Pharmacokinetics of 8-*O*-Acetylharpagide in Mouse Blood by UPLC–MS/MS

Shanjiang Chen[1], Miaoling Huang[2], Zheng Yu[3], Jiamin He[2], Binge Huang[3], Xianqin Wang[2], Jianshe Ma[2] and Congcong Wen[3*]

[1]*Department of Cardiology, Wenzhou Central Hospital, Wenzhou 325000, China*
[2]*Analytical and Testing Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou 325035, China*
[3]*Laboratory Animal Centre, Wenzhou Medical University, Wenzhou 325035, China*

8-*O*-Acetylharpagide is the main active component of the herb *Ajuga decumbens*, which possesses anti-tumor, anti-virus, and anti-inflammation properties. In this study, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was used to measure the concentration of 8-*O*-acetylharpagide in mouse blood, with subsequent investigation of the pharmacokinetics of the drug after intravenous or oral administration. Shanzhiside methyl ester was used as an internal standard, and the acetonitrile precipitation method was used to process the blood samples. Chromatographic separation was achieved using an ultra-performance liquid chromatography ethylene-bridged hybrid (UPLC BEH) column (2.1 mm × 50 mm, 1.7 μm) with a gradient methanol–water mobile phase (containing 0.1% formic acid). The flow rate was 0.4 mL/min, and the elution time was 5.0 min. 8-*O*-Acetylharpagide was quantitatively measured using electrospray ionization (ESI) tandem mass spectrometry in multiple reaction monitoring (MRM) mode with positive ionization. The result indicated that, within the range of 5–500 ng/mL, the linearity of 8-*O*-acetylharpagide in mouse blood was satisfactory ($r > 0.995$), and the lower limit of quantification (LLOQ) was 5 ng/mL. Intra-day precision relative standard deviation (RSD) of 8-*O*-acetylharpagide in blood was lower than 9%, and the inter-day precision RSD was lower than 13%. The accuracy range was between 94.3% and 107.1%, average recovery was higher than 91.3%, and the matrix effect was between 100.8% and 110.8%. This analytical method was sensitive and fast with good selectivity and was successfully applied to perform pharmacokinetic studies of 8-*O*-acetylharpagide in mice. The bioavailability of 8-*O*-acetylharpagide was 10.8%, and the analysis of the primary pharmacokinetic parameters after oral and intravenous administration indicated that 8-*O*-acetylharpagide had a significant first pass effect after oral administration.

Keywords: 8-*O*-acetylharpagide, pharmacokinetics, bioavailability, mouse, UPLC–MS/MS

Introduction

The herb *Ajuga decumbens* Thunb. (family Lamiaceae) is called bugleweed or carpet bugle and is found in China, Korea, Japan, Taiwan, and the Ryukyu Islands. There are two similar species also found in the same general geographical location, *Ajuga nipponensis* Makino and *Ajuga taiwanensis* Nakai ex Murata [1, 2]. Modern pharmacological research indicates that 8-*O*-acetylharpagide in *A. decumbens* has various pharmacological activities, such as antitumoral, antiviral, and anti-inflammatory [3–5]. A pharmacokinetics study was performed to quantitatively investigate the absorption, distribution, metabolism, and excretion (ADME) of 8-*O*-acetylharpagide in vivo and also focus on the metabolic mechanism, interaction within a complex drug system, and dynamic investigation of the material basis of the drug's effect [6, 7]. Thus, research on the pharmacokinetics of 8-*O*-acetylharpagide is very important for the clinical application of *A. decumbens*.

There have been few literatures reported for determination 8-*O*-acetylharpagide for pharmacokinetics in rats or beagle dogs [8, 9]. Wen et al. [8] established a sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for determination of 8-*O*-acetylharpagide in rat plasma and processed plasma with ethanol. Then, they compared the pharmacokinetics of pure 8-*O*-acetylharpagide and an *A. decumbens* Thunb. extract in rats by oral administration. Wen et al. [9] applied LC–MS/MS to investigate the pharmacokinetics of 8-*O*-acetylharpagide in beagle dogs after oral administration of an extract of *A. decumbens*. Currently, the pharmacokinetics of 8-*O*-acetylharpagide in mice have not been reported.

