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A UFLC/MS/MS method for simultaneous quantitation of alisol A and alisol B 23-acetate from Alisma orientale (Sam.) Juz. in rat plasma

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ABSTRACT

A sensitive and reliable ultra fast liquid chromatography tandem mass spectrometry (UFLC-MS/MS) method has been developed and validated for simultaneous quantitation of alisol A and alisol B 23-acetate from Alisma orientale (Sam.) Juz. in rat plasma using diazepam as an internal standard (IS). The plasma samples were extracted by liquid–liquid extraction with methyl tert-butyl ether and separated on a Venusil MP C18 column (100 mm × 2.1 mm, 3.0 mm) (Venusil, China) using gradient elution with the mobile phase consisting of methanol and 0.1% acetic acid in water at a flow rate of 0.4 ml/min. The two analytes were monitored with positive electrospray ionization by multiple reaction monitoring mode (MRM). The lower limit of quantitation was 5.00 ng/ml for alisol A and 5.00 ng/ml for alisol B 23-acetate. The calibration curves were linear in the range of 5.00–2500 ng/ml for alisol A and 5–2500 ng/ml for alisol B 23-acetate. The mean extraction recoveries were above 63.8% for alisol A and 68.0% for alisol B 23-acetate from biological matrixes. Both intra-day and inter-day precision and accuracy of analytes were well within acceptance criteria (15%). The validated method was successfully applied to the pharmacokinetic study of alisol A and alisol B 23-acetate in rat plasma after oral administration of alcohol extract of Alismatis Rhizoma.

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1. Introduction

Alisma orientale (Sam.) Juz. (Alismataceae) is distributed in China, Japan, Mongolia and Russia [1]. The dried rhizome of this plant is a common traditional Chinese medicine, known as Alismatis Rhizoma (Zexie in Chinese) with the function of removing dampness and promoting water metabolism according to the theory of Traditional Chinese Medicine [2]. Alismatis Rhizoma has been commonly used for the treatment of various diseases, including hyperlipidemia, diabetes, hypertension, and urological diseases [3–5]. Protostane-type triterpenes are the principal active constituents of Alismatis Rhizoma and more than 50 unique protostane-type triterpenes have been isolated from this herbal drug [6–8]. As the bioactive "marker compounds" of Alismatis Rhizoma, alisol A and alisol B 23-acetate (Fig. 1), which are the two major active triterpenoid compounds isolated from Alismatis Rhizoma, have particularly been the focus of research in recent years because of their relatively high levels in Alismatis Rhizoma and various pharmacological activities. They were demonstrated to possess diuretic, anti-inflammatory, anti-allergic effects, and hypocholesterole-mic effects [9–11]. Furthermore, it has been demonstrated that alisol B 23-acetate induces cell death of hepatoma and leukemia cells [12,13].

However, as far as we are aware, there are few reports on the pharmacokinetic study of alisol A and alisol B 23-acetate, despite that the study of the pharmacokinetics is essential to illuminate the mechanism of action. In previous studies, the pharmacokinetics of Alismatis Rhizoma was evaluated by HPLC and LC-MS methods [14–16]. The previous experiments had shown that HPLC and LC-MS did not provide satisfactory lower limits of quantification (LLOQ) and also took long analysis time. While the UFLC-MS/MS method can overcome these barriers, it is more fast, selective and sensitive. Thus, a UFLC-MS/MS method for the simultaneous determination of alisol A and alisol B 23-acetate in rat plasma after oral administration of Alismatis Rhizoma extract has been developed and validated for the first time in the work discussed in this paper. This will be helpful for improving clinical therapeutic efficacy and further pharmacological studies of alisol A and alisol B 23-acetate, meanwhile it can facilitate the further research and reasonable usage of Alismatis Rhizoma.

