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ORIGINAL ARTICLE

Study on Pharmacokinetics and Tissues Distribution of Neomangiferin, Mangiferin, Timosaponin BII, Timosaponin BIII, and Timosaponin AIII after Oral Administration of *Anemarrhenae Rhizoma* Extract in Rats

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ABSTRACT

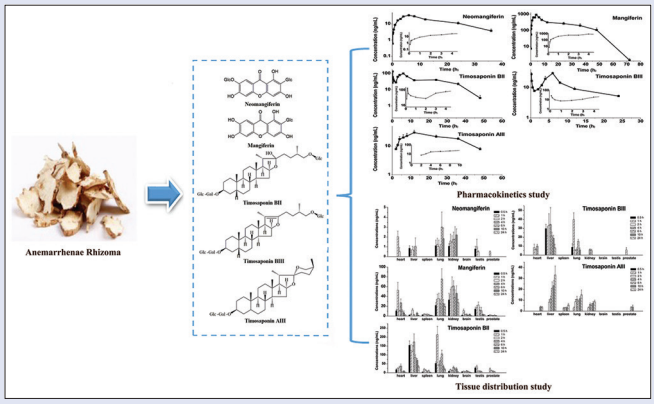
Background: *Anemarrhenae rhizoma* (AR) is widely used for the treatment of febrile diseases, cough, and diabetes in traditional Chinese medicines. AR mainly contains flavonoids and steroidal saponins, such as neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII, which showed various biological activities. **Objective:** The main objective of the study is to establish an ultra-high-performance liquid chromatography-tandem mass spectrometry (MS/MS) method to determine the concentrations of five bioactive constituents in rats' plasma and various tissues. **Materials and Methods:** The analytes were separated on a C_{18} reversed-phase column. A triple-quadrupole MS/MS equipped with an electrospray ionization source was used as a detector. The main pharmacokinetic parameters were estimated with Drug and Statistics 2.0 Software Package. **Results:** Neomangiferin and mangiferin exhibit poor oral absorption and slow clearance from the body. Timosaponin BII and timosaponin BIII could be quickly absorbed into the blood circulation and showed double plasma concentration peaks. Timosaponin AIII exhibited a single peak in the plasma concentration-time plot and pharmacokinetic parameters of timosaponin AIII indicated slower absorption, longer body residence time, and slower elimination than timosaponin BII and timosaponin BIII. The five analytes were widely distributed to most of the tissues. Neomangiferin and mangiferin exhibited the maximum concentration in the lung at 6 h after oral administration, the highest levels of timosaponin BII and timosaponin BIII were also observed in the lung at 1 h after oral administration, and the maximum concentration of timosaponin AIII was observed in the liver. **Conclusion:** The findings of the present study might be helpful to better understand the pharmacokinetics and distribution of AR bioactive constituents *in vivo*, which would facilitate the clinical application of AR.

Key words: *Anemarrhenae rhizoma*, herbal medicine, pharmacokinetics, tissue distribution, traditional Chinese medicine, ultra-high-performance liquid chromatography-tandem mass spectrometry

SUMMARY

- Contents of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII in rats' plasma and various tissues were determined by UPLC-MS/MS
- Neomangiferin and mangiferin exhibit poor oral absorption. Timosaponin BII and timosaponin BIII could be quickly absorbed into the blood circulation and timosaponin AIII showed slower absorption than timosaponin BII and timosaponin BIII
- The five analytes were widely distributed to most of the tissues.

Abbreviations used: HMs: Herbal medicines; ADME: Absorption, Distribution, Metabolism, and Excretion; AR: *Anemarrhenae rhizoma*; UHPLC-MS/MS: Ultra-high-performance liquid chromatography-tandem mass spectrometry; IS: Internal standard; SD: Sprague–Dawley rats; QC: Quality control; ESI: Electrospray ionization; MRM: Multiple reaction monitoring; LLOQ: Lower limit of quantification; RSD: Relative standard deviation; C_{max}: Peak concentration; T_{max}: The time to reach C_{max}; $t_{1/2}$: Half-life; AUC_{0-t}: Area under the curve of 0-t time; $AUC_{0-\infty}$: Area under the plasma concentration-time curve extrapolated to infinity; MRT_{0-t}: Total body mean residence time; *CLz/F*: Apparent oral clearance; *Vz/F*: Apparent volume of distribution; TCM: Traditional Chinese medicine.



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INTRODUCTION

Herbal medicines (HMs) are gaining increasing interest worldwide owing to their promising efficacy and minimal side effects. Although they are well-known and widely used in some Eastern countries, HMs are considered complementary and alternative medicines in Western countries because of their unknown bioactive components and mechanisms of action, as well as the lack of safety data.[1] Pharmacokinetic (Absorption, Distribution, Metabolism, and Excretion [ADME]) studies are essential for modern drug development because they are crucial to the final clinical success of a drug candidate.[2] However, data on the ADME and pharmacokinetic properties of most of HMs are lacking or scant. Therefore, it is necessary to investigate the *in vivo* pharmacokinetic and ADME properties of HMs to ensure their clinical efficacy and safety.

Anemarrhenae rhizoma (AR), the dried rhizome of *Anemarrhena asphodeloides* Bge. (Liliaceae), has been used in traditional Chinese medicine (TCM) for thousands of years for the treatment of febrile diseases, fever, cough, and diabetes.[3] Phytochemical investigations have revealed that compounds isolated from AR include steroidal saponins, flavonoids, lignans, and fatty acids.[4] Recent pharmacological studies showed that constituents of AR, such as mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII, exhibited various biological activities, including antidiabetic,[5] antidementia,[6] antidepressant,[7] anti-inflammatory,[8] and antioxidant activities.[9] Thus, they were considered the principal components responsible for the main pharmacological activities of AR; in addition, timosaponin BII and mangiferin were selected as the marker components for quality control of AR in the Chinese pharmacopeia.[10]

To understand the underlying mechanisms of the beneficial effects of AR in preclinical studies and clinical practice, extensive studies were conducted to analyze its multiple active ingredients in biological fluids, as a single compound, in the mixture extracted from AR, or in AR-constituted formulations by using high-performance liquid chromatography (HPLC)-ultraviolet,[11,12] HPLC-mass spectrometry (MS),[13,14] and HPLC-tandem mass spectrometry (HPLC-MS/MS) methods.[15-21] However, most previous studies focused on the comparison of the plasma pharmacokinetics after administration of different botanical extracts or using different animal models. The absorption[22,23] and metabolism[24-27] of the pure compounds were also extensively studied. No study has investigated the simultaneous pharmacokinetics and tissue distribution of multiple bioactive components after oral administration of AR extract although the effects of drugs on target organs are related to their concentrations in respective tissues. In this study, a rapid ultra-HPLC-MS/MS (UHPLC-MS/MS) method was developed for the determination of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII in rat plasma and tissues. The method was validated and successfully applied to study the pharmacokinetics and tissue distribution of the five compounds after oral administration of AR extract.

MATERIALS AND METHODS

Chemicals and reagents

Authentic standards of mangiferin, timosaponin BII, and ginsenoside-Rg1 (internal standard [IS]) (all with purity >98%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), whereas those of neomangiferin, timosaponin BIII, timosaponin AIII, and liquiritigenin (IS) (all with purity >98%) were purchased from Chengdu Must Bio-technology Co., Ltd., (Chengdu, China). Acetonitrile (LC/MS grade) was purchased from E. Merck (Merck, Darmstadt, Germany). Formic acid (purity, 99%; UHPLC grade) was purchased from Anaqua Chemical Supply (ACS, Houston, USA). Purified water was obtained using a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). All other reagents were of analytical grade.