Compared with LC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) is more sensitive [10], possessing significant advantages in pharmacokinetic investigations of chemical drugs. The superior separation and analysis capacity is ideal for analyzing the in vivo metabolism of complicated traditional Chinese medicine (TCM) components and complex compound systems [11, 12]. In this study, we established an analytical method to detect the concentration of 8-*O*-acetylharpagide in mouse blood via different routes and investigated the pharmacokinetics in vivo, which enabled us to calculate the bioavailability of 8-*O*-acetylharpagide. This study can provide a scientific basis for 8-*O*-acetylharpagide drug formulation and clinical research. Our method utilized a more appropriate mobile phase and internal standard than a previously reported LC–MS/MS method [8, 9]. Furthermore, the UPLC–MS/MS method for quantitative determination of 8-*O*-acetylharpagide was faster and more sensitive than traditional HPLC.

Materials and Methods

Experimental Materials. 8-*O*-Acetylharpagide (purity >98%, Figure 1A) and shanzhiside methyl ester (purity >98%, Figure 1B) were purchased from Chengdu Mansite Pharmaceutical Co., Ltd. HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). HPLC-grade
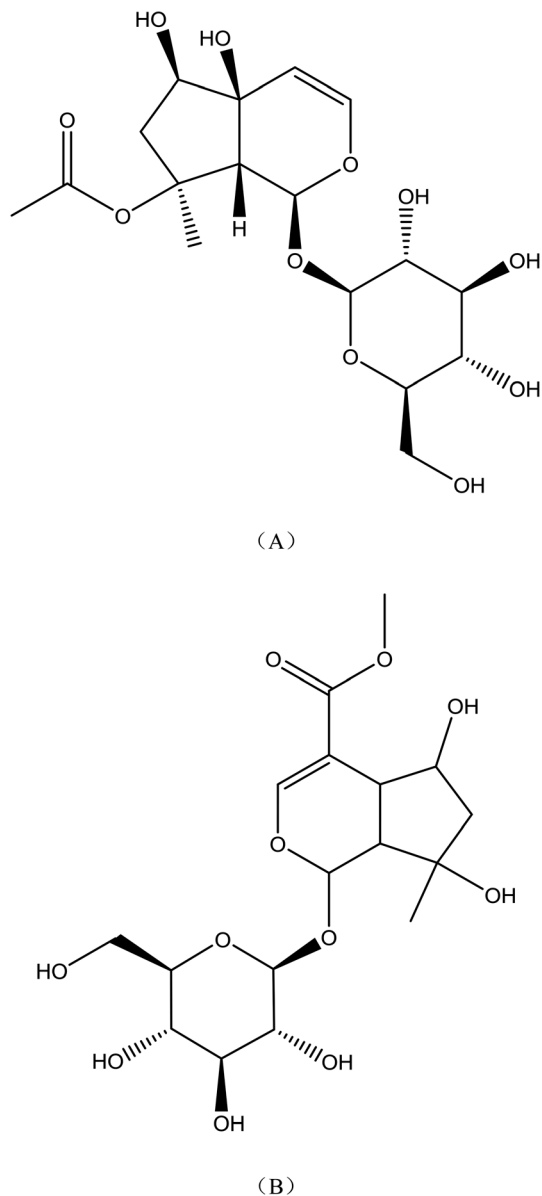
（A）

（B）

Figure 1. Chemical structures of (A) 8-*O*-acetylharpagide and (B) shanzhiside methyl ester

formic acid was obtained from Tedia (Ohio, USA). Ultrapure water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA). Institute for Cancer Research (ICR) mice (body weight, 20–22 g) were obtained from the Animal Experimental Center of Wenzhou Medical University.

Equipment. An ACQUITY I-Class UPLC and XEVO TQS-micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) was used in this study. Masslynx 4.1 software (Waters Corp.) was applied to collect the data and control the equipment.

An ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm) was used to separate 8-*O*-acetylharpagide and the internal standard in mouse blood. The temperature was set at 40 °C. The mobile phase was composed of methanol and water (containing 0.1% formic acid), and a gradient elution was performed with a flow rate of 0.4 mL/min. The gradient elution was as follows: 0–0.2 min, methanol 10%; 0.2–1.5 min, linear methanol from 10% to 80%; 1.5–2.0 min, methanol 80%; 2.0–2.5 min, linear methanol from 80% to 10%; and 2.5–5.0 min, methanol 10%. The total run time was 5.0 min.

