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Short Communication

Pharmacokinetics and enterohepatic circulation of jervine, an antitumor steroidal alkaloid from *Veratrum nigrum* in rats

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ABSTRACT

Jervine, a novel steroidal alkaloid from *Veratrum nigrum* L., exhibits both antitumor effect and potential toxicity. The aim of study was to characterize the pharmacokinetic behaviors and enterohepatic circulation of jervine in rats. A rapid and simple ultra-high performance liquid chromatography-tandem mass spectrometric method was developed and validated for quantification of jervine and alpinetin (internal standard) in rat plasma. After extraction from rat plasma by a simple protein-precipitation method, the analyte was separated on a C_{18} column (2.1 mm × 50 mm, 1.7 μm) using water with 0.1% formic acid and acetonitrile as the mobile phase delivered at a flow rate of 0.4 mL/min. Jervine and alpinetin were determined in the positive mode with multiple reaction monitoring (MRM) of the ion transitions at *m/z* 426.3 → 108.8 and *m/z* 271.0 → 166.9, respectively. Molecular docking method was used to investigate the binding of jervine to p-glycoprotein and dehydroepiandrosterone sulfotransferase. The method was well validated within acceptance limits including specificity, matrix effect, recovery, precision, accuracy, and stability, and was successfully applied to the pharmacokinetic study of jervine after oral and intravenous administration to rats. Jervine presented a small volume of distribution, fast absorption, high oral bioavailability, and enterohepatic circulation. The enterohepatic circulation was first observed in veratrum alkaloids, and was further investigated by molecular docking studies, which was related to the binding of jervine to p-glycoprotein and dehydroepiandrosterone sulfotransferase. The pharmacokinetic properties and enterohepatic circulation of jervine in rats provided a significant basis for the drug-drug interaction and toxicity study in the future.

1. Introduction

Jervine is a steroidal alkaloid originated from the dried rhizome of *Veratrum nigrum* L. (VN). Traditionally, this herbal medicine has been used for the treatment of hypertension, epilepsy, and lymphangitis [1]. The veratramine-type steroidal alkaloids are the major bioactive and neurotoxic components in VN [2], and are primarily metabolized through hydroxylation, methylation and sulfation [3,4]. As a typical veratrum alkaloid, jervine demonstrates a variety of pharmacological effects such as anti-inflammatory, anti-tumor, and anti-platelet, and anti-adipogenic ones [5,6]. The anti-adipogenesis property of it is properly achieved by activating the LKB1-AMPKα-ACC axis [7]. In addition, jervine is reported to be poisonous [8] and cross-react with digoxin [9]. Therefore, the characterization of jervine pharmacokinetic profile is essential for the evaluation of its efficacy, safety, and potential interactions with other drugs. Due to the narrow therapeutic index, a variety of veratrum alkaloids have been investigated for their pharmacokinetic characteristics [10]. However, up to now, few studies have evaluated the pharmacokinetic profile of monomer of jervine.

There are just a few reports that separated and determined jervine from veratrum plants by the high performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) [11] or ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method [12]. Chen et al. [11] developed a sensitive hydrophilic interaction liquid chromatography (HILIC) method to determine pseudojervine, veratrosine, jervine, veratramine, veramarine and veratroylzygadenine in rat plasma with LLOQ at 1 μg/L for jervine. Carlier et al. [12] developed an original UPLC-MS/MS method to determine thirty-nine toxic principles of plant origin in the blood

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using a standard solid phase extraction [12]. However, their work did not obtain the pharmacokinetic characteristics of monomer of jervine. In this study, a simple and rapid UPLC-MS/MS method was established for the quantitation of jervine in rat plasma and fully validated. The method required a chromatographic run time of 4 min, and achieved a lower limit of quantitation (LLOQ) of 0.25 ng/mL. The method was then successfully applied to the pharmacokinetic study of jervine after oral as well as intravenous administration in rats. Furthermore, the enterohepatic circulation was first investigated in veratrum alkaloids by molecular docking studies.

2. Material and methods

2.1. Materials

Jervine (MUST-16080504, purity > 98%) and alpinetin (MUST-17053004, IS, purity > 98%) were purchased from Chengdu Munster Biotechnology Co., Ltd (Chengdu, China). Chromatographic grade methanol, formic acid, and acetonitrile were purchased from Merck (Merck, Darmstadt, Germany). Ultra-pure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade.