AR samples were collected from Anguo, Hebei Province, China. The voucher specimens were identified by Professor Tu-Lin Lu at the Nanjing University of Chinese Medicine. Voucher specimens (No. 20171020) were deposited at the College of Pharmacy of Nanjing University of Chinese Medicine. AR (100 g) was extracted with 70% ethanol (1:10, w/v) at 75°C twice for 2 and 1.5 h, separately. The solution obtained by filtration was concentrated to approximately 100 mL (equivalent to 1 g/mL raw materials) and stored at 4°C until use the next day. The contents of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII in AR extract were 3.5, 36.5, 66.7, 15, and 16.7 mg/g, respectively.

Animals

Male Sprague–Dawley (SD) rats, weighing 220–250 g, were supplied by the Experimental Animal Center of Nanjing University of Chinese Medicine. They were kept in environmentally controlled breeding room for 7 days prior to the experiments and fed with standard laboratory food. Rats were fasted for 12 h before oral gavage, and water was provided *ad libitum*. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council [U.S.]), Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011), and related Ethical Regulations of Nanjing University of Chinese Medicine.

Preparation of standard solutions, calibration, and quality control samples

Stock solutions of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, timosaponin AIII, and IS were prepared separately in acetonitrile-water (70:30, v/v) to yield a concentration of 2 mg/mL. A series of working solutions were prepared by mixing and diluting the appropriate volume of the stock solution of each compound with acetonitrile-water (70:30, v/v). The IS working solution of ginsenoside-Rg1 (50 ng/mL) and liquiritigenin (60 ng/mL) was prepared in a similar manner. All solutions were stored at 4°C.

Calibration work solutions and QC samples were prepared by adding the diluted stock solutions to blank rat plasma and various tissue homogenates (10:90, v/v). The concentrations of the final plasma-derived working solutions of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII ranged from 0.5–120 ng/mL, 0.5–1600 ng/mL, 2–600 ng/mL, 5–120 ng/mL, and 3–60 ng/mL, respectively. The final standard concentrations of the tissue samples ranged from 0.5–24 ng/mL, 0.5–500 ng/mL, 2–300 ng/mL, 5–100 ng/mL, and 3–60 ng/mL for neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII, respectively. For method validation, QC plasma samples of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII were prepared separately at three concentrations (1, 20 and 100 ng/mL; 1, 300 and 1500 ng/mL; 5, 80 and 400 ng/mL; 10, 20 and 100 ng/mL; and 5, 10 and 50 ng/mL, respectively). For tissue homogenate samples of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII, the three concentrations prepared were 1, 5 and 20 ng/mL; 1, 40 and 400 ng/mL; 5, 25 and 250 ng/mL; 10, 20 and 100 ng/mL; and 5, 10 and 50 ng/mL, respectively.

Sample preparation

Biological samples were stored at −20°C and thawed at room temperature before use. IS solution (5 μL, 50 ng/mL ginsenoside-Rg1 and 60 ng/mL

liquiritigenin) and 195 μL of acetonitrile were added to 100 μL of the plasma or tissue homogenate. The mixture was vortexed for 3 min and centrifuged at 12,000 ×*g* for 5 min. The supernatant was transferred into a 1.5-mL centrifuge tube and evaporated to dryness under a flow of nitrogen gas. The residue was redissolved in 100 μL of the mobile phase, vortexed for 1 min, and centrifuged at 12,000 rpm for 5 min. Then, the supernatants were transferred to vials and 5 μL of each was injected into the UHPLC-MS/MS system.

Instrumentation and ultra-high-performance liquid chromatography-tandem mass spectrometry conditions

The UHPLC system (Shimadzu, Kyoto, Japan) consisted of a LC-30AD binary pump, autosampler (Model SIL-30SD), online degasser (DGU-20A5R), and column temperature controller compartment (CTO-30A). Separation of the analytes was performed on a Waters BEH-C_{18} column (100 mm × 2.1 mm, 1.7 μm). The mobile phase was composed of 0.1% formic acid aqueous solution (A)-acetonitrile (B) with a gradient elution program (0–0.5 min, 5%–23% B; 0.5–2 min, 23%–45% B; 2–5 min, 45%–80% B; 5–5.5 min, 80% B; 5.5–5.8 min, 80%–90% B; 5.8–6 min, 95% B; 6–6.5 min, 95%–5% B; and 6.5–7.5 min, 5% B). The flow rate was set at 0.3 mL/min and the injection volume was 5 μL.

Mass spectrometric detection was carried out using a triple quadrupole 5500 instrument (AB Sciex, Concord, Ontario, USA) with an electrospray ionization (ESI) source operated in the negative-ion mode. The scan mode was set at multiple reaction monitoring (MRM), and the selected monitor ions were m/z 583.2/301.1 for neomangiferin, m/z 421.1/331.1 for mangiferin, m/z 919.5/757.6 for timosaponin BII, m/z 947.5/901.5 for timosaponin BIII, m/z 785.5/739.5 for timosaponin AIII, m/z 845.6/799.5 for ginsenoside-Rg1, and m/z 255.1/119 for liquiritigenin. The chemical structure and mass spectrum of the analytes and IS are shown in Figure 1. The main working parameters for the ion source were as follows: ion spray voltage, 4.5 kV; ion source temperature, 500°C; curtain gas, 35 psi; ion source gas 1, 35 psi; and ion source gas 2, 35 psi. Analyte concentrations were determined using the MultiQuant software 2.1.

Method validation

Assay validation was performed according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidelines (FDA, Center for Drug Evaluation and Research, 2001).

Specificity

Specificity was evaluated by analyzing six different sources of biological samples (plasma various tissue homogenates), blank biological matrix samples spiked with neomangiferin, mangiferin, timosaponin BII,