Nitrogen was used as the desolvation gas (800 L/h) and nebulizing gas. The capillary voltage was set at 2.3 kV, the ion source temperature was 150 °C, and the desolvation temperature

was 400 °C. As shown in Figure 2, 8-*O*-acetylharpagide was quantitatively measured using electrospray ionization (ESI) tandem mass spectrometry in multiple reaction monitoring (MRM) mode with positive ionization. The MRM transitions were m/z 429.1 → 203.0 (cone voltage, 22 V; fragmentor voltage, 26 V) and 407.2 → 209.0 (cone voltage, 36 V; fragmentor voltage, 12 V) for 8-*O*-acetylharpagide and the internal standard, respectively.

Preparation of Control Solutions. 8-*O*-Acetylharpagide and shanzhiside methyl ester stock solutions at a concentration of 1.0 mg/mL were prepared with methanol. A series of standard solutions of 8-*O*-acetylharpagide was obtained by diluting the stock solution with methanol to concentrations of 50, 100, 200, 500, 900, 1000, 2000, 4500, and 5000 ng/mL. Shanzhiside methyl ester at a concentration of 50 ng/mL in acetonitrile was prepared from shanzhiside methyl ester stock solution diluted with acetonitrile. All the solutions were stored at 4 °C.

Standard Curve Preparations. Moderate amounts of working solutions of 8-*O*-acetylharpagide were added to blank mouse blood to prepare standard solutions at concentrations of 5, 10, 20, 50, 60, 200, and 500 ng/mL, with a concentration range of 5–200 ng/mL. Quality control (QC) samples at concentrations of 8, 80, and 450 ng/mL were prepared using the same method.

Sample Processing. A 20-microliter blood sample was added to a 1.5 mL Eppendorf (EP) tube, further diluted with acetonitrile (100 μL, containing the internal standard shanzhiside methyl ester at 50 ng/mL), vortexed for 1.0 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant (80 μL) was transferred to a vial, and 2 μL was used for injection.

Method Validation. The method validation was conducted according to the Analytical Procedures and Methods Validation for Drugs and Biologics by the U.S. Food and Drug Administration (FDA). The validation included selectivity, matrix effect, linearity, precision, accuracy, recovery, and stability [13, 14].

The selectivity of the UPLC–MS/MS method was evaluated by analyzing blank blood, blank blood spiked with 8-*O*-acetylharpagide and internal standard, and mouse blood samples.

A series of standard solutions with different concentrations (concentration range 5–500 ng/mL) was prepared using the standard working solution. Using the same conditions for the measured samples, the area of each peak was measured. The ratios of drug peak area to internal standard peak area vs. sample concentration were used to draw the standard curve, which was used to evaluate the linearity.

Precision and accuracy of the method were evaluated by measuring the concentrations of 3 QC samples (8, 80, and 450 ng/mL) 6 times. The precision was expressed as the relative standard deviation (RSD). The intra-day and inter-day precision was assessed by measuring the QC samples at three concentrations within three consecutive days. The accuracy was evaluated by coincidence level between average concentrations of the QC samples (at 3 concentration levels) and actual values within three consecutive days.

The recovery was assessed by comparison of peak areas between the QC samples (at 3 concentrations) and the standard samples. Three solutions at low, medium, and high concentrations (8, 80, and 450 ng/mL) were prepared by spiking standard solutions into processed blank blood. The matrix effect was evaluated by comparison of the peak area obtained for the 3 solutions and the standard solutions (8, 80, and 450 ng/mL) diluted with acetonitrile–0.1% formic acid (1:1, v/v).

