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Original research paper

Identification and pharmacokinetics of saponins in *Rhizoma Anemarrhenae* after oral administration to rats by HPLC-Q-TOF/MS and HPLC-MS/MS

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Rhizoma Anemarrhenae is a well-known herbal medicine with saponins as its commonly regarded major bioactive components. It is essential to classify the properties of saponins which are associated with their toxicity and efficacy. In this study, 25 compounds were identified by HPLC-Q-TOF/MS in the extract of *Rhizoma Anemarrhenae* and 8 saponins were detected in rat plasma by HPLC-MS/MS after oral administration of this extract. These were neomangiferin, mangiferin, timosaponin E1, timosaponin E, timosaponin B-II, timosaponin B-III, timosaponin A-III and timosaponin A-I. A sensitive and accurate HPLC-MS/MS method was developed and successfully applied to a pharmacokinetic study of the abovementioned eight saponins after oral administration of the *Rhizoma Anemarrhenae* extract to rats. The method validation, including specificity, linearity, precision, accuracy, recovery, matrix effect and robustness, met the requirements of the intended use. The pharmacokinetic parameter, T_{max} value, ranged from 2 to 8 h for these eight saponins whereas their elimination half-life ($t_{1/2}$) ranged from 4.06 to 9.77 h, indicating slow excretion. The plasma concentrations of these eight saponins were all very low, indicating a relatively low oral bioavailability. All these results provide support for further clinical studies.

Keywords: Rhizoma Anemarrhenae, saponin, identification, pharmacokinetics, HPLC-Q-TOF/MS, HPLC-MS/MS

Rhizoma Anemarrhenae, the dried rhizome of *Anemarrhena asphodeloides*, is a well-known traditional herbal medicine in China, Korea, and Japan (1). As a typical herbal medicine, *Rhizoma Anemarrhenae* has been extensively used for the treatment of inflammatory and other diseases, including cough, fever, allergies, Alzheimer's disease and diabetes (2–5). Modern pharmacological research has also revealed its antidepressant, antidiabetic, anti-inflammatory, antiplatelet aggregation, and antipyretic effects (6, 7). Previous studies have shown that *Rhizoma Anemarrhenae* contains steroidal saponins, flavonoids,

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polysaccharides, lignans, *etc*. (4, 5, 8, 9), among which steroidal saponins are commonly regarded as the major bioactive components (10). These components have been demonstrated various pharmacological properties including blood coagulation (11), attenuation of senile dementia (12, 13), tumour progression (1), inflammation (4, 14–17), and osteoporosis (18). It is worth noting that over-administering some saponins can also cause cardiovascular disorders and even death (19).

Several previous studies have investigated the pharmacokinetics of timosaponin B-II and timosaponin A-III, two important steroidal saponins in *Rhizoma Anemarrhenae*, and showed slow absorption and low bioavailability after administration (20, 21). However, few systematic studies have addressed the pharmacokinetic characteristics of the total saponins in *Rhizoma Anemarrhenae* after the administration of its saponin extract (22–24).

The aim of this study was to identify the main saponins in rat plasma after oral administration of *Rhizoma Anemarrhenae* saponin extract using high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF/MS), and to elucidate their pharmacokinetic characteristics by high-performance liquid chromatography-tandem triple quadrupole mass spectrometry (HPLC-MS/MS). Wang *et al*. (25) have established this HPLC-TOF/MS method. Here we applied it to the identification of chemical components in *Rhizoma Anemarrhenae asphodeloides* saponin extract and those absorbed into the blood. Also, here, an HPLC-MS/MS method was developed, validated and applied the first time, for quantitation of 8 saponins in the extract before administration, and afterward for the pharmacokinetic assays.

EXPERIMENTAL

Chemicals and reagents

Standards of neomangiferin, mangiferin, timosaponin E1, timosaponin E, timosaponin B-II, timosaponin B-III, timosaponin AIII, timosaponin A-I and the internal standard (IS), carbamazepine, were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China). Their structures were fully characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), and their purities were shown to be over 98.5 %.

Acetonitrile and methanol were of HPLC grade and were purchased from Honeywell (USA). HPLC-quality water was purified using a Milli-Q water purification system (Millipore, USA). All other reagents were of analytical grade.

The crude drug *Rhizoma Anemarrhenae* was purchased from the Shanghai Huayu Medicine Corporation (China) and authenticated by Prof. L. N. Sun, School of Pharmacy, Second Military Medical University, Shanghai, China.