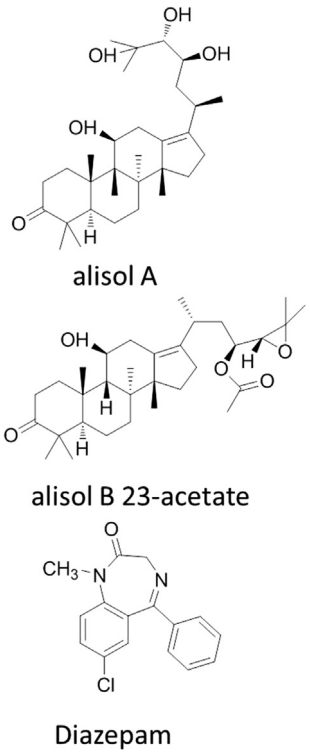

Fig. 1 – Chemical structures of alisol A (a), alisol B 23-acetate (b), and diazepam (IS) (c).

2. Materials and methods

2.1. Chemicals and materials

The dried rhizomes of *Alisma orientale* (Sam.) Juz. were purchased from Shenyang Tongrentang Drug Company (Shenyang, China) and identified by associate professor Ying Jia (Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China). Alisol A (purity >98%) and alisol B 23-acetate (purity >98%) were purchased from Chengdu Must Biotechnology Co.Ltd (Chengdu, People's Republic of China). Diazepam (purity >98%) (Fig. 1), used as the internal standard (IS) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol (high-performance liquid chromatography (HPLC) grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade reagents such as acetic acid, methyl tert-butyl ether were provided by Shandong Yuwang Industrial Co.Ltd. (Yucheng, China). All other reagents were of analytical purity. Distilled water prepared with demineralized water was employed throughout the experiment.

2.2. Animals

Six male pathogen-free Sprague-Dawley rats (220–250 g) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, People's Republic of China). The animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol was approved by the Animal Ethics Committee of the institution.

2.3. Instrumentation and chromatographic conditions

The UFLC-ESI-MS/MS system was performed using an XR LC-20AD Prominence™ UFLC system equipped with a binary pump, a degasser, an autosampler and a thermostatted column compartment (Shimadzu, Japan) and a QTRAP™ 4000 MS/MS system from Applied AB Sciex equipped with a turbo ion spray source (Foster City, CA, USA). All the operations, the acquiring and analysis of data were controlled by Analyst (version 1.6, AB Sciex, USA).

The compounds were separated on a Venusil MP C18 column (100 mm × 2.1 mm, 3.0 mm) (Venusil, China) protected by a high pressure column pre-filter (2 μm) (Shimadzu, Japan) at

Table 1 – List of selected MRM parameters, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) for each analyte and IS.

Analyte	Q1Mass(Da)	Q3Mass(Da)	DP(V)	EP(V)	CE(V)	CXP(V)
Alisol A	491.3	383.4	120	10	17	12.6
Alisol B 23-acetate	515.6	437.5	120	10	21.8	10
Diazepam	284.7	193.1	80	10	43	10

30 °C. The mobile phase was composed of methanol (A) and 0.1% acetic acid in water (B). The 8.0 min UFLC gradient program was as follows: 80% A → 100% A at 0.01 → 4.00 min; 100% A at 4.01 → 5.00 min; 80% A at 5.01 → 8.00 min. Efficient and symmetrical peaks were obtained at a flow rate of 0.4 ml/min with a sample injection volume of 5 μl.

The detection of the analytes was in the multiple reaction monitoring mode (MRM) using an electrospray positive ionization (ESI^+). The electrospray source and the mass spectrometer were adjusted to the following parameters: curtain gas, 20 psi; gas 1, 50 psi; gas 2, 50 psi (all gases: nitrogen) with a source temperature of 500 °C. The ion spray voltage was set at 5500 V for positive monitoring. The main MS parameters are listed in Table 1.

