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Original Research Paper

A rapid and sensitive liquid chromatography–tandem mass spectrometric method for the determination of hederasaponin B in rat plasma: Application to a pharmacokinetic study

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ABSTRACT

A rapid, simple and sensitive ultra-high performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method was developed and validated for the determination of hederasaponin B, an active triterpenoid saponin widely existed in *Hedera helix* L. Plasma samples were processed by protein precipitation with acetonitrile and separated on a Thermo Hypersil GOLD C_{18} (2.1 mm × 50 mm,1.9 μm) at flow rate of 0.3 ml/min, with a gradient elution consisting of acetonitrile and water containing 0.1% (v/v) formic acid at 30 °C and detected by electrospray ionization mass spectrometry in the positive multiple reaction monitoring (MRM) mode. The linearity was found to be within the concentration range of 0.5–5000 ng/ml with a lower limit of quantification of 0.5 ng/ml. The absolute oral bioavailability of hederasaponin B was 0.24 ± 0.49%. This indicated that the concentration-time course of the hederasaponin B existed a double-peak phenomenon. This method was further applied to the determination of hederasaponin B in rat plasma and showed good practicability, for the first time, after intragastric (25 mg/kg) and intravenous (2 mg/kg) administration in rats.

1. Introduction

Hedera helix L., an important herbal medicine, is an evergreen cirrus belonging to the family of *Araliaceae*. In addition to landscaping and greening, the leaf extractions had been reported to have anti-inflammatory [1,2], anti-fungal [3], anthelmintic [4], anti-bronchial [5] properties and increased β_2-adrenegic responsiveness [6,7] activity. Phytochemical investigations revealed that triterpene saponins were primary active components of *H. helix* [8,9]. As we all know, triterpene saponins in *Araliaceae* played an important role in the aspect of anti-inflammation

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and anti-cancer. Hederasaponin B as a member of this kind of active triterpene saponins had drawn increasing attention, especially effective in anti-cancer, anti-inflammation and anti-virus fields. Recently, Lin-Tao Han determined that five triterpenoid saponins isolated from *Anemone flaccid* including hederasaponin B could induce apoptosis activity in Hela cells [10]. It was also reported that the expressions of EV71C3 and C4aVP protein were inhibited in the presence of 50 μg/ml hederasaponin B, which had significant anti-viral effects on hand, foot and mouth disease (HFMD) [11]. According to Liu's work, hederasaponin B decreased pro-inflammatory cytokine levels and significantly inhibited TNF-α and IL-6 production in LPS-treated RAW264.7 cells [12]. In addition, the superoxide generation induced by arachidonic acid (AA) was suppressed by hederasaponin B effectively [13]. In consideration of numerous bioactivities of hederasaponin B above, it is essential to understand the pharmacokinetic and bioavailability characteristics. To develop a validated analytical method suitable for hederasaponin B determination in relevant biological samples is now an urgent task.

Therefore, a simple and reliable method for pharmacokinetic study of hederasaponin B is badly in need to understand the drug safety profiles and interpret the mechanism of effectiveness and toxicity. However, in the past decades, researchers paid more attention to the quantity or quality of components in *H. helix* [14–16]. Only few literatures demonstrated the pharmacokinetic mechanism of the active ingredients such as α-hederin [17], hederacoside C [18]. Unfortunately, we found no information about pharmacokinetic study of hederasaponin B. Thus, a novel, sensitive and simple ultra-high performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method in MRM mode was developed for determination of hederasaponin B in plasma, and pharmacokinetic studies were devised to determine its bioavailability in rats, for the first time. It was expected that the results of this work would provide some references for the apprehension of the action mechanism and further clinical efficacy studies of hederasaponin B.

2. Experimental

2.1. Materials, reagents and animals

Hederasaponin B and hederacoside D (IS) were purchased from spring & autumn biological engineering Co. Ltd. (Nangjing, China). Acetonitrile of HPLC grade was obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). All the other reagents were of analytical grade.