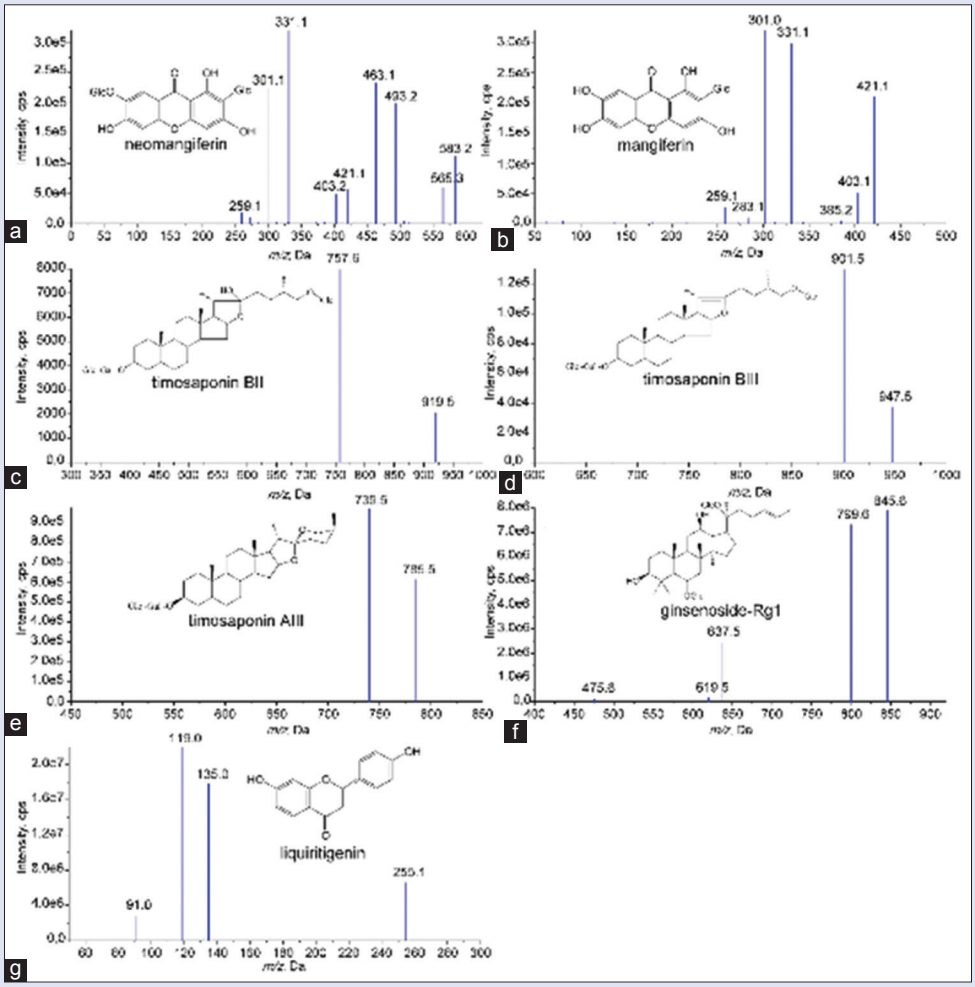

Figure 1: Molecular structures and tandem mass spectrometry (MS/MS) spectra of neomangiferin (a), mangiferin (b), timosaponin BII (c), timosaponin BIII (d), timosaponin AIII (e), ginsenoside-Rg1 (f, IS), and liquiritigenin (g, IS)

timosaponin BIII, timosaponin, and IS, and actual biosamples obtained after oral administration of AR extract. Effects of endogenous substances present in rat plasma and tissues were investigated. Cross-interference between analytes and IS was established by injecting biological samples containing five analytes or IS separately to see if the mixture solution of analytes and IS is acceptable.

Linearity and lowest limit of quantification

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of the analytes to IS versus the nominal concentration (x) of the analytes with weighted ($1/x^2$) least-squares linear regression. The lower limit of quantification (LLOQ) was determined as the lowest concentration of the calibration curve (S/N >12). The procedure was repeated five times to ensure that the precision and accuracy were <20%.

Intra- and inter-day precision and accuracy

Accuracy and precision of the method were assessed by analyzing the QC samples at low, medium, and high concentrations and calculating their concentrations using the calibration curve constructed each day. Intraday precision of the method was estimated by determining six replicates of each QC sample at each concentration level, whereas interday precision was determined by analyzing the QC samples over three consecutive days. The relative standard deviation (RSD) values were used to evaluate the precision (acceptable, within 15%). Accuracy was assessed by comparing the measured concentration with its true value (accepted error, within ±15% for all QC samples).

Extraction recovery and matrix effect

The extraction recovery was evaluated by comparing the mean peak areas of the processed QC samples at three levels with those of the corresponding standard solutions spiked with blank biological matrix. The matrix effect was assessed in a similar fashion. Analytes of all the five compounds were added to the extract of precipitated blank plasma and tissue homogenates to prepare three concentration levels. Their peak areas were compared with those obtained by adding the same concentrations of the analytes into acetonitrile. The extraction recovery and matrix effect of IS were measured at the working concentration in the same manner. The matrix effect was considered negligible if the values were below ±15%.

Stability

The short-term stability was determined by exposing the samples to room temperature for 4 h, whereas the long-term stability was evaluated by keeping the samples at the storage temperature (−20°C) for 30 days. The freezing and thawing stability was assessed after three freeze-thaw cycles. In addition, the postpreparative stability was investigated in an autosampler for 24 h after preparation. QC samples were considered stable if the average deviation was within ± 15% of the actual value.

Pharmacokinetic study

The validated method was applied to study the pharmacokinetics of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII after oral administration of AR extract in rats. Six male SD rats were housed individually under normal conditions. They were fasted overnight before the experiment with free access to water. Then, 2 mL/kg AR extract (approximately 7 mg/kg neomangiferin, 73 mg/kg mangiferin, 133.4 mg/kg timosaponin BII, 30 mg/kg timosaponin BIII, and 33.4 mg/kg timosaponin AIII) was administered to the rats by gastric perfusion. Blood samples (~0.3 mL) were collected into 1.5-mL heparinized polythene tubes at 0 (before administration), 0.083, 0.167, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, and 72 h after dosing. They were immediately centrifuged at 4000 rpm for 10 min and the plasma was stored at −20°C until analysis.

Tissue distribution study

Male SD rats were randomly divided into six groups (6 animals each), in the tissue distribution study. After administration of AR extract as described above, tissues, including the heart, liver, spleen, lung, kidney, brain, prostate, and testis, were isolated at 0.167, 2, 4, 6, 10, and 24 h, respectively, after administration. Tissue samples were rapidly weighed, rinsed with ice-cold normal saline solution to remove the blood or content, and blotted on filter paper. Each weighed tissue sample was cut into small slices and homogenized in ice-cold physiological saline solution. The obtained tissue homogenates were immediately stored at −80°C until analysis.

Data analysis

The pharmacokinetic parameters, including the area under the blood concentration-time curve from time zero to the last measured concentration (AUC_{0-t}), the area under the plasma concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), total body mean residence time (MRT), and elimination half-life ($t_{1/2}$), were calculated using noncompartmental methods by Drug and Statistics software (version 2.0 Mathematical Pharmacology Professional Committee of China). The maximum plasma concentration (C_{max}) and time to reach the maximum concentration (t_{max}) were directly obtained from the experimental data. Data were presented as the means ± standard error (SE).

RESULTS AND DISCUSSION

Optimization of mass spectrometric and chromatographic conditions

Under the optimized ESI conditions, all analytes and IS exhibited higher sensitivity in the negative-ion mode, compared to that in the positive-ion mode. MRM was used for quantitative analysis of neomangiferin (m/z 583.2 → m/z 301.1), mangiferin (m/z 421.1 → m/z 331.1), liquiritigenin (IS, m/z 255.1 → m/z 119), timosaponin BII (m/z 919.5 → m/z 757.6), timosaponin BIII (m/z 947.5 → m/z 901.5), timosaponin AIII (m/z 785.5 → m/z 739.5), and ginsenoside Rg1 (IS, m/z 845.6 → m/z 799.6). Other parameters, such as ion source temperature, desolvation gas flow, nebulizer gas pressure, and capillary voltage, were also optimized to improve the responses of all compounds. Chromatographic conditions, including the type of reversed-phase chromatographic column, mobile phase composition, choice of additives, column temperature, and mobile phase flow rate, were optimized to achieve short retention time, symmetric peak shape, and satisfactory ionization. In the LC system, 0.1% formic acid was added to the mobile phase because an acidified mobile phase could minimize peak tailing, improve the resolution, and facilitate ionization.

