Pharmacokinetics and Bioavailability of Hapepunine in Mice by Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry

Lianguo Chen¹, Qingwei Zhang², Yijing Lin², Xiaojie Lu², Zuoquan Zhong², Jianshe Ma³,
Congcong Wen^{3*} and Cheng Ding^{4*}

¹Department of Pharmacy, Wenzhou People's Hospital, Wenzhou 325000, China

²Shanghai Institute of Pharmaceutical Industry, China State Institute of Pharmaceutical Industry, Shanghai 201203, China

³Laboratory Animal Center, Wenzhou Medical University, Wenzhou 325035, China

⁴The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, China

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An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was established to determine the hapepunine in mouse blood, and the pharmacokinetics of hapepunine after intravenous (1.0 mg/kg) and intragastric (2.5, 5, and 10 mg/kg) administrations was studied. Delavinone was used as an internal standard. The UPLC ethylene bridged hybrid (BEH) C18 column was used for chromatographic separation. The mobile phase consisted of acetonitrile and 0.1% formic acid with a gradient elution flow rate of 0.4 mL/min. Multiple reaction monitoring (MRM) mode was used for quantitative analysis of hapepunine in electrospray ionization (ESI) positive interface. Proteins from mouse blood were removed by acetonitrile precipitation. The verification method was established in accordance with the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. In the concentration range of 1–1000 ng/mL, the hapepunine in the mouse blood was linear ($r^2 > 0.995$), and the lower limit of quantification was 1.0 ng/mL. In the mouse blood, the intra-day precision coefficient of variation (CV) was less than 12%, the inter-day precision CV was less than 14%. The accuracy ranged from 91.7% to 109.3%. The average recovery was higher than 76.7%, and the matrix effect was between 86.0% and 106.4%. The UPLC–MS/MS method was sensitive, rapid, and selective and was successfully applied to the pharmacokinetic study of hapepunine in mice. The absolute bioavailability of hapepunine was 22.0%.

Keywords: hapepunine, pharmacokinetics, bioavailability, mouse, UPLC–MS/MS

Introduction

Fritillaria species was one of the important drug sources often used for depressing coughs [1, 2]. Some steroidal alkaloids of the ceveratrum and jerveratrum alkaloid groups were isolated [3]. The bulbs of *F. maximowiczii* (Rinyou-Baimo) grown in northeast China were expected to be a substitute for the bulbs of other *Fritillaria* species, *F. thunbergii* (Setu-Baimo), *cirrrosa*, *unibracteata*, and *taipaiensis* (Sen-Baimo), since pharmacological tests provided evidence that they also could be used to treat coughs [4, 5]. Therefore, the chemical constituents of these species were investigated, and hapepunine was one of the main active components in *maximowiczii*. To better understand the pharmacology of hapepunine, it was necessary to investigate its pharmacokinetics. Pharmacokinetic study was a discipline that quantitatively investigates the absorption, distribution, metabolism, and excretion of drugs in vivo [6, 7]. Therefore, it was necessary to establish an analytical method to monitor the concentration of hapepunine.

To the best of our knowledge, the pharmacokinetics of hapepunine had not been reported. In this paper, an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was established to determine hapepunine in mouse blood, the pharmacokinetics of hapepunine

after intravenous and intragastric administration was studied, and the absolute bioavailability was obtained.

Experimental

Chemicals and Animals. Hapepunine (purity >98%, Figure 1A) and delavinone (internal standard, purity >98%, Figure 1B) were purchased from Chengdu Mansite Pharmaceutical Co., Ltd. Chromatographically pure acetonitrile and methanol were purchased from Merck Co., Ltd. (Darmstadt, Germany). Ultrapure water was prepared using a Millipore Milli-Q purification system (Bedford, MA, USA). Institute of Cancer Research (ICR) mice (body weight 20–22 g) were purchased from the Animal Experimental Center of Wenzhou Medical University.

Instrument and Conditions. ACQUITY I-Class UPLC and XEVO TQS-micro triple quadrupole mass spectrometers (Waters Corp., Milford, MA, USA) were used for the determination of hapepunine in mouse blood. Masslynx 4.1 software (Waters Corp.) was used for data acquisition and instrument control.

The column was UPLC ethylene bridged hybrid (BEH) C18 (2.1 mm × 50 mm, 1.7 μm), and the column temperature was set to 30 °C. The mobile phase consisted of acetonitrile and 0.1% formic acid with a gradient elution at a flow rate of 0.4 mL/min and an elution time of 4 min. The gradient elution conditions were as follows: 0–0.2 min, acetonitrile 10%; 0.2–1.5 min, acetonitrile 10%–85%; 1.5–2.0 min, acetonitrile

*Author for correspondences: bluce494949@163.com (C.W.), charles0022@sina.com (C.D.).

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Figure 1. Chemical structure of hapepunine (A) and delavinone (internal standard, B)

85%; 2.0–2.5 min, acetonitrile 85%–10%; and 2.5–4.0 min, acetonitrile 10%.