SPF degree Sprague Dawley rats (male, 200 ± 20 g) were provided by Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were housed in controlled conditions (temperature, 22 ± 2 °C; humidity 50 ± 10%; 12 h light/dark cycle) and received a standard rat chow and tap watered ad libitum for a week prior to experiments. All animal experiments were carried out according to the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University; the procedure was approved by the Animal Ethics Committee of the institute.

2.2. Instruments and conditions

The UPLC–MS/MS analysis was carried out on an Acquity UPLC-Class system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ-S mass spectrometer (Waters Corp., Milford, MA, USA). A Thermo Hypersil GOLD C_{18} (2.1 mm × 50 mm, 1.9 μm) was employed and the column temperature was maintained at 30 °C. Gradient elution (delivered at 0.3 ml/min) was employed using acetonitrile (mobile phase A) and 0.1% formic acid in water (mobile phase B). The gradient started at 20% A, followed by linear increased to 60% A from 0 to 3 min, then quickly returned to initial 20% A and maintained till 4 min for column balance. The auto-sampler was conditioned at 4 °C and the injection volume was 2 μl.

A mass spectrometer with an electrospray ionization (ESI) interface in a positive ion mode (ESI) was used for quantitative analysis. The capillary voltage was set at 3.0 kV, and the source and desolvation temperatures were set at 150 °C and 350 °C respectively. Nitrogen was used as the desolvation and cone gas with a flow rate of 700 and 60 l/h. The quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 1227.27→492.77 for hederasaponin B, and m/z 1097.48 → 493.75 for hederacoside D (IS), respectively. The results were shown in Table 1 and Fig. 1.

2.3. Sample pretreatment

A simple and rapid protein precipitation method was used for the preparation of plasma samples. An aliquot of 100 μl of each plasma was spiked with 10 μl of IS (2 μg/ml) and mixed for 30 s. Then, 300 μl of acetonitril was added, and the mixture was vortexed for 1 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was transferred to a tube and evaporated to dryness at 40 °C under a slight stream of nitrogen. Then, the residue was reconstituted with 100 μl of mobile phase, and a 2 μl aliquot of the supernatant was injected into the UPLC-MS/MS for analysis after centrifugation at 12,000 rpm for 5 min.

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

Stock solutions of hederasaponin B and IS were prepared in methanol at 1.0 mg/ml and 100 μg/ml, respectively. The standard stock solution was then serially diluted with methanol to obtain working standard solutions in a concentration range of 5–50,000 ng/ml, for hederasaponin B. The IS solution was similarly diluted to 2 μg/ml with methanol. Calibration standard samples of hederasaponin B were obtained by spiking 10 μl of the appropriate working solutions to 100 μl blank plasma. Quality control (QC) samples at low, medium and high

Table 1 – MRM transition in positive ion mode cone voltage and collision energy for the determination of the hederasaponin B and IS.

Analytes	Transition	Cone voltage(V)	Collision energy (eV)
Hederasaponin B	1227.27→492.87	60	58
Hederacoside D	1097.48→492.75	55	58