Selection of internal standard

It is necessary to use IS to get high accuracy and precision when LC is equipped with a mass spectrometer as a detector. It is difficult to use one IS for simultaneous quantification of multiple analytes of different types; therefore, multiple IS are required. Liquiritigenin and ginsenoside Rg1 were selected as the IS of xanthone and steroid saponin because of the resemblance of their chromatographic behavior, extraction efficiency, and ionization properties to that of the analytes.

Selection of sample preparation procedures

Protein precipitation method using acetonitrile as the reagent was selected to pretreatment biological samples, which could achieve give

good and consistent recovery for all the analytes in our study as reported previously.[16,21] Different dilution ratios (2, 3, and 5 times of plasma or tissue homogenate) were compared, and it was found that precipitation with two times acetonitrile produced optimum recovery and little matrix effects for the analytes.

Method validation

Specificity

Under optimized conditions, no significant interfering peaks were observed in the chromatograms of all tested matrices at the retention times of the analytes and IS. Moreover, we found no evidence of cross-interference among the analytes and IS. Thus, the method exhibited good specificity. The typical MRM chromatograms of all components in the plasma and liver tissue are shown in Figure 2.

Linearity of calibration curve and lower limit of quantification

The calibration curves of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII in all matrices showed good linearity over the concentration ranges tested (all correlation coefficients >0.9945). The LLOQs of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII were 0.5, 0.5, 2, 5, and 3 ng/mL, respectively, which indicated that the method was sensitive for the quantitative analysis of the five analytes. At the LLOQ, S/Ns of each analyte in all matrices were all above 15. The intraday precision (RSD, %) of five analytes at LLOQs did not exceed 16.42% and the accuracy value (RE, %) did not exceed ± 13.63%. The regression equations, linear ranges, correlation coefficients (r), and LLOQs are shown in Table 1.

Intra- and inter-day precision and accuracy

The accuracy, intra-, and inter-day precision of the five analytes in the plasma and liver samples are shown in Table 2. The data of other tissues are shown in Supplementary material [Table S1]. At each concentration, the precision RSD did not exceed 12.73%, and the accuracy RE value did not exceed ± 10.90% for all the analytes, indicating that this method was accurate, reproducible, and reliable.

Extraction recovery and matrix effect

The extraction recovery and matrix effects of each QC sample concentration and IS in plasma and liver samples are listed in Table 3. The data of other tissues are shown in Supplementary material [Table S2]. Results showed that the extraction recoveries were >79.43% at different concentrations in various samples. For the matrix effects, all ratios ranged from 95.48%–108.59% to 86.25%–109.99% for the plasma and tissue samples, respectively, which indicated that the matrix did not significantly affect the analytes and IS.

Stability

The stability results (RE) of the five analytes in the plasma and liver samples under different conditions are summarized in Table 4. The

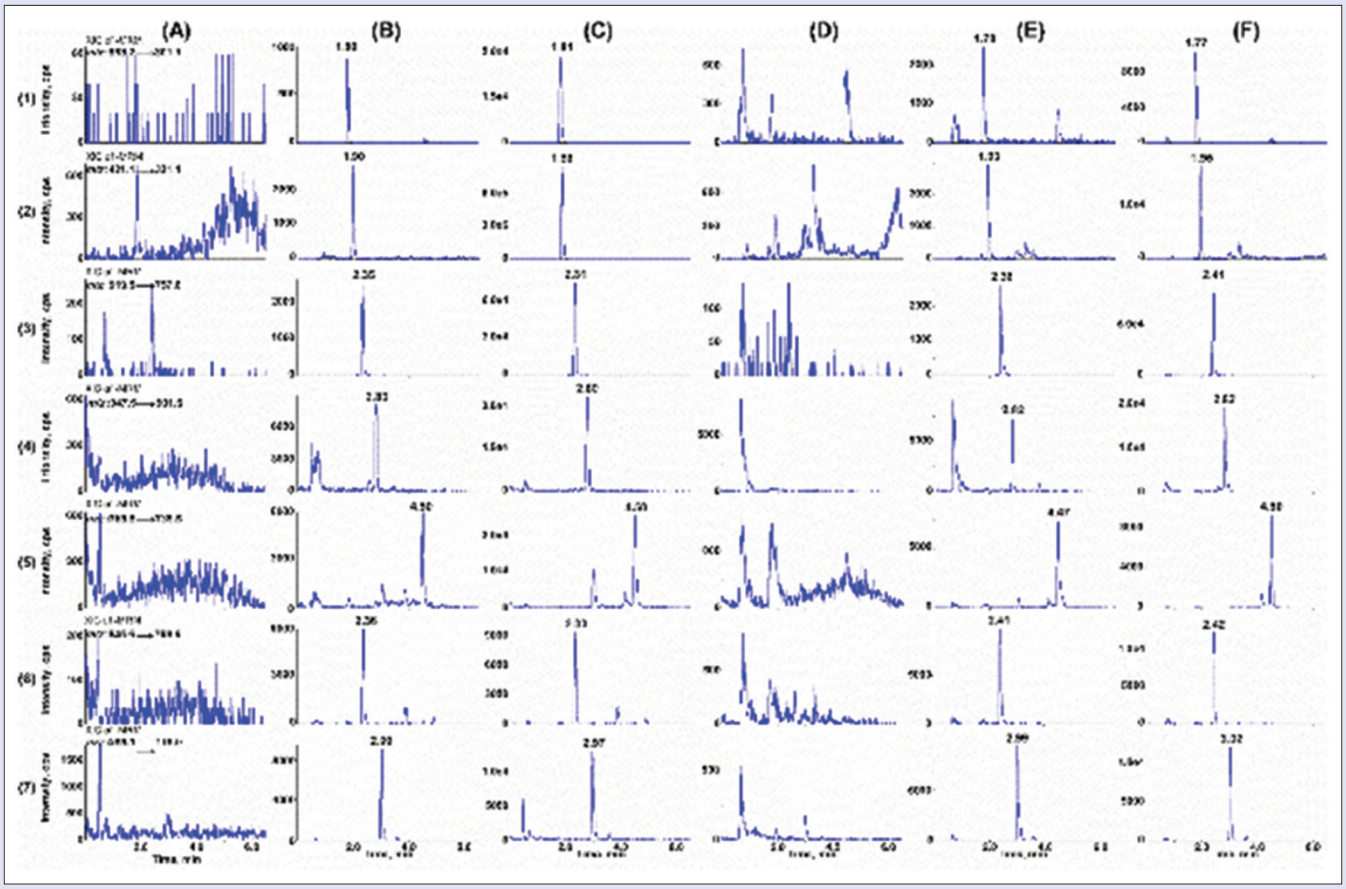

Figure 2: Representative multiple reaction monitoring chromatograms of neomangiferin (1), mangiferin (2), timosaponin BII (3), timosaponin BIII (4), timosaponin AIII (5), ginsenoside-Rg1 (6, internal standard), and liquiritigenin (7, internal standard) in rat plasma and liver homogenates. (A) Blank plasma; (B) blank plasma spiked with the five analytes at lower limit of quantification and internal standard; (C) plasma sample obtained 4 h after drug administration; (D) blank liver homogenates; (E) blank liver homogenates spiked with the five analytes at lower limit of quantification and internal standard; (F) liver homogenate obtained 4 h after drug administration