2.4. *Preparation of Alismatis Rhizoma extract*

The crude drug (100 g) was extracted twice by refluxing with 95% ethanol (1:10, w/v), 1 h for each time. After removing the ethanol under reduced pressure, the residue was dissolved in aqueous solution of 0.5% CMC-Na to get the Alismatis Rhizoma extract with an Alismatis Rhizoma concentration of 2 g/ml (containing 1.4 mg/g alisol A and 0.6 mg/g alisol B 23-acetate).

2.5. *Preparation of standard solution*

The stock solution of alisol A, alisol B 23-acetate and diazepam (IS) were prepared in acetonitrile at the concentration of 500.0 μg/ml, 100.0 μg/ml and 10.0 μg/ml, respectively. They were further diluted with acetonitrile to make a series of mixed working solutions at the concentration of 40.0–200*e4 ng/ml for alisol A and 40.0–200*e4 ng/ml for alisol B 23-acetate; IS was diluted to a concentration of 50.0 ng/ml with acetonitrile as working solution. All the stocks were stored at 4 °C.

2.6. *Sample preparation*

All the plasma samples were extracted by liquid–liquid extraction technique. Aliquots (200 μl) of plasma were spiked with 25 μl of IS, 25 μl of methanol and 20 μl of 1% acetic acid by vortexing for 30 s, then the mixture were extracted with 800 μl of methyl tert-butyl ether by vortex-mixing for 3 min. After centrifugation at 13,000 rpm, 4 °C for 8 min, the organic layer was quantitatively transferred to a clean centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 50 μl of methanol and vortex-mixed for 3 min, then sonicated for 3 min followed by being centrifuged at 13,000 rpm for 5 min; finally, an aliquot of 5 μl of the solution was injected into the LC-MS/MS system for analysis.

2.7. *Method validation*

The method was validated according to the currently accepted US-FDA Bioanalytical Method Validation Guidance and European Medicines Agency Guideline on Bioanalytical Method Validation with respect to selectivity, matrix effect, lower limit of quantification, calibration curve, accuracy and precision, recovery and stability [17].

Selectivity was assessed by comparing chromatograms obtained from six different batches of blank plasma of six rats, with those obtained from corresponding standard plasma samples spiked with analytes, IS, and a plasma sample after oral administration of Alismatis Rhizoma extract. The matrix effect was measured at three QC levels by comparing the peak response of blank plasma extracts spiked with the analytes (A) with that of pure standard solution containing equivalent amounts of the compounds (B). The ratio (A/B × 100)% was used to evaluate the matrix effect.

The linearity of the assay was assessed by analyzing the calibration curves of alisol A (5.00, 10.00, 20.00, 100.0, 500.0, 1000 and 2500 ng/ml) and alisol B 23-acetate (5.00, 10.00, 50.00, 250.0, 500.0, 1250, 2500 ng/ml) in plasma using least-squares linear regression of the analyte-to-IS peak-area ratios versus the nominal concentration of the calibration standard with a weighed factor ($1/x^2$). The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with acceptable accuracy within ±20% and precision below 20%.

Quality control sample (QC samples) at low, medium and high concentrations (at 25.00, 300.0, and 4000 ng/ml for alisol A and 12.50, 150.0, and 2000 ng/ml for alisol B 23-acetate) were analyzed on three separate occasions with six replicates at each concentration per occasion to determine accuracy and precision. Precision was expressed as the relative standard deviation (RSD, %) and accuracy was defined as relative error (RE, %). The recoveries of alisol A and alisol B 23-acetate were determined at three QC levels with six replicates by comparing the peak response from extracted samples (C) with those from post-extracted blank plasma samples spiked with the analytes (A) at the same concentration. The ratio (C/A × 100)% was used to evaluate the recovery. Recovery of the IS was determined in the same way at the concentration of 5.00 ng/ml.

2.8. *Stability testing*

Stability studies in plasma samples were also conducted at three QC levels under several different storage conditions: at room temperature for 24 h, at −20 °C for at least 7 days, after three freeze-thaw cycles, and 8 h after prepare at 4 °C during the analysis process.