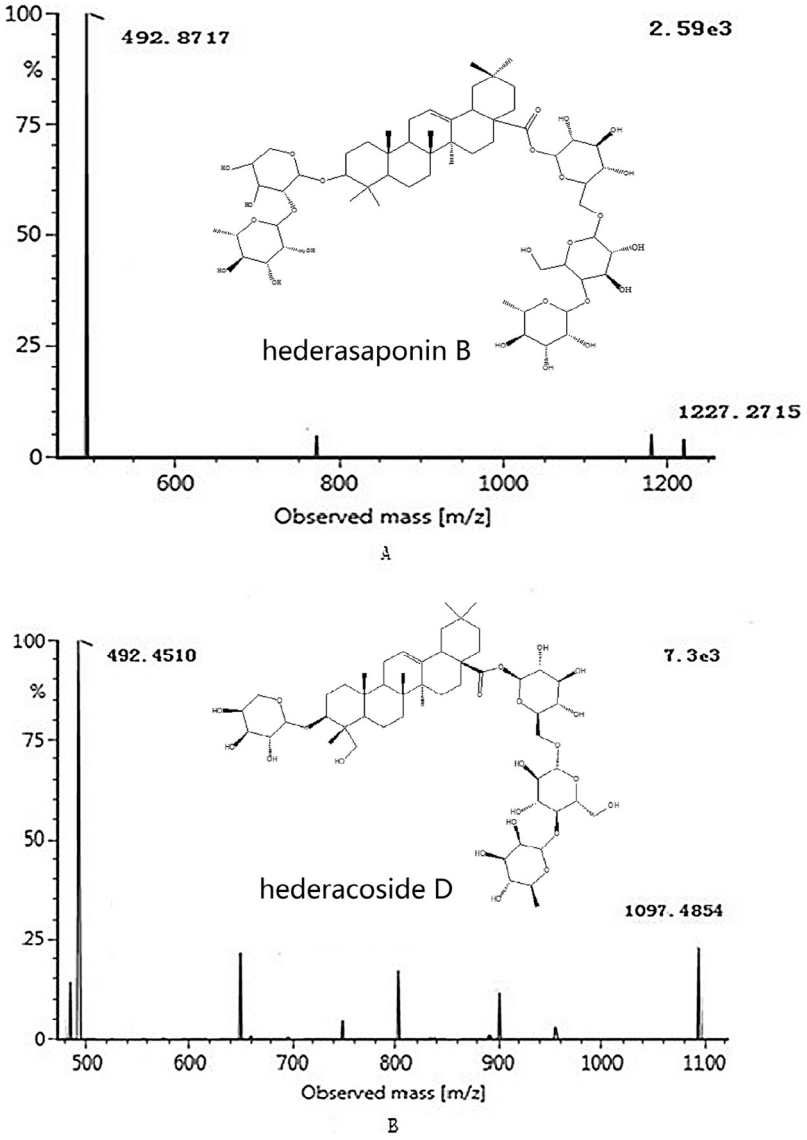

Fig. 1 – MS/MS spectra of hederasaponin B(A) and hederacoside D (IS) (B).

concentrations (1, 100 and 4000 ng/ml) were prepared separately in the same fashion. All stock solutions, working solutions and quality control samples were stored at –20 °C until used.

2.5. *Method validation*

2.5.1. *Specificity*

Specificity of this method was determined by comparing chromatograms of blank plasma from six different rats, blank plasma spiked with hederasaponin B and IS, and plasma samples obtained after administration of hederasaponin B.

2.5.2. *Linearity and LLOQ*

Calibration curves were constructed using 8 calibration standards over the range of 0.5–5000 ng/ml by plotting the peak area ratio of hederasaponin B to the IS (y) versus the nominal concentrations of hederasaponin B (x) in plasma. The linearity was assessed using a weighted ($1/x^2$) least square regression. The criteria for the calibration curve included a correlation coefficient (r) of >0.99. The lower limit of quantification (LLOQ) which the signal-to-noise ratio was determined to be more than 10 (S/N > 10) was defined as the reproducible lowest possible drug concentration that could be determined with both accuracy and precision within ±20% .

2.5.3. *Extraction recovery and matrix effect*

The recovery was determined at three QC levels with six replicates by comparing the peak areas from plasma samples spiked before extraction with those spiked after extraction. The matrix effect was determined by examining the ratio of the peak areas dissolved in the blank matrix extract to that in standard solution containing equivalent amounts of the compounds. The procedure was repeated 6 times.

2.5.4. *Precision and accuracy*

The precision and accuracy of the method were assessed by performing 6 replicates of QC samples spiked with low, medium and high concentrations against calibration standards on the

same day and on 3 consecutive days. The precision at each QC concentration was expressed as relative standard deviation (RSD%) and the accuracy as error (RE%).

2.5.5. *Stability*

The stability tests of analytes in rat plasma were under different storage conditions: 6 h at room temperature, under autosampler condition (4 °C) for 12 h, frozen for 2 weeks and three freeze-thaw cycles. All stability testing QC samples were determined by using a calibration curve of freshly prepared standards. The results were evaluated by RE% and RSD%.