Table 1: Regression equations, linear ranges, correlation coefficients (r), and lower limit of quantification of the five analytes in rat plasma and tissues

Matrix	Analyte	Standard curves	Linear ranges (ng/mL)	*r*	LLOQ (ng/mL)
Plasma	Neomangiferin	y=2.45411× + 0.00492	0.5-120	0.9970	0.5
	Mangiferin	y=3.22052× + 0.14185	0.5-1600	0.9950	0.5
	Timosaponin BII	y=1.30004× + 0.01023	2-600	0.9948	2
	Timosaponin BIII	y=6.18199× + 0.01287	5-120	0.9945	5
	Timosaponin AIII	y=11.35168× + 0.14888	3-60	0.9948	3
Heart	Neomangiferin	y=0.46646× + 0.00477	0.5-24	0.9960	0.5
	Mangiferin	y=0.52531× + 0.02717	0.5-500	0.9966	0.5
	Timosaponin BII	y=0.15392× + 0.00751	2-300	0.9961	2
	Timosaponin BIII	y=0.72960× + 0.02345	5-100	0.9959	5
	Timosaponin AIII	y=1.57698× + 0.05821	3-60	0.9958	3
Liver	Neomangiferin	y=0.41154× + 0.00454	0.5-24	0.9961	0.5
	Mangiferin	y=0.49856× + 0.01436	0.5-500	0.9971	0.5
	Timosaponin BII	y=0.15168× + 0.00608	2-300	0.9984	2
	Timosaponin BIII	y=0.67239× + 0.02685	5-100	0.9994	5
	Timosaponin AIII	y=1.43605× + 0.23735	3-60	0.9985	3
Spleen	Neomangiferin	y=0.56108× + 0.00588	0.5-24	0.9973	0.5
	Mangiferin	y=0.60904× - 0.00333	0.5-500	0.9967	0.5
	Timosaponin BII	y=0.15547× + 0.01375	2-300	0.9966	2
	Timosaponin BIII	y=0.77248× + 0.01539	5-100	0.9994	5
	Timosaponin AIII	y=1.58659× + 0.27704	3-60	0.9995	3
Lungs	Neomangiferin	y=0.59078× + 0.00575	0.5-24	0.9972	0.5
	Mangiferin	y=0.57790× + 0.01903	0.5-500	0.9956	0.5
	Timosaponin BII	y=0.17716× + 0.02162	2-300	0.9958	2
	Timosaponin BIII	y=0.80161× + 0.02108	5-100	0.9968	5
	Timosaponin AIII	y=1.61621× + 0.11655	3-60	0.9973	3
Kidneys	Neomangiferin	y=0.45398× + 0.00841	0.5-24	0.9957	0.5
	Mangiferin	y=0.43318× + 0.01514	0.5-500	0.9951	0.5
	Timosaponin BII	y=0.16247× - 0.00106	2-300	0.9951	2
	Timosaponin BIII	y=0.85483× - 0.01306	5-100	0.9982	5
	Timosaponin AIII	y=1.70419× + 0.22338	3-60	0.9952	3
Brain	Neomangiferin	y=0.44741× + 0.00363	0.5-24	0.9988	0.5
	Mangiferin	y=0.40456× + 0.01353	0.5-500	0.9959	0.5
	Timosaponin BII	y=0.16804× + 0.00788	2-300	0.9975	2
	Timosaponin BIII	y=0.81896× - 0.00754	5-100	0.9993	5
	Timosaponin AIII	y=1.39859× + 0.14804	3-60	0.9957	3
Prostate	Neomangiferin	y=0.58761× + 0.00982	0.5-24	0.9952	0.5
	Mangiferin	y=0.56207× + 0.04436	0.5-500	0.9961	0.5
	Timosaponin BII	y=0.16591× + 0.05159	2-300	0.9957	2
	Timosaponin BIII	y=0.89709× + 0.03740	5-100	0.9954	5
	Timosaponin AIII	y=1.63834× + 0.79028	3-60	0.9975	3
Testis	Neomangiferin	y=0.49186× - 0.00570	0.5-24	0.9955	0.5
	Mangiferin	y=0.43990× + 0.04048	0.5-500	0.9952	0.5
	Timosaponin BII	y=0.15046× + 0.03206	2-300	0.9967	2
	Timosaponin BIII	y=0.93735× + 0.04040	5-100	0.9958	5
	Timosaponin AIII	y=1.40488× + 0.33445	3-60	0.9973	3

LLOQ: Lower limit of quantification

data of other tissues are shown in Supplementary material [Table S3]. All values were within 15%, indicating that the five analytes, were stable under the tested conditions.

Pharmacokinetic study

The validated UHPLC-MS/MS method was successfully applied to study the pharmacokinetics of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII after oral administration of AR extract. The corresponding pharmacokinetic parameters, calculated using noncompartmental analysis, are listed in Table 5. Figure 3 shows the mean plasma concentration-time curves ($n = 6$).

The analytes were divided into two groups: C-glycosyl xanthones (neomangiferin and mangiferin) and steroid saponins (timosaponin BII, timosaponin BIII, and timosaponin AIII). Neomangiferin and mangiferin exhibited similar pharmacokinetic parameters and concentration-time curves owing to their similar chemical structures. However, the t_{max} values of neomangiferin and mangiferin were 6.33 ± 1.51 h and 4.33 ± 0.82 h, respectively, which indicated that they might have different absorption rates after oral administration of AR extract (2 g/kg) in rats. The C_{max} and mean AUC $_{(0-t)}$ values were as follows: neomangiferin, 35.36 ± 12.21 μg/L and 551.15 ± 153.20 μg h/L and mangiferin, 945.68 ± 278.06 μg/L and 15897.84 ± 3730.93 μg h/L, which correlated well with a dose of 7 mg/kg for neomangiferin and 73 mg/kg for mangiferin. The MRT and $t_{1/2}$ values of neomangiferin and mangiferin did not differ significantly from each other, indicating their similar elimination rate and long duration *in vivo*. The apparent oral clearance (*CLz*/*F*) and apparent volume of distribution (*Vz*/*F*) of neomangiferin were 12.91 ± 5.02 L/kg and 171.01 ± 62.80 L/h/kg, whereas the *CLz*/*F* and *Vz*/*F* of mangiferin were 4.71 ± 1.19 L/kg and 71.19 ± 36.94 L/h/kg, respectively. These findings suggested that neomangiferin and mangiferin exhibit poor oral absorption and slow clearance from the body, and this was possibly attributed to their poor membrane permeability.[28]

Table 2: Accuracy, intra- and inter-day precision of the five analytes in quality control samples prepared in rat plasma and liver homogenates (*n*=6)