The stability of 8-*O*-acetylharpagide in blood was analyzed by storing the QC samples (at 3 concentrations, 8, 80, and 450 ng/mL) in vials under 3 conditions: short-term storage (2 h at room temperature), long-term storage (−20 °C, 30 days), and after freezing and thawing (−20 °C to room temperature). The
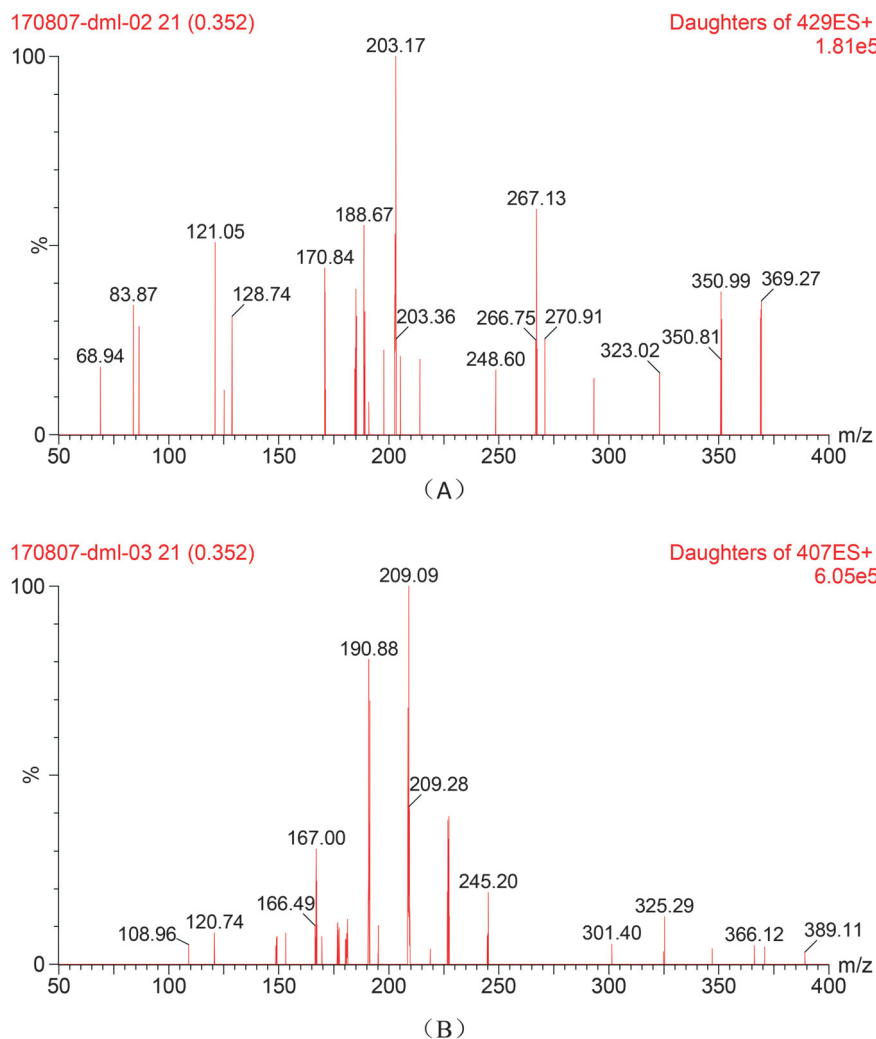
Figure 2. Mass spectra of (A) 8-*O*-acetylharpagide and (B) shanzhiside methyl ester

stability was evaluated by comparing their peak areas with those of freshly prepared standard samples (3 concentrations, 8, 80, and 450 ng/mL).

Pharmacokinetics. Twelve mice were divided into 2 groups and numbered from 1 to 12. All experiment procedures were approved by the Animal Care and Use Committee of Wenzhou Medical University. Four milligrams of 8-*O*-acetylharpagide was accurately weighed and then dissolved in purified water to prepare a fresh 1.0 mg/mL solution. Mice nos. 1–6 were given 3 mg/kg 8-*O*-acetylharpagide by intravenous administration, and nos. 7–12 were given 15 mg/kg 8-*O*-acetylharpagide by oral administration. At 0.0833, 0.5, 1, 1.5, 2, 3, 4, 8, and 12 h, blood (20 μL) was withdrawn from the caudal vein, collected in a 1.5 mL EP tube and stored at −20 °C.

The blood was processed as described in section *Sample processing* and then measured by UPLC–MS/MS. The area under the curve (AUC), mean residence time (MRT), clearance rate (CL), apparent distribution volume (V), maximum plasma concentration (C_{max}), and half-life ($t_{1/2}$) were analyzed with the non-compartmental model using DAS 2.0 software (China Pharmaceutical University). The bioavailability equation is as follows: absolute bioavailability (%) = (AUC$_{PO}$ × D_{IV}/AUC$_{IV}$ × D_{PO}) × 100.