2.2. Animals

Twelve male Sprague-Dawley (SD) rats (230 ± 20 g) were purchased from the Experimental Animal Center of Wenzhou Medical University. The animals were cared under a controlled environment at 25 ± 2 °C, a relative humidity of 50 ± 10% and a 12 h day/light cycle for one week, and fed standard laboratory food and water *ad libitum*. Prior to the experiment, the rats were fasted overnight but had unrestricted access to water. All experimental procedures complied with the "Principles of Laboratory Animal Care" and were approved by the Animal Ethics Committee of Wenzhou Medical University (20 July 2017, no: 2017-257). The animal experiments were carried out according to the European Community guidelines for the use of experimental animals.

2.3. Analytical conditions

Chromatography was performed on a Waters Acquity-UPLC (Waters Corp., Milford, MA, USA). Separation was achieved on an ACQUITY UPLC C_{18} (2.1 mm × 50 mm, 1.7 μm) with the column temperature of 40 °C. The mobile phase consisted of A (water with 0.1% formic acid, v/v) and B (acetonitrile), and delivered at a flow rate of 0.4 mL/min. The gradient elution was performed as follows: the gradient was maintained at 10% B from 0 to 0.2 min, increased to reach 80% at 1.5 min, maintained at 80% B until 2 min, rapidly returned to 10% B at 2.2 min, and then maintained until 4 min. A total of 2 μL of the sample was injected into the UPLC system for analysis.

Mass spectrometric (MS) quantitation was conducted on a Waters Micromass Quattromicro triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA) in the positive mode. The MS parameters were optimized as follows: desolvation gas, nitrogen, 1000 L/h; cone gas, nitrogen, 50 L/h; capillary voltage, 1.93 kV; cone voltage, 33 V; source temperature, 148 °C; desolvation temperature, 597 °C. The quantitation of jervine and IS was achieved by the multiple reaction monitoring (MRM) mode. The MRM transitions for jervine were *m/z* 426.3 → 108.8, and for IS were *m/z* 271.0 → 166.9. The collision-induced dissociation energies of jervine and IS were optimized to 32 and 42 eV, respectively. All data were analyzed using MassLynx software (version 4.1, Waters Corp., Milford, MA, USA).

2.4. Preparation of stock solutions, calibration standards and quality control (QC) samples

The stock solutions of jervine and IS were prepared at 200 μg/mL in methanol and stored at 4 °C. A series of jervine working solutions with different concentrations were obtained by diluting the stock solutions with methanol. The calibration standards and QC samples were prepared by adding 10 μL of jervine working solution into 100 μL of blank rat plasma. The concentrations of the calibration standards for jervine ranged from 0.25 to 1000 ng/mL. The QC samples contained jervine at final concentrations of 0.25 ng/mL (LLOQ), 0.5 ng/mL, 50 ng/mL, and 800 ng/mL, respectively. All samples were stored at 4 °C until analysis.

2.5. Sample preparation

A total of 100 μL plasma was first placed in the centrifuge tube, and spiked with 10 μL of IS working solution (2 μg/mL). Then, the mixture was immediately vortexed for 30 s and extracted with 290 μL of acetonitrile by vortex for 2 min. Next, the extracted samples were centrifuged at 14900 *g* for 15 min. Finally, 2 μL of supernatant was injected for the UPLC–MS/MS analysis.

2.6. Method validation

The specificity was evaluated through comparing the chromatograms of blank rat plasma, blank plasma spiked with jervine and IS, and plasma samples after oral and intravenous administration to observe the interference from endogenous substances at the retention time of jervine and IS. The linearity was evaluated by thirteen jervine concentrations (0.25–1000 ng/mL). The calibration curves were constructed by plotting the peak area ratio of jervine to the corresponding IS versus the concentrations of jervine in rat plasma. The linear regression was calculated by the weighted linear least-squares regression (1/x). The lower limit of quantitation (LLOQ) and limit of detection (LOD) were defined as the signal/noise ratio 10 and 3, respectively.

