Determination of cepharanthine in rat plasma by LC–MS/MS and its application to a pharmacokinetic study

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ABSTRACT
Context: Cepharanthine (CPA) has been reported to possess a wide range of pharmacological activities. Objective: This study investigates the pharmacokinetic characteristics after oral or intravenous administration of CPA by using a sensitive and rapid LC–MS/MS method. Materials and methods: A sensitive and rapid LC–MS/MS method was developed for the determination of CPA in Sprague–Dawley rat plasma. Twelve rats were equally randomized into two groups, including the intravenous group (1 mg/kg) and the oral group (10 mg/kg). Blood samples (250 μL) were collected at designated time points and determined using this method. The pharmacokinetic parameters were calculated. Results: The calibration curve was linear within the range of 0.1–200 ng/mL (r = 0.999) with the lower limit of quantification at 0.1 ng/mL. After 1 mg/kg intravenous injection, the concentration of CPA reached a maximum of 153.17 ± 16.18 ng/mL and the t1/2 was 6.76 ± 1.21 h. After oral administration of 10 mg/kg of CPA, CPA was not readily absorbed and reached Cmax 46.89 ± 5.25 ng/mL at approximately 2.67 h. The t1/2 was 11.02 ± 1.32 h. The absolute bioavailability of CPA by oral route was 5.65 ± 0.35%, and the bioavailability was poor. Discussion and conclusions: The results indicate that the bioavailability of CPA was poor in rats, and further research should be conducted to investigate the reason for its poor bioavailability and address this problem.

INTRODUCTION
Cepharanthine (CPA), a bisococlaurine alkaloid isolated from Stephania cepharantha Hayata (Menispermaceae), has been reported to possess a wide range of pharmacological activities (Azuma et al. 2006; Chea et al. 2007; Desgrouas et al. 2014). CPA has been widely used for the treatment of many acute and chronic diseases, such as venomous snakebite, alopecia areata, exudative otitis media, and endotoxic shock (Furusawa & Wu 2007; Kusaka et al. 2011; Rogosnitzky & Danks 2011). CPA also exhibits potent anticancer activity in vitro for different cancer cells (Biswas et al. 2006; Harada et al. 2009; Seubwai et al. 2010; Ikeda et al. 2011; Chen et al. 2012). Some research articles (Ikeda et al. 2005; Seo et al. 2009; Li et al. 2011; Zahedi et al. 2011; Han et al. 2014) have reported that CPA could modulate the activity of P-glycoprotein (P-gp) through downregulation of the expression MDR1, and therefore, combined with other drugs, it could be used for overcoming the multidrug resistance phenomenon in cancer therapy.

Due to the potent pharmacological activities of CPA, it is of great significance to investigate the pharmacokinetic properties of CPA. Hao et al. (2010) have developed a sensitive and reliable LC–MS/MS method to determine the concentration of CPA in human plasma, and investigate the pharmacokinetics profiles of CPA after single intravenous administration of 50 mg CPA. However, to the best of our knowledge, there is little data available regarding the oral bioavailability of CPA. Investigating the bioavailability of CPA in rats will be helpful for the development of preparations and pharmacological investigations, and it could also provide a basis for the rational drug use and prediction of drug toxicity reaction. To enhance the development potential of CPA, there is an urgent need to investigate the pharmacokinetic profiles of CPA, especially its bioavailability characteristics.

This study investigates the pharmacokinetic characteristics after oral or intravenous administration of CPA by using a sensitive and rapid LC–MS/MS method.

MATERIALS AND METHODS
Chemicals and reagents
CPA (purity >98%) and rutin (purity >98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid was purchased from Anaqua Chemicals Supply Inc. Limited (Houston, TX). All other chemicals were of analytical grade or better.

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**Instrumentation and conditions**

The analysis was performed on an Agilent 1290 series liquid chromatography system (Agilent Technologies, Palo Alto, CA) and an Agilent 6470 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatographic separation of CPA and rutin was performed on Waters X-Bridge C18 column (3.0 × 100 mm, i.d.; 3.5 μm, Milford, MA) at room temperature (25°C). The mobile phase was water (containing 1 mM ammonium formate and 0.05% formic acid) and methanol (35:65, v:v) with isotropic elution at a flow rate of 0.3 mL/min, and the analysis time was 1.5 min. The injection volume was 2 μL and the auto-sampler temperature was maintained at 25°C.