2.6. *Pharmacokinetic study*

10 male Sprague Dawley (SD) rats with free access to food and water, then, fasted for 12 h prior to experiments were randomly divided into two groups with 5 in each. The hederasaponin B was prepared in normal saline solution. The method was applied to the pharmacokinetic studies of hederasaponin B by two different administrations: intravenous (2.0 mg/kg) and intragastric (25 mg/kg). Blood samples (about 0.3 ml) were collected in heparinized tubes via the postorbital venous plexus veins from each rat at 0 (pre-dose), 0.083, 0.167, 0.25, 0.3, 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 24 and 36 h after intragastric administration and at 0, 0.05, 0.083, 0.167, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 9 and 12 h after intravenous injection. All blood samples were immediately centrifuged at 10,000 rpm for 10 min, and the plasma was transferred into clean tube and stored at –80 °C prior to analysis, until analyzed. The pharmacokinetic parameters were calculated by drug and statistics (DAS) software 2.0 (Shanghai, China) pharmacokinetic program.

3. Results and discussion

3.1. *Method development*

3.1.1. *Optimization of chromatographic and mass conditions*

In order to optimize MS condition, a standard solution of the hederasaponin B and IS were directly infused along with the mobile phase into the mass spectrometer with electrospray ionization (ESI) as the ionization source. Compared with the response observed in positive ion mode and negative mode, we discovered that hederasaponin B and IS had stronger signal intensity and lower background noise in positive ion mode. Therefore, detection was performed in positive ion mode in this study. The full-scan ESI-MS (positive ionization mode) of hederasaponin B and IS showed the protonate molecular ions $[M+Na]^+$ at m/z 1227.27, and 1097.48, respectively, because of the good sensitivity, reproducibility and response stability. Thus, $[M+Na]^+$ were chosen as the precursor ions for MS/MS analysis. The full-scan product ion spectra of analytes were provided in Fig. 1.

Chromatographic conditions played a critical role in achieving good chromatographic behavior. In this study, acetonitrile was chosen as the organic phase because of the advantage of higher responses and lower background noise by comparison with methanol. Previous study indicates that low concentration of acid solution can facilitate the protonation and improve the intensity of basic compounds in the positive mode [19]. Different concentrations of formic acid (0.05%, 0.1% and 0.2%) were added to acetonitrile-water as different mobile phases. The shape of peak and intensities of hederasaponin B and IS were improved significantly when 0.1% formic acid was added in the mobile phase. Moreover, isocratic elution was also tested, but it showed shortcomings of asymmetry peak. Finally, gradient elution with acetonitrile –0.1% formic acid in water was selected to be the mobile phase. In addition, compared with other researches [18,20] about constituents similar to hederasaponin B, our study was less consuming time, only about 4 min.

3.1.2. *Optimization of sample preparation*

Sample preparation is an important step for accurate and reliable UPLC–MS/MS assay. Several sample pre-treatment methods were investigated in this study, including protein precipitation and liquid–liquid extraction (LLE). However, the liquid–liquid extraction with ethyl acetate or n-butanol showed limited extraction recovery efficiency, considering the polarity of the analytes. Thus, the protein precipitation method which was simpler and less time consuming was finally used. Different precipitation organic solvents (methanol, acetonitrile or acetone) were evaluated. Results showed that a precipitation with acetonitrile provided a higher recovery and perfect peak shape for both hederasaponin B and IS.

3.1.3. *Selection of internal standard*

According to the US Food and Drug Administration (FDA) guidance, any IS used in biological analysis should be a structurally similar analog of the analytes or a stable labeled compound. In this study, baicalin, scutellarin and hederacoside D were tested. However, baicalin or scutellarin were unsatisfactory choices for the IS, on account of poor peak shape and low response. Therefore, hederacoside D was chosen as the IS, because of the high ionization response in ESI mass spectrometry and a similar chromatographic retention time.