The matrix effect was investigated by comparing the peak area ratios of jervine to IS of post-extraction blank plasma samples (five rats) with those prepared in acetonitrile-water (3:1, v/v) at three concentration levels (0.5, 50, and 800 ng/mL). The extraction recovery was determined by comparing the peak area ratios of jervine to IS spiked before extraction with those spiked after extraction at three concentration levels (0.5, 50, and 800 ng/mL). The intra- and inter-day precision and accuracy were validated by analyzing five individual QC samples (0.25, 0.5, 50, and 800 ng/mL) on one day and over the three consecutive days, respectively. The acceptable values of precision should be within 15% (20% for LLOQ) of the relative standard deviation (RSD), while the allowed accuracy ranged between −15% (−20% for LLOQ) and 15% (20% for LLOQ) recovery (RE). The jervine stability in rat plasma and in the reconstituted solution were assessed by analyzing the QC samples in triplicate at three concentration levels under the following conditions short-term stability, kept at room temperature for 8 h; freeze-thaw stability, analyzed after three freeze–thaw cycles; long-term stability, stored at −20 °C for 15 days; short-laying stability, kept in the autosampler at 4 °C for 24 h.

2.7. Pharmacokinetic study

To perform the pharmacokinetic and bioavailability study, the rats were split into two groups for oral and intravenous administration. In the oral administration group, six rats were orally administered with jervine suspension (prepared in 1% CMC Na) at a dose of 40 mg/kg, and then blood samples of approximately

0.25 mL were collected into heparinized tubes at 10 min, 20 min, 40 min, 2, 8, 12, 18, 24, 30, 48, and 60 h. Six rats were anaesthetized with isoflurane prior to sublingual intravenous administration with jervine prepared in citric acid + water (5 mg/kg), and the blood samples were collected at 5 min, 15 min, 30 min, 1, 2, 4, 8, 12, and 24 h after administration. All blood samples were collected from tail vein and all rats were sacrificed by cervical dislocation after last collection. The collected blood samples were centrifuged at 1721 *g* for 15 min. The supernatant fraction was separated and stored at −20 °C prior to analysis. Pharmacokinetic parameters were estimated by a non-compartmental analysis using the Drug and Statistics software (version 3.0).

2.8. Molecular docking

The molecular structure of jervine was optimized based on MMFF94 force field, and the stop condition was set to the root mean square of potential energy smaller than 0.001 kcal $Å^{-1}$ mol^{-1}. The X-ray crystal structures of p-glycoprotein (P-gp) and dehydroepiandrosterone sulfotransferase (SULT2A1) were downloaded from Protein Data Bank. Hydrogen was added to the model, and its orientation was optimized using the CHARMm force field energy minimization. The ligand position in P-gp and SULT2A1 was used to define the active site cavity. Docking protocol was performed to show the interaction with P-gp and SULT2A1 using AutodockTools. This work was conducted using freely available software called AutoDock Vina [13]. The docking score between known (positive) drugs and P-gp or SULT2A1 proteins was used as the cutoff value in this protocol. If the docking score of jervine and P-gp or SULT2A1 was less than the corresponding cutoff value of positive drugs, and also less than −5.0 kcaL/mol, they were considered to be effective docking [14].

3. Results and discussion

3.1. Analysis condition optimization

The optimization of MS parameters was first performed to develop the method. Authentic standards were used to optimize the MS conditions and select the MRM ion pairs. After comparison, the positive-ionization mode provided a better mass spectrometric response for jervine and IS than the negative mode. The major charge state were $[M+H]^+$ for jervine and IS at *m/z* 426.3 and 271.0, respectively (Figs. 1 A and B). For jervine, two daughter ions with the most and second intense abundances were observed in the full-scan daughter ion mass spectra, including *m/z* 108.7 and 113.8. The MRM transition *m/z* 426.3 → 108.8 was finally chosen as the quantifier ion to obtain a relatively stable mass response. For IS, the most intense daughter ion *m/z* 166.9 was used for quantitation (Figs. 1 A and B). The optimized MS parameters are shown in Section 2.3.