Results and Discussion

Method Optimization. Various methodologies are used to evaluate ESI mass spectrometry positive–negative selection. The ESI positive ion mode was found to be more sensitive than the negative ion mode in our study [15, 16]. We optimized the ionization of 8-*O*-acetylharpagide and found that the fragment ion with *m/z* 203.0 had the highest abundance. The fragment ion *m/z* 209.1 from the internal standard had the highest abundance (Figure 2). Thus, 8-*O*-acetylharpagide *m/z* 429.1 → 203.0 (cone voltage, 22 V; fragmentor voltage, 26 V) and internal standard *m/z* 407.2 → 209.0 (cone voltage, 36 V; fragmentor voltage, 12 V) were used for the quantitative analysis.

Ideally, there should be as much separation in retention times as possible during HPLC of the endogenous substance from the analyte and internal standard [17, 18]. The column and mobile phase play decisive roles in the chromatographic behavior [19, 20]. We tried many mobile phases using the BEH C18 (2.1 mm × 50 mm, 1.7 μm) column, such as acetonitrile–0.1% formic acid, acetonitrile–10 mmol/L ammonium acetate solution (containing 0.1% formic acid), methanol–0.1% formic acid, and methanol–10 mmol/L ammonium acetate solutions (containing 0.1% formic acid). Good chromatographic peaks and retention times were obtained using a mobile phase consisting of methanol–0.1% formic acid with gradient elution. 8-*O*-Acetylharpagide exhibited better retention time and peak shape when using methanol as the organic phase compared with acetonitrile. Thus, we chose the BEH C18 (2.1 mm × 50 mm, 1.7 μm) column and used methanol–0.1% formic acid as the mobile phase. The retention times of 8-*O*-acetylharpagide and the internal standard were 1.85 min and 1.69 min, respectively. The peak shape and retention time for 8-*O*-acetylharpagide and the internal standard were more appropriate than those reported by Wen et al. [8, 9] who used acetonitrile–0.1% formic acid as the mobile phase.
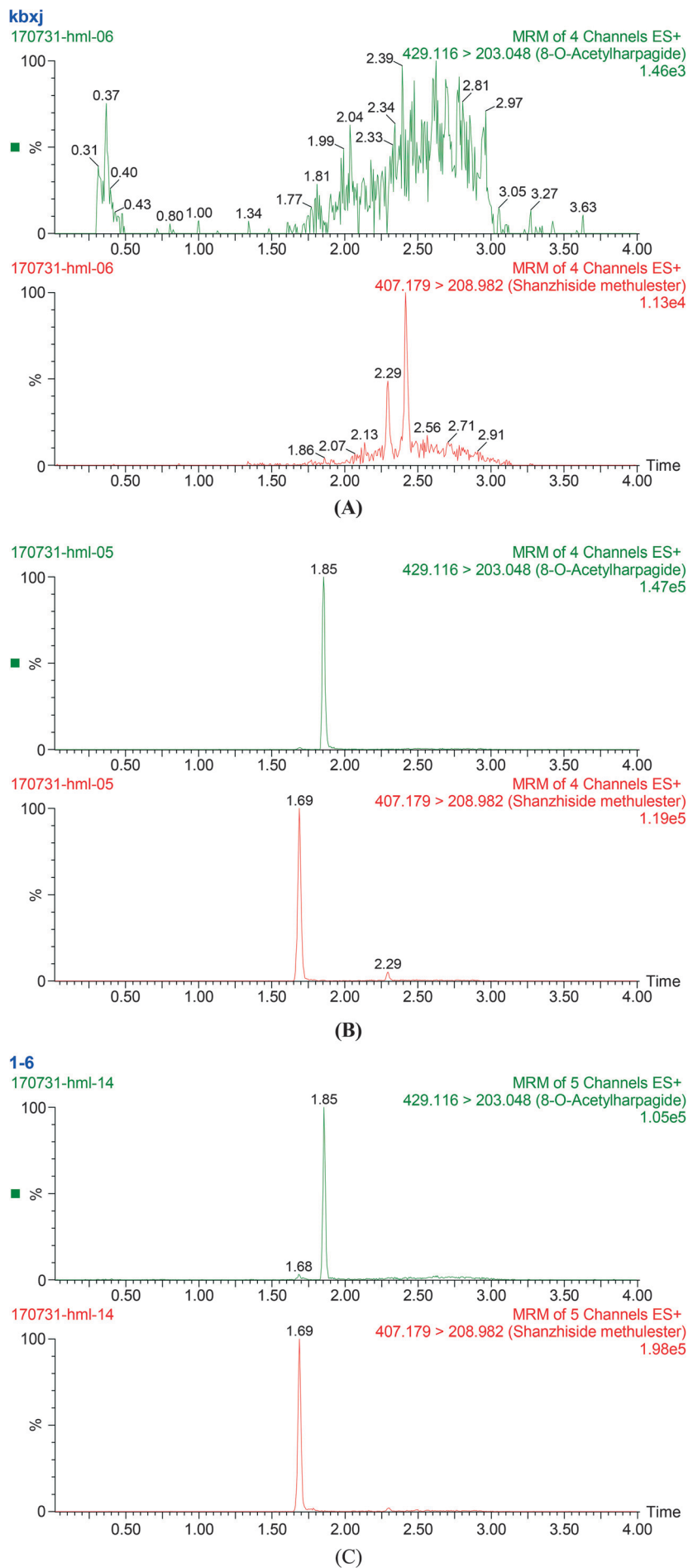
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Figure 3. UPLC–MS/MS chromatograms of 8-*O*-acetylharpagide and shanzhiside methyl ester (internal standard) in blood. (A) Blank blood, (B) blank blood spiked with 8-*O*-acetylharpagide and shanzhiside methyl ester, and (C) blood sample after oral administration