3.2. *Method validation*

3.2.1. *Specificity*

The detection of hederasaponin B and IS by MRM mode was highly selective with no interference from the endogenous substances. Fig. 2 represents typical chromatograms of blank plasma, blank plasma spiked with hederasaponin B at LLOQ and IS, and rat plasma sample collected after intragastric administration of 25 mg/kg hederasaponin B. Typical retention times for hederasaponin B and IS were 2.08 min and 1.79 min, respectively.

3.2.2. *Linearity and LLOQ*

The calibration curves showed good linearity over the concentration range of 0.5–5000 ng/ml in rat plasma. A typical equation of the calibration curves was $y = 0.00435x + 0.00231$ ($r = 0.9928$), where y was the peak area ratio of hederasaponin B to IS and x was the concentration of hederasaponin B. The LLOQ for hederasaponin B in rat plasma was 0.5 ng/ml and the result of accuracy and precision were shown in Table 2, which was sufficient for the pharmacokinetic study. Compared with

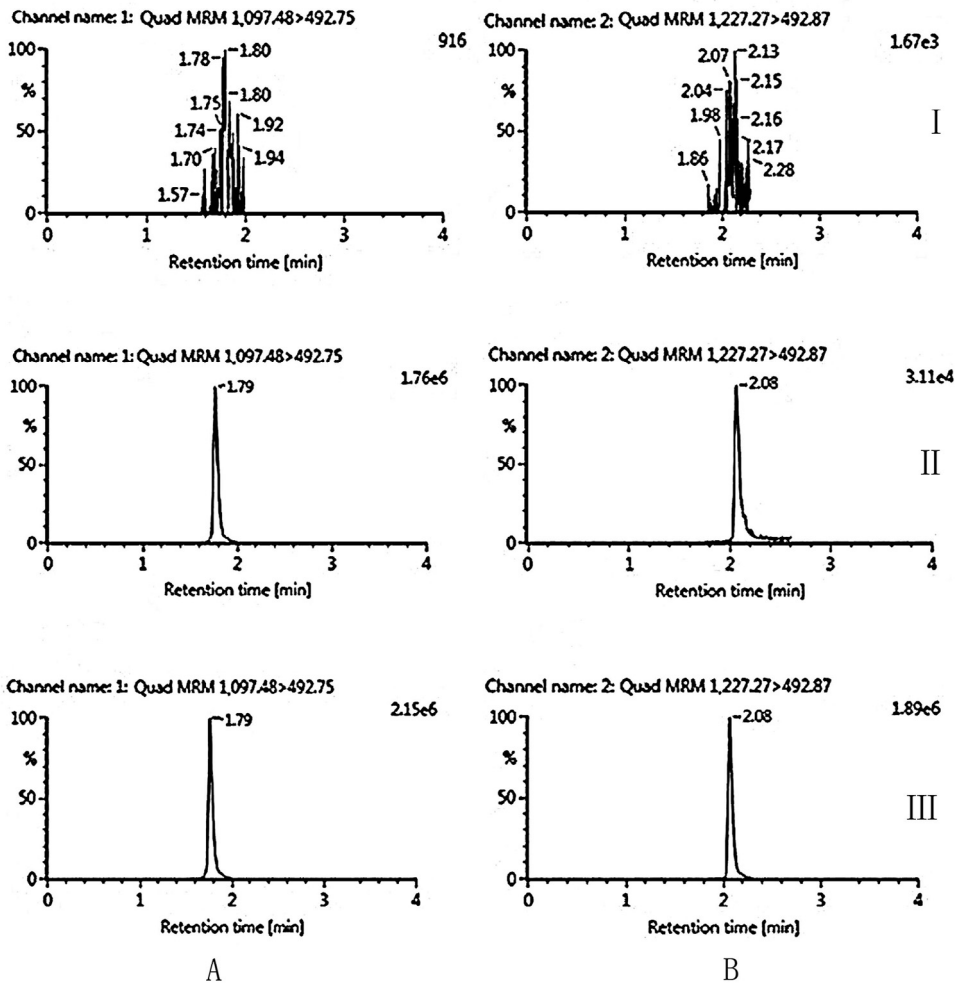

Fig. 2 – Representative MRM chromatograms of IS (A), hederasaponin B (B) in rat plasma: blank rat plasma (I), blank rat plasma spiked with hederasaponin B at LLOQ and IS (II) and rat plasma samples at 1h after drug administration (III).

other hederasaponin researches [17,18], our method could obtain lower LLOQ.