UPLC conditions were then optimized for efficient analysis of jervine. The use of acetonitrile plus water with 0.1% formic acid as the mobile phase resulted in shorter retention time and sharper peaks. The total run time was as short as 4 min. The optimal IS should be the isotopic type of analytes, which exhibited similar structures, mass responses, chromatographic behaviors, matrix effects, and extraction efficiency. However, the available standards of isotopic IS were expensive and unavailable for the most of time. In this experiment, various compounds were evaluated for IS, including nomilin, phellodendrine, and alpinetin. For nomilin, relatively high background noise was observed in spiked rat plasma, and could not be eliminated by chromatographic separation or sample preparation (Fig. 1C). For phellodendrine, the mass response RSD of 20 spiked rat plasma samples was 87.5%, which was not suitable for quantitation (Fig. 1 D). Therefore, alpinetin was selected as IS with similar extraction, chromatographic and mass spectrometric behavior (Figs. 1 E, F and G and Section 2.6 Method validation). Protein precipitation method with acetonitrile was used for preparing plasma samples, which was simpler and more convenient than the extraction method with n-butanol-acetonitrile-ammonium acetate [11] or a standard solid phase extraction [12]. Our pretreatment method also was more economic, which made the high throughput possible. In addition, the retention time of jervine was 1.76 min, shorter than 4.3 min in Ref. [11] or 5.9 min in Ref. [12].

3.2. Method validation

The selectivity of jervine and IS was determined using representative chromatograms of blank plasma, blank plasma spiked with jervine and IS, and rat plasma samples. As shown in Figs. 1 E, F, and G, the established method was highly selective, and free of interferences at the corresponding retention times and peak regions of jervine and IS. The calibration standards for jervine exhibited good linearity with linear correlation coefficient (r) better than 0.99 in the range of 0.25–1000 ng/mL. The LOD and LLOQ for jervine were 0.1 and 0.25 ng/mL in rat plasma, respectively. The matrix effect ranged from 97.3% to 104.5% at 0.5, 50, 800 ng/mL for jervine, indicating that co-eluting endogenous interference from matrices had little influence on the ionization of jervine. The extraction recovery of jervine was in the range of 93.8%–98.6% at three concentration levels, which demonstrated that acetonitrile could extract jervine efficiently and consistently (Table 1). The intra- and inter-day accuracy (RE, %) ranged from −5.0% to −16.0% and −4.7% to −18.3%, respectively. The intra- and inter-day precision (RSD, %) for jervine was less than 8.6%. The precision and accuracy results were within acceptable limits, revealing the established method was precise and accurate for the quantitation of jervine (Table 2). As shown in Table 3, the stability test under different storage conditions suggested that jervine in rat plasma was stable at room temperature for 8 h, at 4 °C for 24 h, and at −20 °C for 15 days, and three freeze–thaw cycles.

3.3. Pharmacokinetic study

The UPLC-MS/MS analytical method was applied to evaluate the pharmacokinetic characteristics of jervine in rats. After intravenous (5 mg/kg) and oral (40 mg/kg) administration of jervine, the mean plasma concentration-time profiles of jervine are shown in Fig. 2. The major pharmacokinetic parameters of jervine are shown in Table 4. For the *i.v.* and *p.o.* administration, the mean volume of distribution for jervine was 44.15 ± 18.11 L/kg and 115.24 ± 19.46 L/kg, respectively, indicating that jervine was mainly distributed in the plasma. In addition, jervine exhibited two peaks at 2 and 24 h, and reached the peak concentrations at 233.30 ± 30.37 and 138.40 ± 19.31 ng/mL, respectively. The oral absolute bioavailability was then calculated with the following formula $F = (AUC_{p.o.} \times Dose_{i.v.})/(AUC_{i.v.} \times Dose_{p.o.}) \times 100\%$. The absolute oral bioavailability was 60.0%, indicating that jervine was absorbed efficiently and experienced little degradation or metabolism by oral administration.

Similar to other veratrum alkaloids [3,10,15], jervine reached the first peak rapidly (<2 h), indicating that jervine was absorbed rapidly from the gastrointestinal tract. The fast absorption may be related to the proper lipophilicity of steroidal alkaloids. With regard to the phenomenon of the second peak, some studies have shown that it is associated with enterohepatic circulation [16], and suggested that it is caused by the different absorption mechanisms of drugs in the gastrointestinal tract [17]. Notably, the phenomenon of

