we should proceed to do the research of tissues distribution, metabolism pathway and metabolites, the relevance of pharmacokinetic and pharmacodynamics of cepharanthine in future human pharmacokinetic studies.

## 4. Conclusion

A HPLC–MS/MS method has been developed for the determination of cepharanthine in human plasma. The assay described here has been found to be specific and accurate in application, no significant interferences caused by endogenous compounds are observed. This simple and sensitive assay is suitable for pharmacokinetic studies of cepharanthine in human subjects.

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