Pharmacokinetics of Ebeiedinone in Mouse Blood by UPLC–MS/MS

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An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was established to determine ebeiedinone in mouse blood, and the pharmacokinetics of ebeiedinone after intravenous (0.5 mg/kg) and oral (2, 4, and 8 mg/kg) administration was studied. Twenty-four mice were randomly divided into 4 groups, 1 group was for intravenous administration (0.5 mg/kg), and other 3 groups were for oral administration (2, 4, and 8 mg/kg), with 6 rats in each group. Yubeinine was used as an internal standard. Multiple reaction monitoring (MRM) mode was used to quantitatively analyzed ebeiedinone m/z 414.4 $\rightarrow$ 91.1 and the internal standard m/z 430.4 $\rightarrow$ 412.3 in the electrospray ionization (ESI) positive interface. In the concentration range of 1–2000 ng/mL, the ebeiedinone 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 15%, and the inter-day precision CV was less than 15%. The accuracy ranged from 85.4% to 114.6%, and the average recovery was higher than 61.3%. The matrix effect was between 87.0% and 106.5%. These data met the pharmacokinetic study requirements of ebeiedinone. The UPLC–MS/MS method was sensitive, rapid, and selective and was successfully applied to the pharmacokinetic study of ebeiedinone in mice. The absolute bioavailability of ebeiedinone was 30.6%.

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

1. Introduction

Fritillaria thunbergii Miq. is the authentic medicinal material of Zhejiang Province [1]. It is also known as Zhebei, Dabei, Xiangbei, Yuanbao, and Zhubei [2–4]. It is a traditional Chinese herbal medicine. It is listed as one of the “Zheba Flavors” and in the Chinese Pharmacopoeia; it has the functions of clearing away heat, relieving cough, reducing toxins, and removing phlegm, and is commonly prescribed in traditional Chinese medicine [5–7]. Zhejiang *Fritillaria* has a long history of application in traditional Chinese medicine, and it has a wide application and satisfactory results [8–10]. *Fritillaria* has active ingredients such as alkaloids, polysaccharides, and total saponins [11], among which alkaloids include verticine, peimine, verticinone, peiminine, zhebeinine, zhebeirine, eduardine, zhebeinone, peimisine, isovericine, and ebeiedinone [1]. Therefore, it was necessary to establish an analytical method to monitor the concentration of ebeiedinone.

There were several gas chromatography methods developed for determination of ebeiedinone in bulbs of *Fritillaria* [12, 13]; however, there were no ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) reported for the determination of ebeiedinone. To the best of our knowledge, the pharmacokinetics of ebeiedinone had not been reported. In this paper, UPLC–MS/MS method was established to determine ebeiedinone in mouse blood, the pharmacokinetics of ebeiedinone after intravenous (IV) and oral (PO) administration was studied, and the absolute bioavailability was obtained.

2. Experimental

2.1. Chemicals and Animals. Ebeiedinone (purity >98%, Figure 1A) and yubeinine (internal standard [IS], 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 by 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.

2.2. Instrument and Conditions. ACQUITY I-Class UPLC with XEVO TQS-micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) was used for determination of ebeiedinone 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 $\times$ 50 mm, 1.7 μ m), 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 was as follows: 0–0.2 min, acetonitrile 10%; 0.2–1.5 min, acetonitrile 10%–85%; 1.5–2.0 min, acetonitrile 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. Multiple reaction monitoring (MRM) mode was used to quantitatively analyzed ebeiedinone m/z 414.4 $\rightarrow$ 91.1 and the internal standard m/z 430.4 $\rightarrow$ 412.3 in

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Figure 1. Chemical structure of ebeiedinone (A) and yubeinine (internal standard, B)

the electron spray ionization (ESI) positive interface (Figure 2).

2.3. Reference Solution Preparation. A stock solution of ebeiedinone (1.0 mg/mL) and yubeinine (1.0 mg/mL) were prepared with methanol–water (50:50), respectively. A series of working solutions with different concentrations (10, 30, 50, 200, 500, 1800, 2000, 4500, 5000, 10,000, 18,000, and 20,000 ng/mL) of ebeiedinone were prepared by diluting an ebeiedinone stock solution with methanol. A solution of the internal standard yubeinine (50 ng/mL) was prepared by

diluting a stock solution of yubeinine with acetonitrile. All solutions were stored at 4 °C.