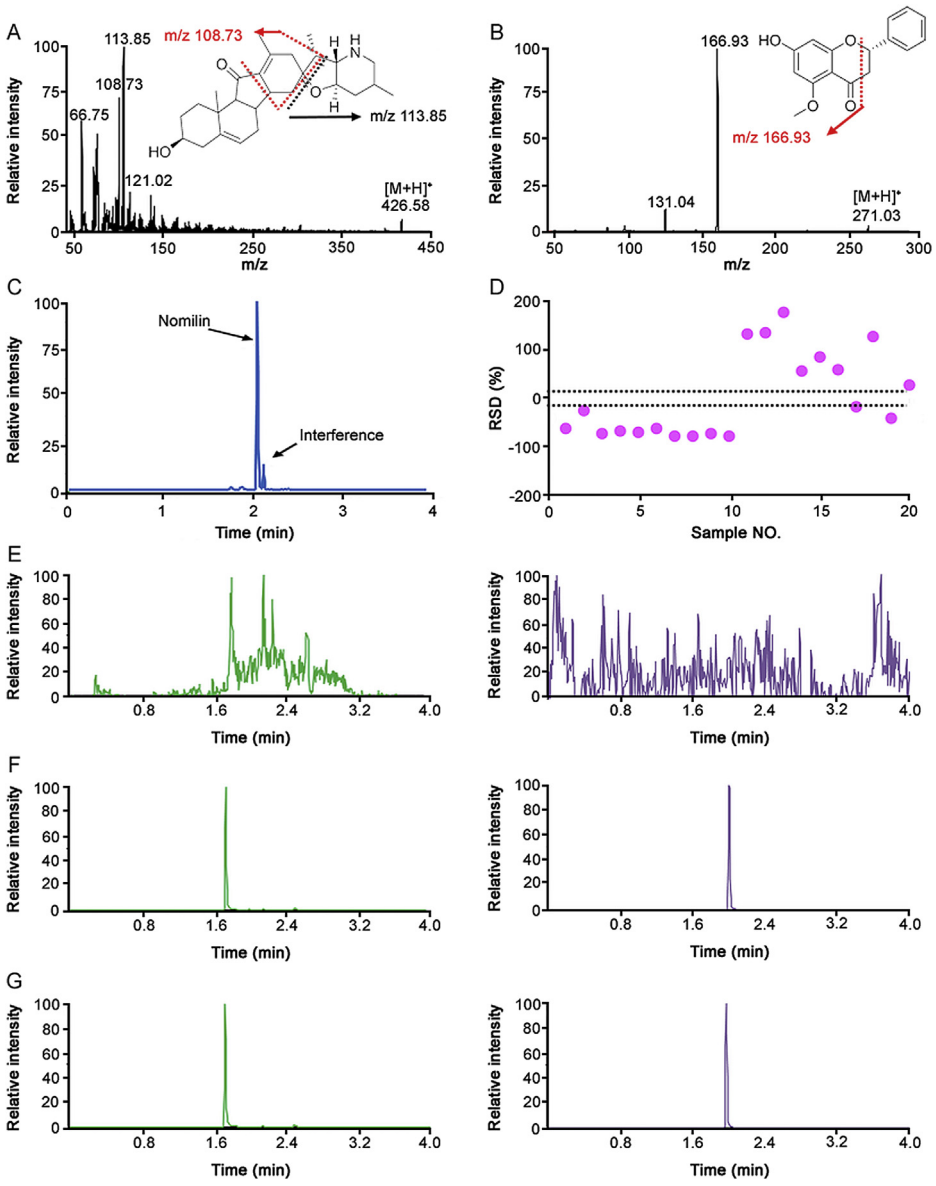

Fig. 1. Chemical structures and MS/MS spectra of (A) jervine and (B) alpinetin. (C) Chromatographic interference of nomilin. (D) RSD of mass response of jervine in 20 plasma samples. Representative MRM chromatograms of (E) blank plasma, (F) blank plasma spiked with jervine (50 ng/mL) and IS and (G) plasma obtained 60 min after sublingual intravenous administration of jervine.

enterohepatic circulation was first observed in jervine, but not other veratrum alkaloids like veratramine [3,10] or cyclopamine [15]. The specific pharmacokinetic behavior of jervine may be related to its particular structure. The appearance of enterohepatic circulation, equivalent to a "autologous administration" process, is bound to improve the oral bioavailability, increase the probability of drug-drug interaction (DDI), and even exhibit potential toxicity. In our study, the oral absolute bioavailability of jervine was 60.0%, which was much higher than that of veratramine (22.5%) [10] or cyclopamine (33%) [15]. It might be due to the enterohepatic circulation. As reported, jervine-type alkaloids were optimal for the inhibition of acetylcholinesterase [18], which was also targeted by drugs for Alzheimer's and Parkinson's diseases [19]. Therefore, the probability of DDI would increase when jervine was used in combination with anti-Alzheimer's or anti-Parkinson's drugs, such as donepezil, rivastigmine, and galanthamine. The veratrum alkaloids like jervine and cyclopamine, have been considered as potent teratogens which inhibit the sonic hedgehog signaling. The approximate median lethal dose concentration for jervine is 120 mg/kg, and its toxicity varies in different species [20]. Additionally, cyclopamine exhibited a dose-dependent teratogenicity, toxicity, and pharmacokinetic behavior [10]. These results suggest that the toxicity of jervine would also increase with the increase of *in vivo* exposure due to the enterohepatic circulation.