The mass scan mode was positive MRM mode, and the mass parameters were optimized using Optimizer software. The precursor ion and product ion are m/z 607.3 → 365.3 for CPA and m/z 610.9 → 355.9 for rutin (internal standard), respectively. The collision energy for CPA and rutin was 30 and 25 eV, respectively. The MS/MS conditions were optimized as follows: fragmentor, 160 V; capillary voltage, 4 kV; Nozzle voltage, 500 V; nebulizer gas pressure (N2), 40 psig; drying gas flow (N2), 10 L/min; gas temperature, 350°C; sheath gas temperature, 400°C; sheath gas flow, 11 L/min.

**Pharmacokinetic study**

**Animals**

Male Sprague–Dawley (SD) rats weighing 220–250 g were provided by the Experimental Animal Center of the Shandong University (Shandong, China). Rats were bred in a breeding room at 25°C, 60 ± 5% humidity, and a 12 h dark–light cycle. Tap water and normal chow were given ad libitum. All the experimental animals were housed under the above conditions for a five day acclimation, and were fasted overnight before the experiments. The study was approved by the Animal Care Committee of the Shandong University (Shandong, China) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2016).

**In vivo pharmacokinetic study**

Rats were fasted for 12 h with free access to water prior to the pharmacokinetic study. Twelve rats were equally randomized to two groups and were treated as followings: intravenous injection of CPA in normal saline was administrated through lateral tail vein at a dose of 1 mg/kg. An oral gavage of CPA dissolved in normal saline containing 0.5% methylcellulose solution was given ad libitum. All the experimental animals were housed under the above conditions for a five day acclimation, and were fasted overnight before the experiments. The study was approved by the Animal Care Committee of the Shandong University (Shandong, China) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2016).

**Plasma sample preparation**

Plasma sample (100 μL) was spiked with 10 μL of the rutin (100 ng/mL), and then the mixture was extracted with acetonitrile (190 μL) by vortexing for 1 min. After centrifugation at 12,500 rpm for 10 min, the supernatant was injected into the LC–MS/MS for the determination.

**Preparation of standard and quality control samples**

A stock solution of CPA was prepared in acetonitrile at a concentration of 2 mg/mL. The stock solution of rutin was prepared in acetonitrile at a concentration of 1 μg/mL. Calibration standards for CPA were prepared in blank rat plasma at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 and 200 ng/mL. The quality control (QC) samples were prepared at low (0.2 ng/mL), medium (10 ng/mL) and high (150 ng/mL) concentrations in the same way as the plasma samples for calibration, and QC samples were stored at −40°C until analysis.

**Method validation**

The method validation assay was performed according to the United States Food and Drug Administration (FDA) guidelines.

**Specificity**

Specificity was investigated by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma to monitor interference of endogenous substances and metabolites.

**Linearity and sensitivity**

To obtain the calibration curve, seven concentrations of the calibration standard were processed and determined as described above. The linearity of calibration curves was constructed by plotting peak area ratios (y) of the analyte to rutin against the nominal concentration (x) of analyte with weighted (1/x²) least square linear regression. The lower limit of detection (LLD) and lower limit of quantification (LLOQ) were determined as the concentration of the analyte with a signal-to-noise ratio at 3 and 10, respectively.

**Precision and accuracy**

The intra-day precision and accuracy of the method were confirmed by determining QC samples at three different concentrations five times on a single day, and the inter-day precision and accuracy were assessed by determining the QC samples over three consecutive days. For each concentration, five replicates were prepared. Relative standard deviation (RSD) and relative error (RE) were used to express the precision and accuracy, respectively.

**Extraction recovery and matrix effect**

The extraction recovery was assessed by comparing peak areas obtained from extracted spiked samples with those originally spiked in the blank plasma samples. The matrix effect was evaluated by comparing the peak areas of the post-extracted spiked QC samples with those of corresponding standard solutions. These procedures were repeated for five replicates at three QC concentration levels.

**Stability**

For sample stability, three levels of QC samples were determined under different conditions, including short-term stability at room temperature for 24 h, long-term stability at −40°C for 30 days and three freeze–thaw cycles at −40°C.

**Data analysis**

The pharmacokinetic parameters, including area under the plasma concentration–time curve (AUC), maximal plasma concentration (Cmax), the time for maximal plasma concentration (Tmax) and...
mean residence time (MRT) were calculated using DAS 3.0 pharmacokinetic software (Chinese Pharmacological Association, Anhui, China).

Results and discussion

Sample preparation

Due to the complex nature of plasma, a sample pretreatment procedure is often needed to remove protein and potential interferences before LC–MS/MS analysis. In this study, protein precipitation, solid phase extraction (SPE) and liquid–liquid extraction were investigated to achieve good resolution and high recovery of analyte from spiked biologic matrices. The recovery of direct protein precipitation method using acetonitrile (88.7 ± 5.8) was better than SPE (82.4 ± 4.7) and liquid–liquid extraction method (76.5 ± 4.2), and the direct protein precipitation method was much easier to operate. Finally, direct protein precipitation method using acetonitrile was selected for biological sample preparation.