Matrix	Analyte	Concentration (ng/mL)	Accuracy (%)		Precision (%)			
			Measured concentration (ng/mL)	RE (%)	Intraday		Interday	
					Measured concentration (ng/mL)	RSD (%)	Measured concentration (ng/mL)	RSD (%)
Plasma	Neomangiferin	1	1.10±0.06	9.62	1.10±0.06	5.61	1.00±0.08	7.96
		20	19.79±0.62	−1.07	19.79±0.62	3.11	20.12±0.99	4.93
		100	102.6±1.89	2.6	102.6±1.89	1.84	100.18±2.61	2.61
	Mangiferin	1	0.99±0.02	−1.15	0.99±0.02	2.18	0.95±0.04	3.91
		300	305.19±13.92	1.73	305.19±13.92	4.56	304.68±11.74	3.85
		1500	1576.65±35.53	5.11	1576.65±35.53	2.25	1522.02±49.63	3.26
	Timosaponin	5	5.06±0.12	1.13	5.06±0.12	2.31	4.98±0.21	4.3
	BII	80	79.84±2.17	−0.2	79.84±2.17	2.71	80.64±2.69	3.34
		400	392.32±6.53	−1.92	392.32±6.53	1.66	399.63±9.31	2.33
	Timosaponin	10	10.02±0.33	0.18	10.02±0.33	6.52	10.15±0.37	3.62
	BIII	20	19.97±1.48	−0.17	19.97±1.48	7.39	20.86±1.61	7.74
		100	102.49±3.91	2.49	102.49±3.91	3.81	101.49±4.6	4.53
	Timosaponin	5	5.18±0.22	3.58	5.18±0.22	4.22	5.29±0.27	5.08
	AIII	10	10.95±0.56	9.5	10.95±0.56	5.11	10.92±0.58	5.36
		50	50.43±1.38	0.86	50.43±1.38	2.73	50.38±1.93	3.84
Liver	Neomangiferin	1	1.04±0.08	3.83	1.04±0.08	7.71	1±0.09	9.5
		5	20.1±1.07	0.49	20.1±1.07	5.33	20.13±0.97	4.83
		20	100.7±1.43	0.7	100.7±1.43	1.42	100.7±1.43	1.7
	Mangiferin	1	1.01±0.13	1.43	1.01±0.13	12.73	1±0.09	9.02
		40	39.43±1.28	−1.42	39.43±1.28	3.24	39.03±2.91	7.46
		400	386.03±12.95	−3.49	386.03±12.95	3.35	386.98±14.22	3.67
	Timosaponin	5	4.99±0.4	0.28	4.99±0.4	7.56	4.99±0.4	8.04
	BII	25	239.37±13.6	−4.25	239.37±13.6	5.68	246.88±10.82	4.38
		250	2574.91±66.33	3	2574.91±66.33	2.58	2551.7±69.66	2.73
	Timosaponin	10	9.92±0.51	−0.8	9.92±0.51	5.17	10.06±0.57	5.68
	BIII	20	37.99±1.8	−5.02	37.99±1.8	4.74	38.62±2.66	6.89
		100	436.3±27.77	9.07	436.3±27.77	6.37	429.24±21.1	4.92
	Timosaponin	5	4.7±0.32	−5.92	4.70±0.32	6.83	4.88±0.35	7.13
	AIII	10	37.11±0.75	−7.22	37.11±0.75	2.01	38.27±1.24	3.25
		50	381.67±25.47	−4.58	381.67±25.47	6.67	378.79±24.78	6.54

RSD: Relative standard deviation; RE: Relative error

Table 3: Extraction recovery and matrix effect of the five analytes in quality control samples prepared in rat plasma and liver homogenates (*n*=6)

Matrix	Analyte	Concentration (ng/mL)	Extraction recovery (%)	RSD (%)	Matrix effect (%)	RSD (%)
Plasma	Neomangiferin	1	84.78	3.71	96.98	4.35
		20	85.64	3.21	101.18	7.01
		100	86.45	3.12	96.76	3.93
	Mangiferin	1	86.22	4.27	105.98	5.79
		300	88.69	1.32	107.39	1.92
		1500	87.58	2.08	104.8	3.21
	Timosaponin	5	88.28	4.53	100.76	6.29
	BII	80	87.21	1.23	105.22	3.30
		400	88.07	1.43	108.59	1.21
	Timosaponin	10	86.97	6.98	103.88	4.64
	BIII	20	85.99	3.95	97.89	9.40
		100	87.50	2.24	99.06	6.35
	Timosaponin	5	86.81	9.44	101.89	7.91
	AIII	10	86.94	2.84	103.24	5.20
		50	87.95	2.19	104.27	4.95
	Ginsenoside	50	80.56	6.21	102.52	4.26
	Liquiritigenin	60	78.54	5.25	95.48	3.26
Liver	Neomangiferin	1	85.82	4.37	103.39	9.64
		5	87.59	2.05	97.59	3.95
		20	85.38	8.89	103.63	10.64
	Mangiferin	1	86.19	6.79	99.3	5.97
		40	86.10	8.82	104.78	11.56
		400	88.11	6.98	107.2	9.43
	Timosaponin	5	85.88	6.24	100.39	7.08
	BII	25	85.65	8.4	109.99	9.04
		250	85.17	10.56	107.11	11.31

Contd...

Table 3: Contd...

Matrix	Analyte	Concentration (ng/mL)	Extraction recovery (%)	RSD (%)	Matrix effect (%)	RSD (%)
	Timosaponin	10	83.04	7.44	93.73	11.18
	BIII	20	84.93	2.64	105.43	2.99
		100	85.02	7.07	107.21	7.10
	Timosaponin	5	87.08	7.09	101.35	6.88
	AIII	10	86.40	3.00	105.24	2.54
		50	86.27	8.70	107.57	8.78
	Ginsenoside	50	84.65	8.42	103.52	7.15
	Liquiritigenin	60	88.62	7.21	98.38	5.52

RSD: Relative standard deviation

Table 4: The stability of the five analytes in rat plasma and liver homogenates (*n*=6)