3.4. Molecular docking

To explain the mechanism shown by jervine, the enterohepatic circulation was docked by transport of jervine to the absorption site. As reported, P-gp is a related enzyme that regulates the transportation of drugs from plasma, bile, into the intestine, and the reabsorption into the portal blood [21], while SULT2A1 takes part in the sulfation of endogenous compounds like bile acids [22]. Thus, P-gp and SULT2A1 were selected as target enzymes for the

Table 1
Matrix effect and extraction recovery for the assay of jervine and IS in rat plasma (n = 5).

Spiked plasma concentration (ng/mL)	Extraction recovery (%, mean ± SD)	Matrix effect (%, mean ± SD)
0.5	98.6 ± 2.8	104.5 ± 1.2
50	97.5 ± 7.9	101.1 ± 0.8
800	93.8 ± 0.6	97.3 ± 3.4

Table 2
Intra- and inter-day accuracy and precision for the determination of jervine in rat plasma ($n = 5$).

Spiked concentration (ng/mL)	Intra-day			Inter-day		
	Concentration (ng/mL, mean ± SD)	Precision (%)	Accuracy (%)	Concentration (ng/mL, mean ± SD)	Precision (%)	Accuracy (%)
0.25	0.19 ± 0.01	8.6	84.0	0.20 ± 0.00	3.2	81.7
0.5	0.44 ± 0.03	7.7	88.2	0.46 ± 0.01	3.8	92.2
50	57.18 ± 1.97	3.4	114.4	56.80 ± 0.98	1.7	113.6
800	839.90 ± 16.69	1.9	105.0	834.71 ± 35.63	4.2	104.3

Table 3
The stability for the determination of jervine in rat plasma ($n = 3$).

Storage conditions	Concentration (ng/mL)	Measured (ng/mL, mean ± SD)	Accuracy (%)	RSD (%)
Room temperature for 8 h	0.5	0.48 ± 0.01	96.0	4.9
	50	50.34 ± 2.30	101	4.5
	800	748.27 ± 10.86	93.5	1.4
Three freeze/thaw cycles	0.5	0.46 ± 0.02	92.0	6.1
	50	55.57 ± 3.24	111	5.8
	800	797.56 ± 14.20	99.7	1.7
Keeping at 4 °C for 24 h	0.5	0.45 ± 0.03	90.0	14.7
	50	55.31 ± 1.34	111	2.4
	800	740.02 ± 13.03	92.5	1.7
Long-term stability (at −20 °C for 15 days)	0.5	0.47 ± 0.03	94.0	8.3
	50	57.54 ± 3.61	115	6.2
	800	796.76 ± 8.67	99.6	1.0