2.4. Standard Curve Preparation. The blank mouse blood was mixed with an appropriate amount of standard working solution to prepare the blood standard curve of ebeiedinone, and the concentration of ebeiedinone in the blood of mice was 1, 5, 20, 50, 200, 500, 1000, and 2000 ng/mL. The standard curve ranges from 1 to 2000 ng/mL. Quality Control (QC) samples were prepared in the same manner as the standard curve, with 4 blood concentrations (1, 3, 450, and 1800 ng/mL).

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

2.6. Method Validation. The verification method was established in accordance with the US Food and Drug Administration (FDA) bioanalytical method validation guidelines [14–19]. Validation projects included selectivity, matrix effects, linearity, precision, accuracy, recovery, and stability [20–24].

The selectivity of the method was evaluated by analyzing 6 lots of different sources of blank mouse blood, blank blood spiked with ebeiedinone, and the IS.

Calibration curves were obtained by analyzing spiked calibration samples on 3 separate days. Peak area ratios of ebeiedinone to IS were plotted against ebeiedinone concentrations. Standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration ($1/x$) in the concentration range of 1–2000 ng/mL for mouse blood. The lower limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank mouse blood was extracted and then spiked with the analyte at 1, 3, 450, and 1800 ng/mL ($n = 6$). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect.

Accuracy and precision were evaluated by the determination of QC samples at 4 concentration levels (1, 3, 450, and 1800 ng/mL) in 6 replicates over 3 validation. The precision is expressed in terms of coefficient of variation (CV).

Figure 2. UPLC–MS/MS of ebeiedinone and yubeinine (internal standard) in mouse blood

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

Concentration (ng/mL)	Accuracy (%)		Precision (CV%)		Matrix effect (%)	Recovery (%)
	Intra-day	Inter-day	Intra-day	Inter-day		
1	114.6	85.4	14.1	11.1	87.0	65.6
3	99.4	109.7	12.9	14.5	88.5	61.3
180	107.1	108.2	10.8	9.2	93.6	70.8
1800	96.6	100.8	6.4	10.8	106.5	64.1

The recovery of ebeiedinone was evaluated by comparing the peak area of extracted QC samples with those of the reference QC solutions reconstituted in blank mouse blood extracts ($n = 6$).

The stability of ebeiedinone in mouse blood was evaluated by analyzing 3 replicates of the blood samples (3, 450, and 1800 ng/mL) exposed to different conditions. These results were compared with that of the freshly-prepared blood samples. Short-term stability was determined after the exposure of the spiked samples to room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the UPLC autosampler at room temperature for 24 h. Freeze–thaw stability was evaluated after 3 complete freeze–thaw cycles (-20 to 25 °C) on consecutive days. Long-term stability was assessed after storage of the standard spiked blood samples at -20 °C for 20 days [25, 26].

2.7. Pharmacokinetic Study. About 8.0 mg of ebeiedinone was dissolved in purified water containing 0.01% HCl and prepared into a 1.0 mg/mL drug solution, freshly prepared before the experiment. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University. Twenty-

four mice were randomly divided into 4 groups, 1 group was for intravenous administration (0.5 mg/kg), and the other 3 groups were for oral administration (2, 4, and 8 mg/kg), with 6 rats in each group. Then, 20 μ L of blood was obtained in a 1.5-mL Eppendorf tube from tail vein at 5 min, 0.5, 1, 1.5, 2, 3, 4, and 8 h after intravenous or oral 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 = oral administration AUC/intravenous AUC $\times 100\%$ [27–30].

3. Results and Discussion

3.1. Method Optimization. ESI positive and negative selection was often evaluated in methodological studies [31–36]. Ebeiedinone was a basic compound, more suitable for ESI positive detection. Our experiments verified that the ESI positive ion mode was more sensitive than the negative one.