Nitrogen was used as the desolvation gas (800 L/h) and the cone gas (50 L/h). The capillary voltage was set to 2.0 kV, the ion source temperature was 150 °C, and the desolvation temperature was 400 °C. The multiple reaction monitoring (MRM) model was quantitatively analyzed for hapepunine m/z 430.5 $\rightarrow$ 111.9 and internal standard m/z 414.4 $\rightarrow$ 98.1 in electrospray ionization (ESI) positive interface (Figure 2).

Reference Solution Preparation. Stock solutions of hapepunine (1.0 mg/mL) and delavinone (1.0 mg/mL) were prepared with methanol–water (50:50), respectively. A series of concentration working solutions (10, 30, 50, 200, 500, 1800, 2000, 5000, 9000, and 10,000 ng/mL) of hapepunine was prepared by diluting a hapepunine stock solution with methanol. A solution of the internal standard delavinone 50 ng/mL in acetonitrile was prepared by diluting stock solution of delavinone with acetonitrile. All solutions were stored at 4 °C.

Standard Curve Preparation. The blank mouse blood was mixed with an appropriate amount of standard working

Figure 2. Mass spectrum of hapepunine (A) and delavinone (internal standard, B)

solution to prepare the blood standard curve of hapepunine, and the concentration of hapepunine in the blood of mice was 1, 5, 20, 50, 200, 500, and 1000 ng/mL. The standard curve ranges from 1 to 1000 ng/mL. Quality-control (QC) samples were prepared in the same manner as the standard curve, with 4 blood concentrations (1, 3, 180, and 900 ng/mL).

Sample Processing. The 20 μ L blood sample was added to a 1.5-mL Eppendorf tube, and then 100 μ L acetonitrile (containing 50 ng/mL delavinone) was added, vortexed for 1.0 min, and centrifuged at 4 °C for 1 min at 13,000 rpm. The 80 μ L of the supernatant was taken into the inner tube of the sample bottle, and 2 μ L was injected into UPLC–MS/MS for analysis.

Method Validation. The verification method was established in accordance with the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. Validation projects included selectivity, matrix effects, linearity, precision, accuracy, recovery, and stability [8–12].

Pharmacokinetic Study. About 8.0 mg hapepunine was dissolved in purified water containing 0.01% HCl and prepared into a 1.0 mg/mL drug solution, freshly prepared before the experiment. Twenty-four mice were randomly divided into 4 groups, one group was for intravenous administration (1.0 mg/kg), and other three groups were for intragastric administration (2.5, 5.0, and 10.0 mg/kg), with 6 rats in each group. Then, 20 μ L blood was obtained in a 1.5 mL Eppendorf tube from tail vein, 5 min, 0.5, 1, 1.5, 2, 3, 4, and 8 h after intravenous or intragastric administration in mice, and was frozen at –20 °C.

DAS 2.0 software (China Pharmaceutical University) was used to analyze pharmacokinetic parameters. The formula for bioavailability was absolute bioavailability = intragastric administration AUC/intravenous AUC $\times$ 100%.

Results and Discussion

Method Optimization. ESI positive and negative selections were often evaluated in methodological studies [13–16].

Figure 3. UPLC-MS/MS of hapepunine and delavinone (internal standard) in mouse blood. (A) Blank blood, (B) blank blood spiked hapepunine (5 ng/mL) and internal standard (50 ng/mL), and (C) a mouse blood sample

Hapepunine was an alkaline compound, more suitable for ESI positive detection. Our experiments verified that ESI positive ion mode was more sensitive than the negative one. We optimized the ionization conditions of hapepunine; the highest abundance of fragment ion was m/z 111.9, and the highest abundance of internal standard fragment was m/z 98.1. Therefore, the quantitative analysis was carried out by

selecting hapepunine m/z 430.5 $\rightarrow$ 111.9 (cone hole voltage of 30 V and collision voltage of 22 V) and internal standard m/z 414.4 $\rightarrow$ 98.1 (cone hole voltage 32 V and collision voltage 25 V).

Liquid chromatography conditions separate the endogenous interfering substances as much as possible from the analyte with internal standard retention time [17–20]. We tried

Table 1. Accuracy, precision, matrix effect, and recovery of hapepunine in the mouse blood

Concentration (ng/mL)	Accuracy (%)		Precision (CV%)		Matrix Effect (%)	Recovery (%)
	Intra- day	Inter- day	Intra- day	Inter- day		
1	94.7	91.7	11.2	13.7	86.0 ± 4.9	85.5 ± 5.2
3	104.8	109.3	7.7	7.2	87.1 ± 6.7	92.0 ± 5.3
180	103.1	93.8	6.8	8.8	101.0 ± 4.6	81.9 ± 2.4
900	97.4	103.5	3.3	4.8	106.4 ± 9.6	76.7 ± 6.9

acetonitrile–0.1% formic acid, acetonitrile–10 mmol/L ammonium acetate (containing 0.1% formic acid), methanol–0.1% formic acid, methanol–10 mmol/L ammonium acetate (containing 0.1% formic acid), and using gradient elution. It was