Matrix	Analyte	Concentration (ng/mL)	Short-term stability		Long-term stability		Freeze and thaw stability		Postpreparative stability	
			Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)
Plasma	Neomangiferin	1	1.02±0.13	1.9	0.99±0.04	−1.3	0.96±0.04	−3.53	1.07±0.1	7.1
		20	22.81±1.9	14.07	21.52±1.1	7.59	20.78±0.33	3.90	20.76±0.75	3.82
		100	99.23±3.69	−0.77	98.06±4.01	−1.94	99.65±1.89	−0.35	98.79±3.63	−1.21
	Mangiferin	1	1.05±0.08	5.07	1.05±0.13	5.43	1.04±0.08	4.27	1.08±0.09	7.6
		300	294.94±7.51	−1.69	299.75±5.59	−0.08	302.30±10.77	0.77	303±10.25	1
		1500	1509.48±28.86	0.63	1507.54±54.22	0.5	1474.35±52.13	−1.71	1471.19±82.24	−1.92
	Timosaponin	5	4.86±0.45	−2.71	5.66±0.24	13.19	5.54±0.36	10.79	5.41±0.3	8.13
	BII	80	80.29±2.85	0.36	80.63±3.12	0.79	81.74±1.93	2.18	82.26±2.58	2.82
		400	408.83±22.48	2.21	412.23±21.9	3.06	395.59±26.12	−1.1	411.00±17.93	2.75
	Timosaponin	10	9.98±0.38	−0.17	10.17±0.95	1.7	9.58±0.49	−4.21	9.44±0.53	−5.59
	BIII	20	19.14±1.4	−4.29	11.43±0.48	9.65	20.42±1.98	2.12	20.54±1.71	2.71
		100	109.13±5.1	9.13	87.07±8.77	5.22	102.81±5.19	2.81	100.70±3.29	0.7
	Timosaponin	5	5.24±0.35	4.81	4.99±0.36	−0.22	5.23±0.46	4.58	4.46±0.28	−10.84
	AIII	10	11.38±1.11	13.84	10.76±0.73	7.57	10.51±0.7	5.06	11.01±0.90	10.06
		50	51.78±2.3	3.55	51±3.35	2	51.49±3.2	2.97	47.57±3.03	−4.85
Liver	Neomangiferin	1	1.02±0.04	1.85	1.06±0.08	6.45	1.05±0.1	5.42	1.05±0.09	4.8
		5	5.28±0.54	5.58	5.39±0.45	7.73	5.50±0.81	9.98	5.20±0.85	4.07
		20	21.49±0.73	7.44	19.13±0.66	−4.36	22.14±1.34	10.7	22.14±0.79	10.68
	Mangiferin	1	1.04±0.08	4.33	0.93±0.06	−7.23	1.01±0.06	0.98	1.12±0.13	11.72
		40	41.22±3.51	3.04	42.11±3.03	5.27	41.2±2.61	2.99	39.08±3.16	−2.3
		400	419.92±14.22	4.98	421.39±9.79	5.35	413.35±23.47	3.34	429.52±16.32	7.38
	Timosaponin	5	4.64±0.56	−7.23	5.26±0.58	5.18	5.45±0.38	9.09	5.13±0.45	2.63
	BII	25	24.62±0.64	−1.51	24.98±0.77	−0.07	24.29±1.40	−2.84	24.80±0.59	−0.81
		250	248.00±8.23	−0.8	263.37±7.04	5.35	252.53±9.51	1.01	255.46±5.06	2.18
	Timosaponin	10	10.98±0.63	9.83	9.63±0.46	−3.68	11.04±0.91	10.4	9.84±0.51	−1.56
	BIII	20	19.35±2.35	−3.25	21.47±0.82	7.34	20.10±2.64	0.49	20.81±3.13	4.06
		100	109.39±7.36	9.39	108.62±4.33	8.62	110.45±6.93	10.44	102.65±4.36	2.64
	Timosaponin	5	5.5±0.34	9.9	5.38±0.55	7.64	5.38±0.61	7.62	4.54±0.42	−9.25
	AIII	10	10.89±0.41	8.84	10.89±0.76	8.86	11.05±1.15	10.44	10.67±0.61	6.72
		50	51.78±2.30	3.55	52.34±2.48	4.68	49.98±3.98	−0.03	49.09±3.82	−1.83

Table 5: Pharmacokinetic parameters of the five analytes in rat plasma (*n*=6, mean±standard deviation)

Parameters	Neomangiferin	Mangiferin	Timosaponin BII	Timosaponin BIII	Timosaponin AIII
C_{max1} (μg/L)	35.36±12.21	945.68±278.06	131.67±31.03	27.63±8.60	31.04±13.24
t_{max1} (h)	6.33±1.51	4.33±0.82	0.17±0.00	0.17±0.00	14±4.9
C_{max2} (μg/L)	-	-	99.03±23.8	29.59±10.12	-
t_{max2} (h)	-	-	5.67±0.82	6.00±0.00	-
$t_{1/2}$ (h)	9.54±2.95	10.60±5.86	13.09±3.51	10.46±5.26	27.83±14.66
$AUC_{(0-t)}$ (μg h/L)	551.15±153.20	15897.84±3730.93	1716.00±439.81	270.62±42.18	997.89±357.41
$AUC_{(0-\infty)}$ (μg h/L)	603.95±206.10	16283.84±3755.68	1936.34±508.23	350.65±85.30	1445.18±471.72
$MRT_{(0-\infty)}$ (h)	16.13±3.78	19.22±3.42	18.96±2.39	16.68±5.71	33.73±8.32
CLz/F (L/kg)	12.91±5.02	4.71±1.19	72.73±17.98	89.42±19.47	24.95±6.88
Vz/F (L/h/kg)	171.01±62.80	71.19±36.94	1361.48±496.10	1267.02±466.57	919.14±265.86

C_{max}: Maximum plasma concentration; t_{max}: Time to reach the maximum concentration; $t_{1/2}$: Elimination half-life; $AUC_{(0-t)}$: Area under the blood concentration-time curve from time zero to the last measured concentration; $AUC_{(0-\infty)}$: Area under the plasma concentration-time curve extrapolated to infinity; $MRT_{(0-\infty)}$: Total body mean residence time; *CLz/F*: Apparent oral clearance; *Vz/F*: Apparent volume of distribution

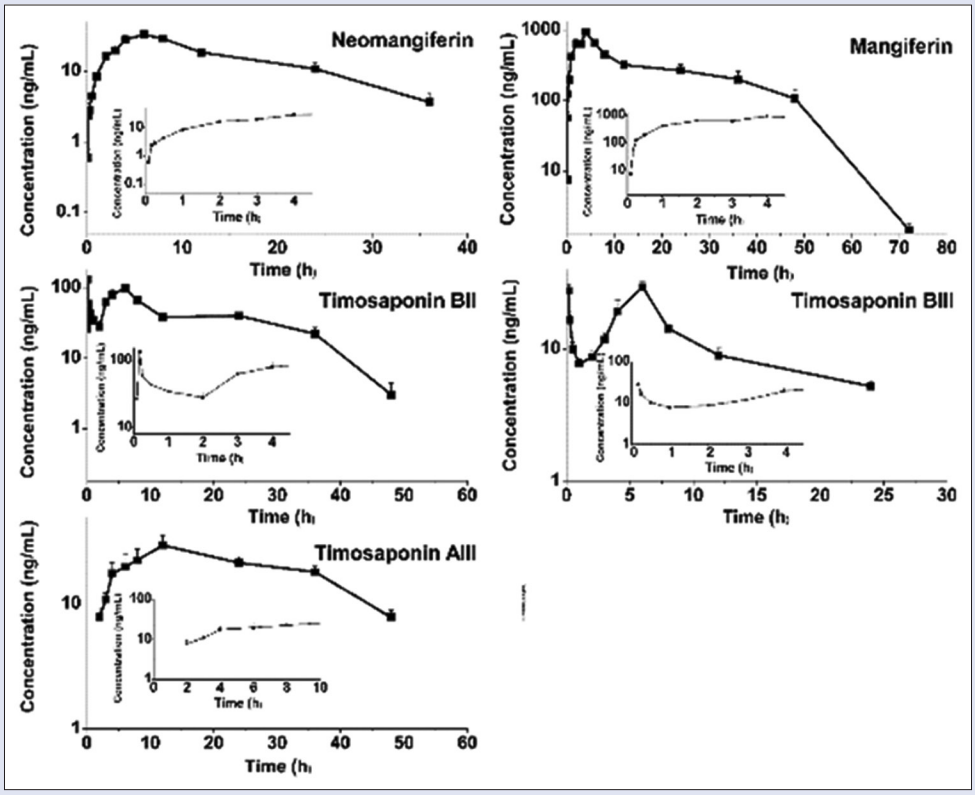

Figure 3: Mean plasma concentration-time curves of the five analytes in rat plasma after oral administration of AR extract ($n = 6$)