2.9. Pharmacokinetic studies

All of the six rats were fasted for 12 h, with free access to water prior to the experiments. After giving 25 g/kg of Alismatis Rhizoma extract (calculated by crude drug) to each rat orally, the blood samples (0.3 ml) obtained from the oculi chorioideae vein of rats at 0, 0.17, 0.33, 0.75, 1, 1.5, 2, 4, 6, 7, 9, 12, 24, and 36 h were placed in heparinized tubes and separated by centrifugation at 4000 rpm for 5 min. The separated plasma samples were finally stored at −20 °C until analysis.

The pharmacokinetic analysis of the two analytes was performed by a non-compartmental approach using the DAS 2.1 software package (Chinese Pharmacological Society) to calculate AUC and $T_{1/2}$. C_{max} and T_{max} were obtained directly from plasma concentration–time curve.

3. Results and discussion

3.1. Optimization of mass and chromatographic conditions

Under the chosen electrospray ionization conditions, greater sensitivity was achieved for alisol A, alisol B 23-acetate and diazepam in the positive mode than the negative mode. The full-scan product ion spectrums of analytes by infusing the standard solutions into the mass spectrometer are shown as Fig. 2. Some parameters such as gas1, gas2, curtain gas, IS Voltage, DP, CE and CXP were optimized. The other parameters were adopted for the recommended value of the instrument.

The mobile phase played an important role in improving peak shape, detection sensitivity and shortening analysis time. The methanol–water and acetonitrile–water systems were investigated. Methanol was used as the organic modifier because it provided higher responses and lower background noise than acetonitrile. With addition of acetic acid to the mobile phase, peak symmetry for both alisol A, alisol B 23-acetate and diazepam was improved. Then 0.05%, 0.1% and 0.2% acetic acid were investigated. 0.1% and 0.2% acetic acid were better than 0.05% acetic acid in the peak symmetry of the analytes, and there was no significant difference between 0.1% and 0.2% acetic acid. The gradient program in the work provided good resolution and shorter analysis time. Finally, methanol–0.1% acetic acid with appropriate gradient program was adopted as the mobile phase for sufficient ionization response, good peak symmetry, and proper retention time for the analytes and IS.

3.2. Sample preparation

Liquid–liquid extraction was investigated in our study. The extractant and pH of buffer play a vital role in liquid–liquid extraction (LLE) method. Among the organic solvents such as methyl tert-butyl ether, ethyl acetate and diethyl ether, methyl tert-butyl ether gave the highest recovery for both alisol A and alisol B 23-acetate. 800 μl, 1000 μl, 1600 μl and 2000 μl of methyl tert-butyl ether was added to 200 μl of plasma samples respectively. With the amount of methyl tert-butyl ether increasing, there was no significant difference in extraction recovery, so 800 μl was chosen. Moreover addition of acetic acid in the extraction procedure increased the recovery of the analytes, whereas there was no obvious increase above 20 μl 1% acetic acid. Finally, the addition of 20 μl of 1% acetic acid and 800 μl of methyl tert-butyl ether into 200 μl of plasma was determined because it provided high recoveries for both the analytes and the IS.