Before UPLC–MS/MS analysis, it is necessary to remove protein and potential interference factors [21]. We used ethyl acetate and diethyl ether to extract the 8-*O*-acetylharpagide, as well as methanol, acetonitrile, and methanol–acetonitrile (1:1, v/v) to precipitate the drug. Finally, we determined that the most optimal method was ethyl acetate extraction followed by acetonitrile precipitation. Direct acetonitrile precipitation is fast and simple, and can achieve good recovery and matrix effect. Thus, we chose this method to process all the blood samples. Given the more complicated components in blood than in plasma, 100 μL acetonitrile was used to process 20 μL blood, which completely eliminated proteins and interfering substances in blood.

The selection of the internal standard is also very important [22]. Here, we used shanzhiside methyl ester as an internal standard because it possesses a structure similar to that of 8-*O*-acetylharpagide. The results indicated that the retention time and mass spectrometry ionization of the internal standard were also similar to that of 8-*O*-acetylharpagide, complying with the requirements for internal standards for UPLC–MS/MS. Wen et al. [8, 9] used cinnamic acid as the internal standard, whose structure is greatly different from 8-*O*-acetylharpagide. Additionally, negative mode ionization was more appropriate for cinnamic acid [23, 24], while positive mode ionization was more appropriate for 8-*O*-acetylharpagide. Thus, it was not appropriate as an internal standard in our study.

Method Validation. Figure 3 illustrates UPLC–MS/MS chromatograms of blank blood samples, blank blood samples spiked with 8-*O*-acetylharpagide, the internal standard, and collected blood samples from the caudal vein. The retention times of 8-*O*-acetylharpagide and the internal standard were 1.85 and 1.69 min, respectively. No obvious impurities or endogenous substances were detected, suggesting good selectivity.

The standard curve of 8-*O*-acetylharpagide in mouse blood was within the range of 5–500 ng/mL. The equation of the standard curve is $y = 0.00540149x + 0.00407162$, $r = 0.9984$, where y is the peak area ratio of 8-*O*-acetylharpagide to the internal standard and x represents the 8-*O*-acetylharpagide concentration in the blood. The lower limit of quantification (LLOQ) of 8-*O*-acetylharpagide in blood was 5 ng/mL, the signal-to-noise ratio was 10, precision was 14.9%, and accuracy was 88.2%. The limit of detection (LOD) of 8-*O*-acetylharpagide in blood was 1.0 ng/mL, and the signal-to-noise ratio was 3.

The UPLC–MS/MS method for quantitative determination of 8-*O*-acetylharpagide was faster and more sensitive than traditional HPLC [14, 25]. With a detection time of only 5.0 min, the procedure was efficient and inexpensive. The LLOQ of 8-*O*-acetylharpagide (5 ng/mL) was relatively low, which resulted in ease of measurement for determining the low concentration at the final time point.

As shown in Table 1, intra-day precision RSD of 8-*O*-acetylharpagide in blood was lower than 9%, and inter-day precision RSD was lower than 13%. The accuracy range was between 94.3% and 107.1%, average recovery was higher than 91.3%, and the matrix effect was between 100.8% and 110.8%. These results suggested that the precision, accuracy, recovery, and matrix effect of the established UPLC–MS/MS method complied with the requirements of pharmacokinetic research for 8-*O*-acetylharpagide.