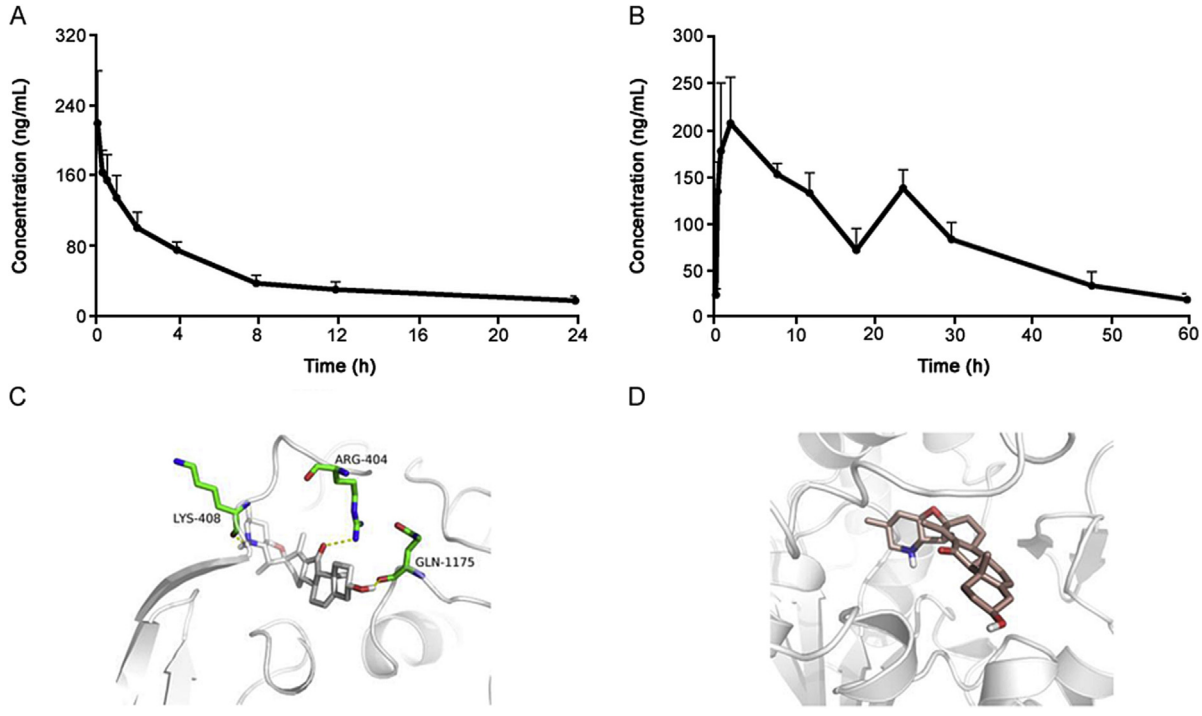

Fig. 2. Mean plasma concentration-time of jervine after sublingual intravenous (A) and oral administration (B) of 5 and 40 mg/kg, respectively (n = 6); 3D docking interactions of jervine to (C) p-glycoprotein (P-gp) and (D) Dehydroepiandrosterone sulfotransferase (SULT2A1).

Table 4
The non-compartmental pharmacokinetic parameters of jervine after oral (*p.o.*) and sublingual intravenous (*i.v.*) administration of 40 and 5 mg/kg, respectively ($n = 6$).

Parameter	*i.v.* (5mg/kg)	*p.o.* (40 mg/kg)
$t_{1/2}$ (h)	8.35 ± 5.15	11.09 ± 2.35
V (L/kg)	44.15 ± 18.11	115.24 ± 19.46
CL (L/h/kg)	4.03 ± 0.80	7.26 ± 0.68
$AUC_{(0-t)}$ (μg/L · h)	1097.27 ± 99.88	5269.16 ± 389.11
C_{max} (ng/L)	138.40 ± 19.31	233.30 ± 30.37
T_{max} (h)	0.13 ± 0.08	1.20 ± 0.73
$AUC_{(0-\infty)}$ (μg/L · h)	1289.67 ± 318.46	5547.95 ± 558.34
F (%)	60.02	

molecular docking. The binding free energy of jervine to P-gp and SULT2A1 were calculated as −8.17 and −10.15 kcaL/mol, respectively. The docking results suggested that potential interactions existed between jevine and target enzymes since the docking results were below −5 kcaL/mol. The 3D interaction between jervine and corresponding active sites are shown in Figs. 2C and D. The molecular docking results, however, need to be validated by further experiments. Therefore, jervine is supposed to interact with P-gp and SULT2A1, and shows the enterohepatic circulation. Collectively, the pharmacokinetic properties of jervine are similar to those of other veratrum alkaloids including a small volume of distribution and fast absorption [3,10,15], while it is the first time that the enterohepatic circulation was speculated in jervine and the mechanism of production needs to be studied further.

4. Conclusion

In this work, a simple and rapid UPLC-MS/MS method was developed for the determination of jervine in rat plasma, and it is the first time that its enterohepatic circulation was speculated and investigated by molecular docking method. Acetonitrile was used to precipitate proteins in rat plasma and LLOQ was 0.25 ng/mL. The retention time of jervine was 1.76 min. The results of molecular docking suggested that jervine was found to interact with P-gp and SULT2A1, and showed the enterohepatic circulation. Our results provide a basis for further guidance on the clinical application of jervine.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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