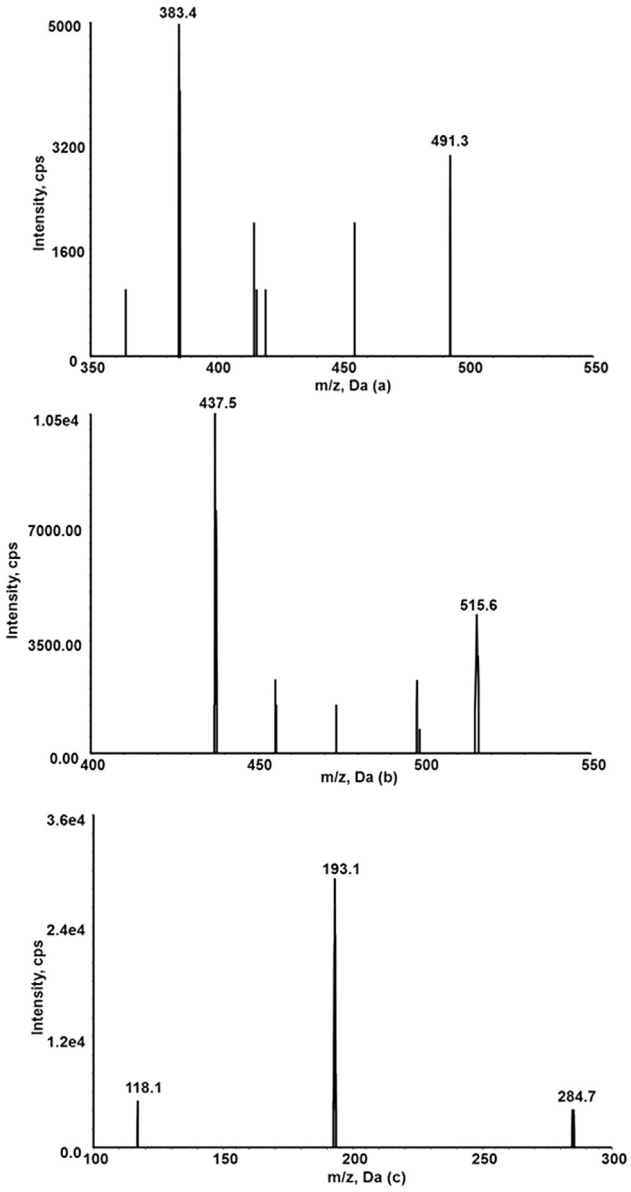

Fig. 2 – The product ion mass spectra of alisol A (a) (*m/z* 491.5/383.4), alisol B 23-acetate (b) (*m/z* 515.6/437.5), and diazepam (IS) (c) (*m/z* 284.7/193.1).

3.3. Method validation

3.3.1. Selectivity and matrix effect

No significant interference or ion suppression was observed at the retention times of alisol A (3.00 min), alisol B 23-acetate (3.17 min), and IS (1.42 min). With the help of UFLC system, high selectivity of MRM mode and appropriate mobile phase, typical chromatograms obtained from blank plasma, blank plasma spiked with analytes and IS, and a rat plasma sample