Chromatography and mass spectrometry

The optimized mass transition ion-pairs for quantification, including precursor and product ions, were \( m/z \) 607.3 → 365.3 for CPA and \( m/z \) 610.9 → 355.9 for rutin, respectively. Blank plasma, plasma spiked with CPA and rutin are shown in Figure 1. No interference substances were observed at the retention time of CPA and rutin in plasma samples.

Method validation

The standard curve for CPA in plasma was linear in the concentration range of 0.1–200 ng/mL \( (y = 0.295x + 0.119, \ r = 0.999) \). The LLOQ and LLOD were 0.1 and 0.038 ng/mL, respectively.
Intra-day and inter-day precision and accuracy were determined by measuring six replicates of QC samples at three concentration levels in rat plasma. The precision and accuracy data are shown in Table 1. These results demonstrated that the precision and accuracy values were well within an acceptable range of 15%.

The mean extraction recoveries determined using three replicates of QC samples at three concentration levels in rat plasma were 87.8 ± 6.5%, 92.4 ± 5.7%, and 85.6 ± 6.5% for 0.2, 10 and 150 ng/mL, respectively.

For ionization, the peak areas of CPA after spiking evaporated plasma samples at three concentration levels were comparable to those of similarly prepared aqueous standard solutions (ranging from 91.6% to 103.8%), suggesting that there was no measurable matrix effect that interfered with CPA determination in rat plasma.

The stability of CPA in plasma was evaluated by analysing three replicates of QC samples containing 0.2, 10 and 150 ng/mL CPA after short-term storage (25°C, 24 h), long-term cold storage (−40°C, 30 days) and within three freeze (−40°C)–thaw (room temperature) cycles. As shown in Table 2, all of the samples displayed 90–110% recoveries after various stability tests. Taken together, the above results show that a rapid, simple and sensitive method for the determination of pharmacokinetic profiles of CPA after intravenous administration of CPA, the limit of quantification was 0.5 ng/mL, and the analysis time was 5 min. The LC–MS/MS method developed in this study was much simpler (direct protein precipitation), sensitive (LLOQ at 0.1 ng/mL) and rapid (1.5 min analysis time) compared with these methods. In addition, we have also investigated the pharmacokinetics profiles of CPA after oral administration, which was not studied previously.

### Pharmacokinetic studies

The validated analytical method was employed to study the pharmacokinetic behaviours of CPA in rats. The mean plasma concentration–time curves of CPA after intravenous or oral administration of CPA are presented in Figure 2.

The pharmacokinetic parameters were calculated using the noncompartmental method with DAS 3.0 pharmacokinetic software (Chinese Pharmacological Association, Anhui, China). The pharmacokinetic parameters are shown in Table 3. The oral bioavailability was calculated by using $AUC_{oral}/dose$ divided by $AUC_{iv/dose}$.

After 1 mg/kg intravenous injection, the concentration of CPA reached the peak plasma concentration of 153.17 ± 16.18 ng/mL and the $t_{1/2}$ was 6.76 ± 1.21 h. After oral administration of 10 mg/kg of CPA, CPA was not readily absorbed and reached $C_{max}$ of 46.89 ± 5.25 ng/mL at approximately 2.67 h. The $t_{1/2}$ was 11.02 ± 1.32 h. These results revealed that CPA was distributed and eliminated slowly in rats. The absolute bioavailability of CPA

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**Table 1.** The intra-day and inter-day precision and accuracy of cepharanthine in plasma samples.

| Analyte    | Plasma samples (ng/mL) | Intra-day                  | Inter-day                  |
|------------|------------------------|----------------------------|----------------------------|
|            |                        | Concentration measured     | Precision (% RSD)          | Accuracy (% RE) | Concentration measured | Precision (% RSD) | Accuracy (% RE) |
|            | (ng/mL)                | (ng/mL)                    |                            |                | (ng/mL)                |                            |                |
| Cepharanthine | 0.2                   | 0.19                       | 6.87                       | -5             | 0.22                   | 5.64                       | 10             |
|            | 10                    | 10.57                      | 5.59                       | 5.70           | 9.14                   | 7.52                       | -8.60          |
|            | 150                   | 141.25                     | 6.38                       | -5.83          | 161.25                 | 8.24                       | 7.50           |