The stability tests for 8-*O*-acetylharpagide in mouse blood were performed at room temperature for 2 h and at −20 °C for 30 days, with freeze–thaw conditions. The results indicated that the variation was within ±13%, and the RSD was within 15%, which denote excellent stability for 8-*O*-acetylharpagide (Table 2).

Pharmacokinetics. In this study, we established a UPLC–MS/MS method to investigate the pharmacokinetics of 8-*O*-acetylharpagide in mice by intravenous or oral administration. Because only 20 μL of mouse blood could be taken at each time point, it was difficult to obtain plasma by centrifugation. We used acetonitrile precipitation to process the blood directly and then used UPLC–MS/MS to measure the 8-*O*-acetylharpagide concentration in the whole blood.

Figure 4 shows the drug concentration–time curves of 8-*O*-acetylharpagide by oral administration (po) and intravenous administration. Figure 4A illustrates that absorption after oral administration is low, so we changed the y axis into a logarithmic scale (log10). Table 3 shows the main pharmacokinetic parameters analyzed by the non-compartment model. Table 3 shows that the AUC for intravenous administration is larger than that for oral administration. The $AUC_{(0-t)}$ for oral (15 mg/kg) and intravenous administration (3 mg/kg) was 110.8 ± 18.1 ng/mL × h and 205.0 ± 54.7 ng/mL × h, respectively. The bioavailability of 8-*O*-acetylharpagide was 10.8%, and the CL was significantly lower than the amount oral administration, suggesting that a portion of the drug was metabolized before entering into the blood. Thus, 8-*O*-acetylharpagide had a significant first-pass effect by oral administration.

Wen et al. [8] compared the pharmacokinetics of a pure extract of 8-*O*-acetylharpagide from *A. decumbens* Thunb. given to rats by oral administration. The extract was administered orally at 15 mg/kg (equivalent to 6 mg/kg of 8-*O*-acetylharpagide), 30 mg/kg, and 60 mg/kg. The pharmacokinetic parameters were compared with that of 8-*O*-acetylharpagide at a dose of 12 mg/kg by oral administration. The result indicated that 8-*O*-acetylharpagide was rapidly absorbed by oral administration in a dose-dependent manner. The pharmacokinetic parameters of 8-*O*-acetylharpagide were similar to those of the extract or the pure form, except for T_{max}. $T_{1/2}$ of the extract (30 mg/kg by oral administration, equivalent to 8-*O*-acetylharpagide 12 mg/kg) was 2.88 ± 0.38 h, and the $AUC_{(0-t)}$ was 1279.56 ± 181.41 ng/mL × h. The $t_{1/2}$ of 8-*O*-acetylharpagide by oral administration (12 mg/kg) was 3.37 ± 0.32 h, and the $AUC_{(0-t)}$ was 1635.03 ± 102.18 ng/mL × h. $T_{1/2}$ of 8-*O*-acetylharpagide

Table 1. Accuracy, precision, matrix effect, and recovery of 8-*O*-acetylharpagide in mouse blood

Concentration (ng/mL)	Accuracy (%)		Precision (RSD%)		Matrix effect (%)	Recovery (%)
	Intra-day	Inter-day	Intra-day	Inter-day		
8	94.3	99.8	8.6	11.4	100.8	92.6
80	100.5	107.1	1.6	12.8	105.8	94.1
450	100.0	98.1	2.9	4.7	110.8	91.3
RSD, relative standard deviation.						