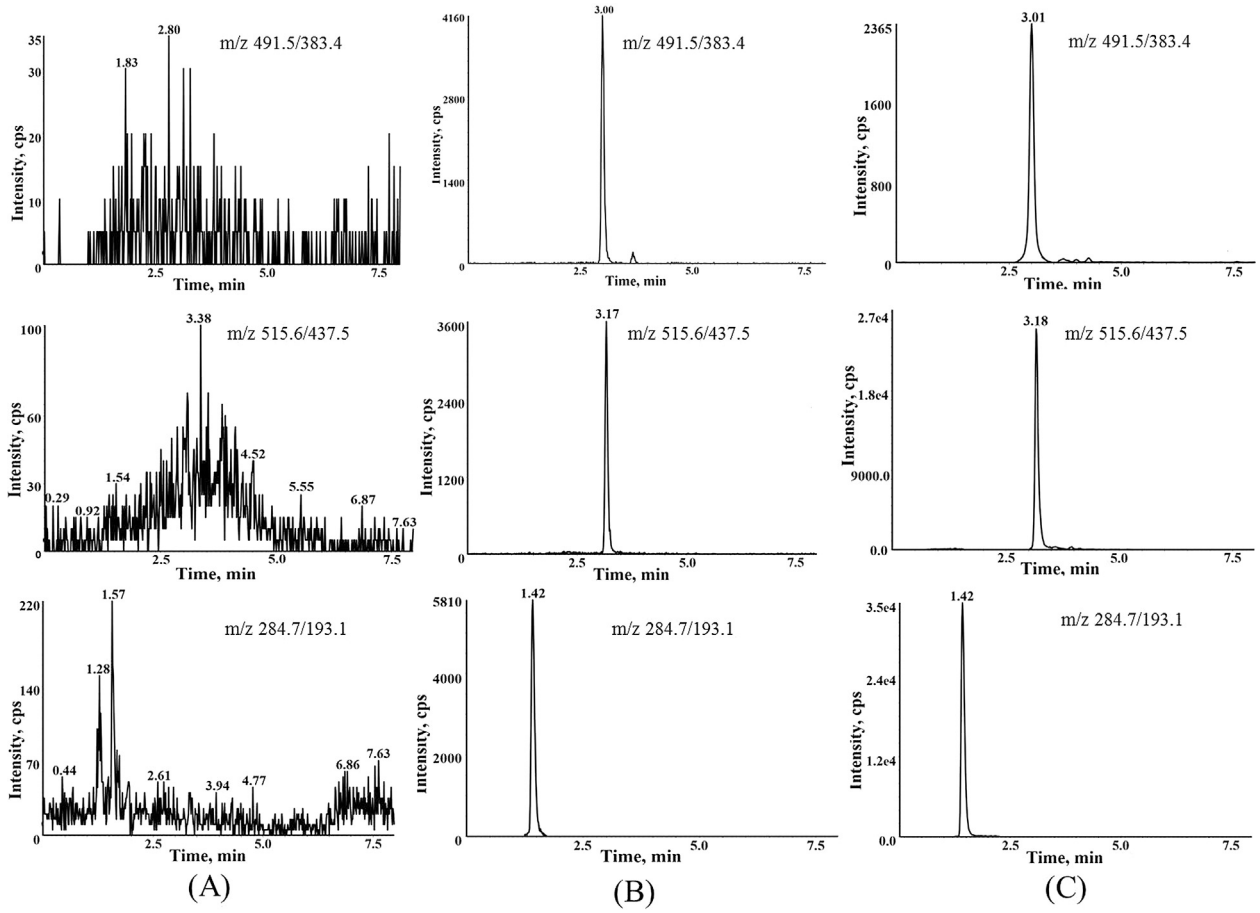

Fig. 3 – A, B and C show the MRM chromatograms of a blank plasma sample, blank plasma spiked with analytes and IS (LLOQ) and plasma sample 1 h after oral administration of Alismatis Rhizoma extract, respectively.

obtained after oral administration of Alismatis Rhizoma are shown in Fig. 3.

For matrix effect, all the ratios listed in Table 2 were within the range 89.7–103.3%, indicating that no significant matrix effect was observed for alisol A and alisol B 23-acetate. The mean matrix effect for the IS was 97.7% at a concentration of 5 ng/ml.

3.3.2. *Linearity and LLOQ*

The linearity was evaluated on three separate occasions with two sets calibration curves per occasion. The calibration curves showed the linearity was good over the concentration range of 5.00–2500 ng/ml for alisol A and 5.00–2500 ng/ml for alisol B 23-acetate. Typical linear regression equations for the calibration curves were as follows: $y = 0.0019x + 0.0012$ ($r = 0.9968$) for alisol A; $y = 0.0034x + 0.0022$ ($r = 0.9977$) for alisol B 23-acetate, where y is the peak area ratio of the analytes to the IS, and x is the plasma concentration of analytes.

The LLOQ of analytes in plasma were 5.00 ng/ml with RSD 6.6% and RE 4.7% for alisol A, 5.0 ng/ml with RSD 7.8% and RE 9.0% for alisol B 23-acetate, for which the signal-to-noise ratios were both above 10. The data above demonstrated that this method was sensitive enough for the pharmacokinetic study of alisol A, alisol B 23-acetate in vivo.

Table 2 – Summary of accuracy, precision, extraction recovery and matrix effect of alisol A and alisol B 23-acetate in rat plasma ($n = 6$).