Timosaponin BII and timosaponin BIII are furostanol saponins with minimal variation in their chemical structures. No significant difference in the t_{max}, MRT, and $t_1/_2$ was observed between timosaponin BII and timosaponin BIII. Timosaponin AIII, a spirostanol saponin, with one less sugar moiety, compared to timosaponin BII, exhibited remarkably different pharmacokinetic parameters. Since the pharmacokinetic behavior of timosaponin AIII was similar to that of its aglycone, sarsasapogenin, the extra sugar moiety in timosaponin BII was considered responsible for the difference in their pharmacokinetics; however, the underlying reasons still need further investigation.[14,29] As shown in Figure 3, timosaponin BII and timosaponin BIII showed double plasma concentration peaks, as previously reported.[19,21] The first peak was observed at approximately 10 min after oral administration, indicating that timosaponin BII and timosaponin BIII could be quickly absorbed into the blood circulation. Then, these saponins were taken up by the hepatocytes by uptake transporters, such as organic anion-transporting polypeptide and a large fraction (approximately 70%) was then excreted into the bile, most probably because of the saturation of the uptake transporters or the metabolism capacity of these saponins in the liver.[30] In contrast to timosaponin BII and timosaponin BIII, the concentration-time curves of timosaponin AIII exhibited a single peak. However, the t_{max}, $t_{1/2}$, and MRT of timosaponin AIII (14.00 ± 4.90 h, 27.83 ± 14.66 h, and 33.73 ± 8.32 h) were longer than that of timosaponin BII (5.67 ± 0.82 h, 13.09 ± 3.51 h, and 18.96 ± 2.39 h) and timosaponin BIII (6.00 ± 0.00 h, 10.46 ± 5.26 h, and 16.68 ± 5.71 h), which indicated a slower absorption, longer body residence time, and slower elimination. In addition, the main pharmacokinetic parameters of the analytes were different between their pure forms[15,17,28,31] and TCM formulas,[14,32] which implied that the complexity of HMs could influence the pharmacokinetic profile of the analytes.

Tissue distribution study

Neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII concentrations were determined in various rat tissues, such as the heart, liver, spleen, lung, kidney, brain, testis, and prostate. The concentrations of the five analytes in rat tissues collected at 0.5, 1, 2, 4, 6, 10, and 24 h after administration of a single oral dose (2 g/kg) of the herbal extract are shown in Figure 4. Results indicated that the five analytes were widely distributed to most of the tissues within the time course examined. Neomangiferin and mangiferin exhibited the maximum concentration in the lung at 6 h after oral administration. Moreover, the relatively high distribution of neomangiferin and mangiferin in the kidney suggested that the kidney might be their primary excretion organ. The concentrations of neomangiferin and mangiferin in the heart, liver, spleen, brain, testis, and prostate were relatively low. Neomangiferin was not detected in the spleen and brain.

The highest levels of timosaponin BII and timosaponin BIII were observed in the lung at 1 h after oral administration. However, the concentrations of timosaponin BII and timosaponin BIII in the liver were remarkably higher than those in other organs, and reached the maximal levels at 4 h. This finding indicated that timosaponin BII and timosaponin BIII might be prone to metabolism in the liver. Timosaponin BII concentration was relatively low in the heart, spleen, kidney, brain, testis, and prostate, whereas timosaponin BIII was not detected in the spleen, brain, and testis. This might be attributable to the low timosaponin BIII content in AR extract, compared to timosaponin BII. Besides, timosaponin BIII was observed in the prostate at 1 h.

The tissue distribution behavior of timosaponin AIII was different from that of timosaponin BII and timosaponin BIII. The maximum concentration of timosaponin AIII was observed in the liver. Moreover, high concentrations of timosaponin AIII were observed in the heart,

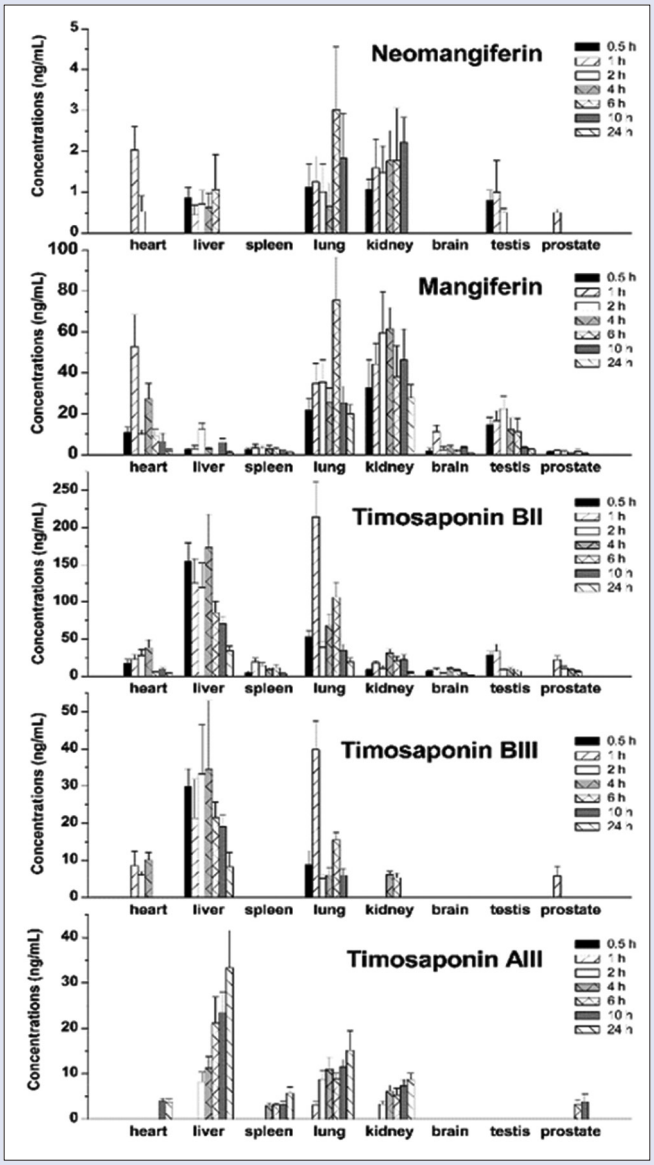

Figure 4: Mean concentrations of the five analytes in various tissues at indicated time points after oral administration of *Anemarrhenae rhizoma* extract ($n = 6$)

spleen, lung, and kidney, indicating that the distribution of timosaponin AIII depended on the blood flow or perfusion rate of the organ. As shown in Figure 4, the concentration of timosaponin AIII gradually increased over 0.5–24 h until it reached a maximum value at 24 h. This is in accordance with the fact that timosaponin AIII has slow absorption and elimination rates *in vivo*. Besides, timosaponin AIII was detected in the prostate in low concentrations and was not detected in the brain and testis.

CONCLUSION

In this study, a simple, rapid, and sensitive UHPLC-MS/MS method was validated for simultaneous determination of neomangiferin, mangiferin, timosaponin BII, timosaponin BIII, and timosaponin AIII in rat plasma and tissues. The established method provided adequate recovery and acceptable accuracy and precision; furthermore, it was successfully applied to study their pharmacokinetics and tissue distribution of targeted five secondary metabolites after oral administration of AR extract in rats. The findings of the present study might be helpful to better understand the pharmacokinetics and distribution of AR *in vivo*, which would facilitate its clinical use and drug discovery.

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Conflicts of interest

There are no conflicts of interest.

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