**Table 2.** Stability of CPA in plasma samples ($n = 3$).

| Analyte | Plasma samples (ng/mL) | Stability (%) | RE (%) |
|---------|------------------------|---------------|--------|
|         |                        | Short-term (24 h at room temperature) | Long-term (30 days at −40°C) | Three freeze (−40°C)–thaw (room temperature) cycles |
| CPA     | 0.2                    | 4.68          | -7.39  | 7.68 |
|         | 10                     | 7.45          | 7.25   | 6.87 |
|         | 150                    | 5.36          | 6.98   | 9.77 |

**Figure 2.** The pharmacokinetic profiles of CPA in rats after intravenous administration of CPA (A) at dosage of 1 mg/kg and oral administration of CPA (B) at 10 mg/kg.
by oral route was 5.65 ± 0.35%, which demonstrated that the drug has poor absorption in rats through oral administration. As reported by Hirai et al. (1995), CPA was a substrate of P-gp, and CPA was transported by P-gp in intestine. Therefore, we suggested that P-gp might hinder its absorption in intestine, and lead to its poor bioavailability in rats (Miao et al. 2016; Wang et al. 2016). The low metabolic stability in liver might also be a reason for its poor bioavailability. Further research should be conducted to investigate the role of P-gp in the transport of CPA in intestine and its effect on the absorption of CPA, the metabolism in liver, while improving its oral bioavailability. As the species differences in the properties of metabolism enzymes between rats and human, it is essential to investigate the pharmacokinetics profiles of CPA in human.

Conclusions

In conclusion, a sensitive and rapid LC–MS/MS method has been developed and successfully applied to determine the concentration of CPA in rat plasma. Using this method, the pharmacokinetic characteristics of CPA in rats were investigated, and the results indicated that the bioavailability of CPA was poor in rats. Further research should be conducted to investigate the reason for its poor bioavailability and address this problem.

Disclosure statement

The authors have declared no conflicts of interest.

References

Azuma M, Ashida Y, Tamatani T, Motegi K, Takamaru N, Ishimaru N, Hayashi Y, Sato M. 2006. Cepharanthin, a bescoquinone alkaloid, prevents destruction of acinar tissues in murine Stréptom’s syndrome. J Rheumatol. 33:912–920.

Biswas KK, Tanchanroen S, Sarker KP, Kawahara K, Hashiguchi T, Maruyama I. 2006. Cepharanthine triggers apoptosis in a human hepatocellular carcinoma cell line (HuH-7) through the activation of JNK1/2 and the down-regulation of Akt. FEBS Lett. 580:703–710.

Table 3. Pharmacokinetic parameters of CPA in rats after intravenous (1 mg/kg) or oral administration (10 mg/kg) of CPA (n = 6, mean ± SD).

| Parameters        | Intravenous | Oral | Parameters |
|-------------------|-------------|------|------------|
| T_max (h)         | –           | 2.67 ± 1.16 |
| C_max (ng/mL)     | 153.7 ± 16.18 | 46.89 ± 5.25 |
| t1/2 (h)          | 6.76 ± 1.21 | 11.02 ± 1.32 |
| AUC(0–∞) (ng·h/mL)| 717.81 ± 158.35 | 406.63 ± 62.57 |
| AUC(0–t) (ng·h/mL)| 721.80 ± 160.76 | 422.26 ± 66.91 |
| MRT(0–t) (h)      | 7.04 ± 0.49 | 10.49 ± 0.62 |
| MRT(0–∞) (h)      | 7.30 ± 0.51 | 12.45 ± 1.20 |
| C_l (L/h/kg)      | 1.431 ± 0.31 | 24.08 ± 2.42 |
| V_l (L/kg)        | 13.79 ± 1.76 | 381.37 ± 61.63 |

C_max: peak plasma concentration; T_max: the corresponding time to reach C_max; t1/2: the terminal elimination half-life; MRT: mean residence time; AUC(0–t): AUC(0–∞): the areas under the plasma concentration–time curve from time zero to the last quantifiable time-point and to infinity; C_l: clearance; V_l: apparent volume of distribution.

Chea A, Hout S, Bun SS, Tabatadze N, Gasquet M, Azas N, Elias R, Balansard G. 2007. Antimalarial activity of alkaloids isolated from Stephania rotunda. J Ethnopharmacol. 112:15–23.

Chen Z, Huang C, Yang YE, Ding Y, Ou-Yang HQ, Zhang YY, Xu M. 2012. Inhibition of the STAT3 signaling pathway is involved in the antitumor activity of cepharanthine in SaOs2 cells. Acta Pharmacol Sin. 33:101–108.