3.2.3. *Recovery and matrix effect*

As shown in Table 2, the extraction recovery was in the range of 85.0–91.8% for hederasaponin B, which could meet the requirements of analysis. Results of matrix effects indicated that no significant matrix effects were observed for the analytes.

3.2.4. *Precision and accuracy*

The results of intra- and inter-day precision and accuracy at three concentration levels of the QC samples (1, 100 and 4000 ng/ml) were summarized in Table 2. The intra- and inter-day RSD were measured to be below 6.3% and 8.4%, and the RE was within 3.9%. The results demonstrated that both the intra- and inter-day assay values were all within the acceptable range.

3.2.5. *Stability*

The data of stability were summarized in Table 3, which indicated that hederasaponin B had no effect on stability in autosampler (12 h) at 4 °C., at room temperature for 6 h, through repeated three freeze-thaw cycles and under the frozen condition at –20 °C for two weeks.

3.3. *Pharmacokinetic application*

The validated UPLC-MS/MS method was successfully applied to a pharmacokinetic study of hederasaponin B in rats. The

Table 2 – Precision, accuracy, recovery and matrix effect of hederasaponin B in rat plasma (n = 6).

Concentration(ng/ml)	Intra-day RSD(%)	Inter-day RSD(%)	Accuracy RE(%)	Recovery (%,mean)	Matrix effect(%,mean)
0.5	8.1	13.5	–13.7	90.2	104.4
1	6.3	8.4	1.8	85.0	101.7
100	4.9	3.5	3.9	91.8	95.7
4000	3.8	5.1	3.4	89.6	96.8

Table 3 – Stability of hederasaponin B under various storage conditions.

Conditions	Concentration (ng/ml)	RSD(%)	RE(%)
Room temperature for 6 h	1	9.4	−4.7
	100	2.8	2.5
	4000	4.1	−1.8
Processed samples in autosampler for 12 h	1	3.6	1.5
	100	5.2	−2.2
	4000	2.8	−1.5
Frozen for 2 weeks	1	8.7	−3.1
	100	4.6	−1.1
	4000	2.4	0.7
Three freeze–thaw cycles	1	9.4	0.5
	100	7.2	−1.3
	4000	5.4	−3.7

Table 4 – Main pharmacokinetic parameters of hederasaponin B after intragastric administration of 25 mg/kg and intravenous administration of 2 mg/kg to rats (mean ± SD, n = 5).

Parameters	25 mg/kg(ig)	2 mg/kg(iv)
Cmax(ng/ml)	35.088 ± 14.244	2720.683 ± 416.196
$AUC_{0\to t}$	255.163 ± 154.091	8342.153 ± 2523.868
$AUC_{0\to\infty}$	264.969 ± 152.538	8586.263 ± 2717.299
$t_{1/2}$	7.683 ± 1.383	2.135 ± 0.354
T_{max}	0.633 ± 0.774	0.083 ± 0

mean plasma concentration-time curves of the hederasaponin B after (A) intragastric administration of 25 mg/kg and (B) intravenous administration of 2 mg/kg to rats were shown in Fig. 3. A non-compartment model was used to calculate the pharmacokinetic parameters. The pharmacokinetic parameters of hedersaponin B in male SD rats were shown in Table 4.

Results showed that hederasaponin B was absorbed very quickly after intragastric administration, which reached the peak concentration (T_{max}) at 0.633 h. Then, the concentration went down with an elimination half time ($t_{1/2}$) of 7.683 h, but at about 5 h, a double-peak phenomenon and "absorption window" were observed. This phenomenon was similar to some other saponins [21,22]. It is acceptable that drug absorption may be affected by membrane permeability, presystemic metabolism, along the gastrointestinal tract, enterohepatic circulation and variable gastric emptying [23,24]. Therefore, further studies should be carried out to ascertain whether this phenomenon is related to enterohepatic circulation or other mechanisms.