Liquid chromatography conditions separate the endogenous interfering substances as much as possible from the analyte and the internal standard at a retention time [37–40]. We tried

Figure 3. Time–blood concentration curve of ebeiedinone in mouse blood after intravenous (0.5 mg/kg) and oral (2, 4, and 8 mg/kg) administration

Table 2. Main pharmacokinetic parameters of ebeiedinone in mice

Parameters	Unit	IV (0.5 mg/kg)	PO (2 mg/kg)	PO (4 mg/kg)	PO (8 mg/kg)
AUC _(0-t)	ng/mL h	1440.0 ± 187.4	2178.4 ± 557.2	3606.6 ± 997.0	5259.7 ± 530.9
AUC _(0-∞)	ng/mL h	1448.6 ± 189.9	2378.0 ± 641.2	3946.0 ± 1267.8	5867.9 ± 987.2
MRT _(0-t)	h	2.8 ± 0.2	3.8 ± 0.5	3.3 ± 0.6	3.7 ± 0.4
MRT _(0-∞)	h	2.9 ± 0.2	4.9 ± 1.4	4.6 ± 2.0	4.8 ± 0.9
t _{1/2z}	h	1.4 ± 0.6	3.1 ± 1.5	3.5 ± 2.8	3.0 ± 0.9
t _{max}	h	0.1	1.6 ± 0.5	2.2 ± 0.8	1.3 ± 0.5
CL _{z/F}	L/h/kg	0.4 ± 0.1	0.9 ± 0.2	1.1 ± 0.4	1.4 ± 0.2
V _{z/F}	L/kg	0.7 ± 0.3	3.8 ± 1.7	4.9 ± 2.6	6.0 ± 1.5
C _{max}	ng/mL	394.3 ± 77.8	471.1 ± 175.7	989.9 ± 173.0	1062.1 ± 161.7
Bioavailability			37.8%	31.3%	22.8%

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}).

methanol–water, methanol–0.1% formic acid, methanol–10 mmol/L ammonium acetate (containing 0.1% formic acid), acetonitrile–water, acetonitrile–0.1% formic acid, and acetonitrile–10 mmol/L ammonium acetate (containing 0.1% formic acid) and using gradient elution. It was found that acetonitrile–0.1% formic acid gave the most satisfactory peak shape and retention time.

3.2. Method Validation. The retention times of ebeiedinone and internal standard were 1.82 and 1.66 min in Figure 2, 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 ebeiedinone in the mouse blood was $Y = 0.0019C + 0.0077$, $r^2 = 0.9984$, where Y is the ratio of the peak area of the ebeiedinone and the internal standard, and C is the concentration of ebeiedinone in mouse blood. The LLOQ in the 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 CV was less than 15%, and the inter-day precision CV was less than 15%. The accuracy ranged from 85.4% to 114.6%, and the average recovery was higher than 61.3%. The matrix effect was between 87.0% and 106.5%. These data met the pharmacokinetic study requirements of ebeiedinone.

For the mouse blood at room temperature for 2 h, –20 °C for 30 days, and freeze–thaw stability test, the variation of ebeiedinone was within ±12%, and the CV was less than 11%, indicating that the stability of ebeiedinone was acceptable.

3.3. Pharmacokinetics Study. In this study, the UPLC–MS/MS method was used to study the pharmacokinetics of ebeiedinone after intravenous and oral administration. UPLC–MS/MS was applied to the quantitative detection of ebeiedinone in mouse blood, which was faster and sensitive than traditional HPLC. It needed only 4 min to complete the analysis of the plasma sample, saving a lot of time and solvent.

The concentration–time curve of ebeiedinone was shown in Figure 3. 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 bioavailability of the ebeiedinone (2, 4, and 8 mg/kg) was 37.8%, 31.3%, and 22.8%, and the average absolute bioavailability was 30.6%. The investigated pharmacokinetics of ebeiedinone could be used to better understand its pharmacology.

4. Conclusion

A sensitive, rapid, and selective UPLC–MS/MS method was developed for the determination of ebeiedinone in the mouse blood, with a linear range of 1–2000 ng/mL and LLOQ of 1.0 ng/mL, and it needed a blood volume of 20 µL and

only 4 min for one sample run. To the best of our knowledge, the pharmacokinetics of ebeiedinone had not been reported. This method was successfully applied to the pharmacokinetics of ebeiedinone in mice after intravenous (0.5 mg/kg) and oral (2, 4 and 8 mg/kg) administration, and the average absolute bioavailability was calculated to be 30.6%.

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