Table 2. Stability of 8-*O*-acetylharpagide in mouse blood

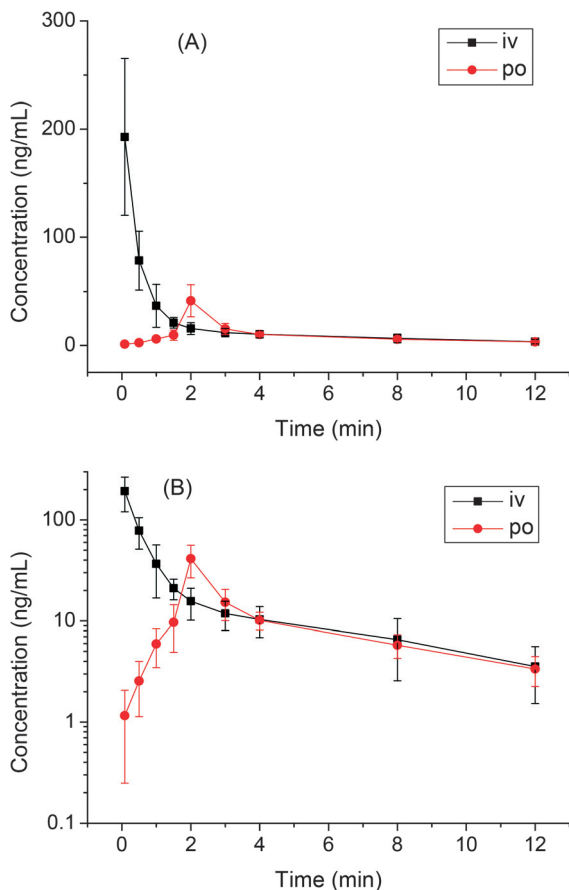| Concentration (ng/mL)	Autosampler ambient		Ambient, 2 h		−20 °C, 30 days		Freeze–thaw	
	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD
8	96.5	5.1	94.1	8.3	92.9	11.9	87.9	14.1
80	96.4	1.6	104.5	7.3	88.2	12.3	112.6	7.6
450	106.4	4.8	99.2	7.5	94.5	9.7	101.1	11.7
RSD, relative standard deviation.								

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Figure 4. Drug–time curves of 8-O-acetylharpagide by oral (po, 15 mg/kg) and intravenous administration (iv, 3 mg/kg). (A) Linear *y* axis; (B) log10 *y* axis

Table 3. Main pharmacokinetic parameters of 8-O-acetylharpagide in mice

Parameters	Unit	po (15 mg/kg)	iv (3 mg/kg)
$AUC_{(0-t)}$	ng/mL*h	110.8 ±18.1	205.0 ± 54.7
$AUC_{(0-\infty)}$	ng/mL*h	132.6 ± 35.4	222.3 ± 56.3
$MRT_{(0-t)}$	h	4.4 ± 0.2	2.4 ± 0.7
$MRT_{(0-\infty)}$	h	6.6 ± 1.8	3.7 ± 1.8
$t_{1/2z}$	h	4.4 ± 1.8	3.9 ± 1.5
$CL_{z/F}$	L/h/kg	159.0 ± 37.8	24.0 ± 7.2
$V_{z/F}$	L/kg	933.7 ± 193.1	132.4 ± 44.5
C_{max}	ng/mL	41.4 ± 14.8	192.7 ± 72.5

AUC, area under the curve; MRT, mean residence time; CL, clearance rate; V, apparent distribution volume; C_{max}, maximum plasma concentration; $t_{1/2}$, half-life; po, oral administration; and iv, intravenous administration.

by oral (15 mg/kg) and intravenous administration (3 mg/kg) was 4.4 ± 1.8 h and 3.9 ± 1.5 h, respectively. $T_{1/2}$ in the rat was similar to that obtained in mice but was slightly delayed in mice, which might be caused by the difference in species.

Healthy female beagle dogs were given an extract of *A. decumbens* at different doses by Wen et al. [9]. The concentrations of 8-O-acetylharpagide in the blood at different time points were measured and analyzed using a non-compartmental model and were determined to be dose-dependent. The peak time of 8-O-acetylharpagide was approximately 1.7 h, much later than that

in rat blood after oral administration of an *A. decumbens* extract, but it was similar to that obtained in mice.

Conclusion

Here, we established a sensitive and rapid UPLC–MS/MS method with good selectivity to measure the amount of 8-O-acetylharpagide in mouse blood. The linear range was 5–500 ng/mL, and the LLOQ was 5 ng/mL. Acetonitrile was used to process 20 μL of whole blood, and the detection time for each sample was 5 min. Our method utilized a more appropriate mobile phase and internal standard than a previously reported LC–MS/MS method. Then, we successfully applied our method to investigate the pharmacokinetics of 8-O-acetylharpagide by oral and intravenous administration, and the bioavailability was 10.8%. The analysis of the main pharmacokinetic parameters indicated that, after administration by oral and intravenous, 8-O-acetylharpagide had a significant first pass effect after oral administration.

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