Analytes	Concentration spiked (ng/ml)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (RE, %)	Matrix effect (%, RSD)	Extraction recovery (%, mean ± SD)
Alisol A	25.00	2.9	6.8	2.5	99.5 ± 3.7	61.80 ± 0.50
	300.0	3.3	7.4	−6.6	94.1 ± 5.5	62.90 ± 1.20
	4000	4.6	3.5	6.3	96.3 ± 4.6	66.80 ± 4.10
Alisol B 23-acetate	12.50	6.9	4.3	−1.4	89.7 ± 3.0	75.40 ± 5.60
	150.0	2.7	7.1	8.1	93.8 ± 5.5	65.10 ± 4.00
	2000	5.9	8.8	6.0	103.3 ± 3.6	63.50 ± 4.50

Table 3 – Stability of alisol A, alisol B 23-acetate and diazepam in rat plasma at different conditions determined by UFLC-MS/MS ($n = 3$).

Analytes	Concentration (ng/ml)	24 h, room temperature		3 freeze-thaw cycles		30 days, −20 °C		8 h, 4 °C	
		RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)
Alisol A	25.00	3.9	6.4	−1.0	3.6	−6.4	4.8	7.2	4.6
	300.0	−2.3	7.5	9.1	7.3	4.5	2.4	9.6	4.0
	4000	−11.4	7.8	6.2	8.3	5.1	7.6	−5.6	2.7
Alisol B 23-acetate	12.50	0.3	6.1	4.2	4.0	6.9	4.5	3.4	1.5
	150.0	5.7	2.1	−3.4	1.9	−6.1	3.7	−7.0	6.8
	2000	−4.6	1.1	−2.0	1.8	3.1	4.2	10.2	5.7

3.3.3. *Precision, accuracy and extraction recovery*

Intra-day precision, inter-day precision, and accuracy for alisol A and alisol B 23-acetate are summarized in Table 2. All results for the samples tested were within the acceptable criteria of ±15%.

The extraction efficiencies at three concentrations (low, medium, high) ranged from 61.80 to 66.80% for alisol A and from 63.50 to 75.40% for alisol B 23-acetate (Table 2), which indicated that recoveries of alisol A and alisol B 23-acetate were consistent, and reproducible at different concentrations. The mean extraction recovery of the IS was 94.5%.

3.3.4. *Stability*

The stability of alisol A and alisol B 23-acetate in rat plasma under different conditions is summarized in Table 3. The results indicate that alisol A and alisol B 23-acetate were all stable in plasma at room temperature for 24 h, at −20 °C for at least 7 days, after three freeze and thaw cycles, and 8 h after prepared at 4 °C during the analysis process.

3.4. *Pharmacokinetic studies*

The developed method has been successfully applied to a pharmacokinetic study of alisol A and alisol B 23-acetate in rat plasma after oral administration of alcohol extract of Alismatis Rhizoma with a concentration of 2 g/ml (containing 1.4 mg/g alisol A and 0.6 mg/g alisol B 23-acetate). The concentration–time curves (mean ± SD) for alisol A and alisol B 23-acetate are presented in Fig. 4 and the corresponding pharmacokinetic data are listed in Table 4. The $T_{1/2}$ and T_{max} in this paper have differences with the existing reports [14,15], this might be caused by the extraction method, enterohepatic circulation and different LLOQ and regions of Alisma orientale (Sam.) Juz.