Desgrous C, Chapus C, Desplas J, Travaille C, Pascual A, Baghdikian B, Ollivier E, Parzy D, Taudon N. 2014. In vitro antiplasmodial activity of cepharanthine. Malar J. 13:327.

Furusawa S, Wu J. 2007. The effects of bescoquinone alkaloid cepharanthine on mammalian cells: implications for cancer, shock, and inflammatory diseases. Life Sci. 80:1073–1079.

Han L, Wang Y, Guo X, Zhou Y, Zhang J, Wang N, Jiang J, Ma F, Wang Q. 2014. Downregulation of MDRI gene by cepharanthine hydrochloride is related to the activation of c-Jun/NFK in K562/ADR cells. Biomed Res Int. 2014:164391.

Hao G, Liang N, Li Y, Li H, Gao H, Liu G, Liu Z. 2010. Simple, sensitive and rapid HPLC–MS/MS method for the determination of cepharanthine in human plasma. J Chromatogr B Anal Technol Biomed Sci. 878:2923–2927.

Harada K, Ferdous T, Itashiki Y, Takii M, Mano T, Mori Y, Ueyama Y. 2009. Cepharanthine inhibits angiogenesis and tumorigenesis of human oral squamous cell carcinoma cells by suppressing expression of vascular endothelial growth factor and interleukin-8. Int J Oncol. 35:1025–1035.

Hirai M, Tanaka K, Shimizu T, Tanigawa Y, Yasuhara M, Hori R, Kakehi Y, Yoshida O, Ueda K, Komano T, et al. 1995. Cepharanthin, a multidrug resistant modifier, is a substrate for P-glycoprotein. J Pharmacol Exp Ther. 275:73–78.

Ikeda R, Che XF, Yamaguchi T, Ushiyama M, Zheng CL, Okumura H, Takeda Y, Shibayama Y, Nakamura K, Jeung HC, et al. 2005. Cepharanthine potently enhances the sensitivity of anticancer agents in K562 cells. Cancer Sci. 96:372–376.

Ikeda R, Vermeulen LC, Lau E, Jiang Z, Sachidanandam K, Yamada K, Kolesar JM. 2011. Isolation and characterization of gemcitabine-resistant human non-small cell lung cancer A549 cells. Int J Oncol. 38:513–519.

Kusaka J, Hagiwara S, Hasegawa A, Kudo K, Koga H, Noguchi T. 2011. Cepharanthine improves renal ischemia-reperfusion injury in rats. J Surg Res. 171:212–217.

Li H, Yan Z, Ning W, Xiao-Juan G, Cai-Hong Z, Jin-Hua J, Fang M, Qing-Duan W. 2011. Using rhodamine 123 accumulation in CD8 cells as a surrogate indicator to study the P-glycoprotein modulating effect of cepharanthine hydrochloride in vivo. J Biomed Biotechnol. 2011:281651.

Miao Q, Wang Z, Zhang Y, Miao P, Zhao Y, Ma S. 2016. In vitro potential modulation of baicalin and baicalein on P-glycoprotein activity and expression in Caco-2 cells and rat gut sacs. Pharm Biol. 54:1548–1556.

National Research Council. 2016. The guide for the care and use of laboratory animals. ILAR J. 57:NP.

Rogosnitzky M, Danks R. 2011. Therapeutic potential of the biscoclaurine alkaloid, cepharanthine, for a range of clinical conditions. Pharmacol Rep. 63:337–347.

Seo S, Hatano E, Higashi T, Nakajima A, Nakamoto Y, Tada M, Tamaki N, Iwaisako K, Kitamura K, Iikai I, et al. 2009. P-glycoprotein expression affects 125I-fluoroedgeuxycoum accumulation in hepatocellular carcinoma in vivo and in vitro. Int J Oncol. 34:1303–1312.

Subbawi W, Vaetewoottacharn K, Hiyoshi M, Suzu S, Puapairoj A, Wongkham C, Okada S, Wongkham S. 2010. Cepharanthine exerts antitumor activity on cholangiocarcinoma by inhibiting NF-kappaB. Cancer Sci. 96:337–347.

Yasuda K, Moro M, Akasu M, Ohnishi A. 1989. Cepharanthin, a multidrug resistant modifier, is a substrate for P-glycoprotein. J Pharmacol Exp Ther. 275:73–78.

Zahedi P, De Souza R, Huyhn L, Piquette-Miller M, Allen C. 2011. Combination drug delivery strategy for the treatment of multidrug resistant ovarian cancer. Mol Pharm. 8:260–269.