Figure 4. Time-blood concentration curve of hapepunine in mouse blood after intravenous (1.0 mg/kg) and oral (2.5, 5, and 10 mg/kg) administrations**Table 2.** Main pharmacokinetic parameters of hapepunine in mice

Parameters	Unit	IV (1.0 mg/kg)	IG (2.5 mg/kg)	IG (5 mg/kg)	IG (10 mg/kg)
AUC _(0–t)	ng/mL·h	413.0 ± 53.2	240.3 ± 5.0	518.3 ± 60.8	729.6 ± 44.3
AUC _(0–∞)	ng/mL·h	433.8 ± 54.5	334.8 ± 39.6	926.0 ± 226.7	896.4 ± 100.7
MRT _(0–t)	h	1.85 ± 0.24	3.37 ± 0.04	3.20 ± 0.09	2.93 ± 0.07
MRT _(0–∞)	h	2.26 ± 0.38	6.40 ± 1.19	9.75 ± 2.76	4.74 ± 0.67
<i>t</i> _{1/2z}	h	1.8 ± 0.4	4.2 ± 0.9	7.0 ± 2.0	3.3 ± 0.5
CL _{z/F}	L/h/kg	2.3 ± 0.3	7.5 ± 0.9	5.7 ± 1.4	11.3 ± 1.2
<i>V</i> _{z/F}	L/kg	6.2 ± 1.8	45.2 ± 5.0	55.0 ± 9.7	52.2 ± 4.6
<i>C</i> _{max}	ng/mL	250.8 ± 53.0	48.1 ± 2.8	165.7 ± 27.4	226.3 ± 30.9
Bioavailability			23.3%	25.1%	17.7%

The area under the plasma concentration–time curve (AUC), the mean residence time (MRT), the half-life (*t*_{1/2}), the plasma clearance (CL), the apparent distribution volume (*V*), and the maximum plasma concentration (*C*_{max}).

found that acetonitrile–0.1% formic acid gave the most satisfactory peak shape and retention time. At the same time, different columns were compared, and finally, the Waters Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm) column was selected. Therefore, the BEH C18 (2.1 mm × 50 mm, 1.7 μm) column with acetonitrile–0.1% formic acid as the mobile phase was used in this work.

Selection of internal standard was very important in the establishment of the method [21, 22]. In this study, delavinone, which had a similar structure to hapepunine, was selected as an internal standard. Results showed that the behavior of chromatographic retention time and the ionization mass spectrometry was similar and met the requirements of internal standard for UPLC–MS/MS analysis.

UPLC–MS/MS was applied to the quantitative detection of hapepunine in mouse blood, which was faster and sensitivity than traditional high-performance liquid chromatography (HPLC). It needed only 4 min to complete the analysis of the blood sample, which saved a lot of time and solvent. In addition, for LLOQ (1.0 ng/mL) was relatively low, which could be used to determine lower blood concentrations at the last sampling time point.

Method Validation. The retention times of hapepunine and internal standard were 1.94 and 1.75 min in Figure 3, respectively. No obvious impurities and endogenous substances interfered with the detection, indicating that the selectivity of the method was good.

The standard curve equation of hapepunine in the mouse blood was $Y = 0.0104C + 0.0123$, $r^2 = 0.9998$, where *Y* represents the ratio of the peak area of the hapepunine and the internal standard, and *C* represents the concentration of hapepunine in mouse blood. The LLOQ in mouse blood was 1.0 ng/mL, with a signal-to-noise ratio of 8. The limit of detection (LOD) was 0.3 ng/mL, with a signal-to-noise ratio of 3.

As shown in Table 1, the intra-day precision coefficient of variation (CV) was less than 12%; the inter-day precision CV was less than 14%. The accuracy ranged from 91.7% to 109.3%, and the average recovery was higher than 76.7%. The matrix effect was between 86.0% and 106.4%. These data met the pharmacokinetic study requirements of hapepunine.

The mouse blood was stored at room temperature for 2 h and –20 °C for 30 days, and subjected to freeze–thaw stability test; the variation of hapepunine was within ±14% and CV was less than 12%, indicating that the stability of hapepunine was acceptable.

Pharmacokinetics Study. In this study, the UPLC–MS/MS method was used to study the pharmacokinetics of hapepunine after intravenous and intragastric administrations. The concentration–time curve of hapepunine was shown in Figure 4. The non-compartmental model was fitted to the main pharmacokinetic parameters, as shown in Table 2. As could be seen from Table 2, the absolute bioavailabilities of the hapepunine (2.5, 5, and 10 mg/kg) were 23.3%, 25.1%, and 17.7%, and the average absolute bioavailability was

22.0%. The investigated pharmacokinetics of hapepunine could be used to better understand its pharmacology.

Conclusion

A sensitive, rapid, and selective UPLC–MS/MS method was developed for the determination of hapepunine in mouse blood, with a linear range of 1–1000 ng/mL and LLOQ of 1.0 ng/mL, and a needed blood volume of 20 µL. The sample was prepared by one-step acetonitrile precipitation. This method was successfully applied to study the pharmacokinetics of hapepunine in mice, and the average absolute bioavailability was calculated to be 22.0%.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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