After oral administration of alcohol extract of Alismatis Rhizoma, alisol A and alisol B 23-acetate in rat plasma exhibit similar pharmacokinetic behavior. Both of the concentration–time curves of alisol A and alisol B 23-acetate showed double-peak phenomenon [14,15]. The concentration–time curves showed that Alismatis Rhizoma was rapidly absorbed in blood, then the concentration decreased slightly and appeared a small peak. Following the first peak, the concentration increased sharply within a short time, subsequently, and the curves showed the second peaks which were much higher than the previous one. The double-peak phenomenon happens very often in the pharmacokinetic study of TCM [18–21]. In this study, the phenomenon might be caused by the following reasons: As for alisol A, simulated gastric fluid has a low pH value of 1.4 approximately. Alisol B 23-acetate contains epoxy heterocyclic which is extremely unstable, and it can start the ring-opening reaction to generate alisol A 24-acetate by rearrangement in acidic condition readily. Meanwhile, Alisol A can be obtained by deacetylation of alisol A 24-acetate in acidic condition [22]. This might be the reason of double peak of alisol A. As for alisol B-23 acetate, the double peak might result from the inter-transformed of alisol B or the effect of enterohepatic circulation [14,22–25]. And further studies need to be investigated.

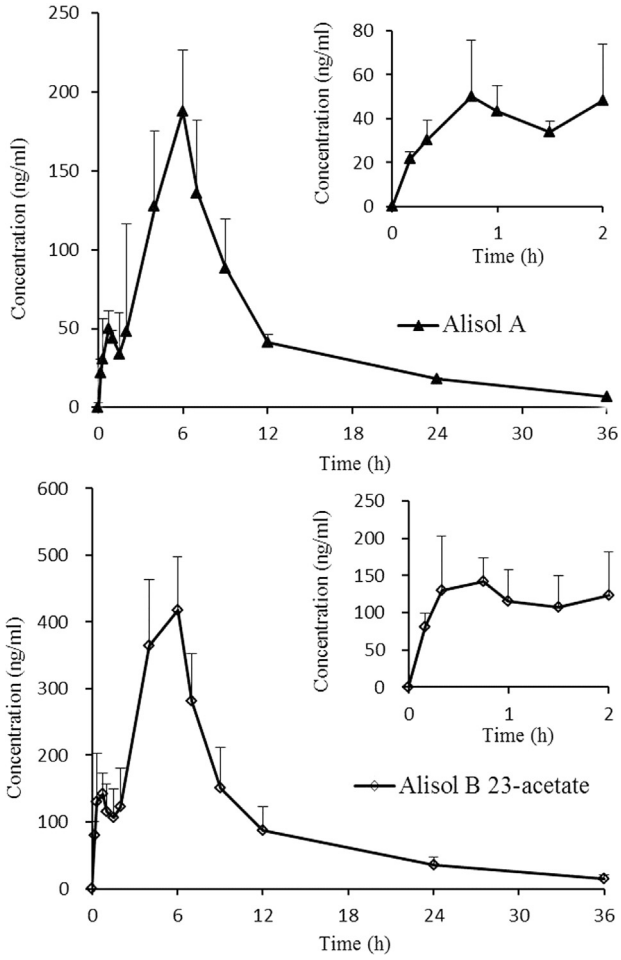

Fig. 4 – The concentration–time curves of alisol A (a) and alisol B 23-acetate (b) in rat plasma after oral administration of Alismatis Rhizoma extract.

Table 4 – Non-compartmental pharmacokinetic parameters for alisol A and alisol B 23-acetate in rat plasma after oral administration of Rhizoma Alismatis extract (mean ± SD, $n = 6$).

Analytes	$AUC_{(0-t)}$(μg h/l)	$AUC_{(0-\infty)}$(μg h/l)	C_{max}(μg/l)	$T_{1/2}$(h)	T_{max}(h)
Alisol A	1549 ± 502	1739 ± 481	192.2 ± 40.9	9.28 ± 2.30	5.67 ± 0.82
Alisol B 23-acetate	3681 ± 679	3866 ± 700	427.7 ± 86.2	8.43 ± 1.97	5.67 ± 0.82

4. Conclusion

In summary, a sensitive and efficient UFLC-MS/MS method for simultaneous determination of alisol A and alisol B 23-acetate in rat plasma has been developed and validated for the first time in the work discussed in this paper. It was applied successfully for the pharmacokinetic studies of oral administration of alcohol extract of Alismatis Rhizoma in rat plasma.

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