The absolute bioavailability is one of the most significant pharmacokinetic parameter for a drug because it is a good indicator for delivery capability of the drug to systemic circulation. The absolute bioavailability (F) is presented as: $F = (AUC_{ig} \times D_{iv})/(AUC_{iv} \times D_{ig}) \times 100\%$. However, similar to other saponins, hederasaponin B showed very low oral bioavailability, only about 0.24 ± 0.49%, which likely resulted from extensive metabolism in the gastrointestinal tract and the poor intestinal absorption [25,26]. Thus, further studies of distribution, metabolism, and excretion of hederasaponin B need to be investigated. In addition, as mentioned above, oral bioavailability of hederasaponin B was very low, and its concentration in blood after intravenous injection reduced rapidly. It indicated that current oral administration and even intravenous injection, are not very appropriate, and formula modification is necessary.

4. Conclusion

A fast, accurate and sensitive UPLC–MS/MS method was established and validated to quantify the concentration of hederasaponin B in rat plasma for the first time. It had been successfully applied to the preliminary pharmacokinetic study of hederasaponin B after intragastric and intravenous administrations. The absolute bioavailability was calculated to be only about 0.2%. However, it is worth expecting that metabolic profile of hederasaponin B and other related investigation will be

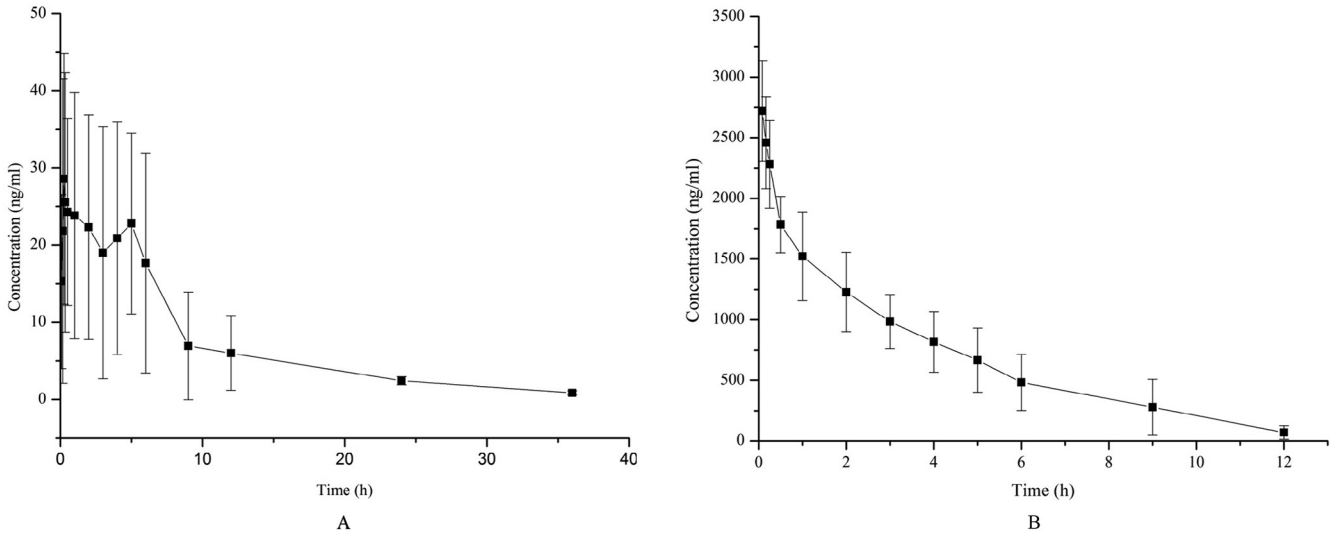

Fig. 3 – Mean plasma concentration–time curves of Hederasaponin B after (A) intragastric administration of 25 mg/kg and (B) intravenous administration of 2 mg/kg to rats.

continued. Moreover, the present pharmacokinetic research of hederasaponin B will provide also helpful information for the development and reasonable usage of it in the future.

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