Preparation of Rhizoma Anemarrhenae *saponin extract*

The dried crude drug was ground in a laboratory mill. One hundred grams of *Rhizoma Anemarrhenae* powder was extracted by ultrasound sonication with 500 mL 70 % ethanol for 30 min and then refluxed for 2 h. After vacuum filtration, another 500 mL 70 % ethanol was added to filter residue for an additional 2 h of reflux extraction and filtered again. The

filtrates from the two extractions were mixed and concentrated to 100 mL (equivalent to 1 g crude herb per mL) for intragastric administration.

To identify the chemical components in *Rhizoma Anemarrhenae*, 1 mL of the *Rhizoma Anemarrhenae* saponin extract was diluted with 25 mL methanol. After 30 min of ultrasound sonication, the sample was vacuum filtered and the filtrate was used for the identification of chemical components in *Rhizoma Anemarrhenae*.

Animals

Male Sprague–Dawley rats aged 8 weeks (220–250 g) were provided by the Experimental Animal Center of the Second Military Medical University (Shanghai, China). Sprague–Dawley rats were bred in a breeding room at 22 ± 2 °C with 50 ± 10 % relative humidity and a 12-h dark-light cycle. All animal experimental procedures conformed to the European Community Guidelines for Animal Experimentation and Welfare. The animal experimentation studies were approved by the Ethics Committee on Animal Research in Second Military Medical University (Shanghai, China). Before the experiments, the animals underwent 1 week of acclimatization with food and water provided *ad libitum* and were fasted with free access to water for 12 h prior to the oral administration. After finishing the animal experiment, the rats were sacrificed by cervical dislocation under anesthesia.

Standard solutions

Stock solutions. – Standard stock solution for any of the eight saponins was prepared by accurately weighing and dissolving the respective standard and internal standard in acetonitrile at the concentration of 200 μg mL^{-1} for each saponin and 100 ng mL^{-1} for the IS, and stored at 4 °C.

Calibration standards and quality control samples. – A series of standard solutions was obtained by diluting the abovementioned standard stock solutions with acetonitrile. The calibration standards were prepared by spiking 20 μL of standard solutions and 180 μL of IS solution into 100 μL of blank rat plasma to obtain the concentrations of 2.5–250 ng mL^{-1} for neomangiferin, mangiferin, timosaponin E1, timosaponin E, timosaponin B-III, timosaponin AIII and timosaponin A-I, and 5–500 ng mL^{-1} for timosaponin B-II. The mixture was then centrifuged at 12000 rpm for 10 min. Then the supernatant was transferred into an injection vial and a 2-μL aliquot was injected into the HPLC-MS/MS system for analysis.

Quality control (QC) samples were prepared by spiking 20 μL of standard solutions and 180 μL of IS solution into 100 μL of blank rat plasma to obtain three concentrations of 10, 50, 250 ng mL^{-1} for timosaponin B-II and 5, 25, 100 ng mL^{-1} for other 7 saponins. The mixture was centrifuged for 10 min at 12000 rpm and the 2-μL aliquot of the supernatant was injected.

HPLC-Q-TOF/MS

For the identification of chemical compounds in *Rhizoma Anemarrhenae* extract and in rat plasma, an Agilent 1290 ultra-high-performance liquid chromatography-tandem quad-

rupole time-of-flight mass spectrometer (HPLC-Q-TOF/MS, Agilent Technology Co., USA) was used, which consisted of a quaternary pump, an on-line degasser, a well-plate autosampler, a thermostatic column compartment and a 6210 TOF mass spectrometer. The chromatographic separations were performed at 30 °C on a Waters Xbridge C_{18} column (3.5 μm, 3.0 × 100 mm, Waters, USA). The mobile phase consisted of (A) water/formic acid (100:0.1, *V*/*V*) and (B) acetonitrile/formic acid (100:0.1, *V*/*V*) with the following gradient elution: 0–3 min, 18–22 % B; 3–8 min, 22–25 % B; 8–10 min, 25–40 % B; and 10–15 min, 40–60 % B. The flow rate was set at 400 μL min^{-1}. The sample injection volume was 2 μL. The parameters of the ion source were as follows: acquisition mode, positive mode; capillary voltage 3500 V; drying gas (N_2) temperature 350 °C; drying gas flow rate, 10 L min^{-1}; nebulizer gas (N_2) pressure 241 kPa; the fragmentor voltage 180 V and skimmer voltage 60 V. Mass spectra in the full-scan mode were recorded between *m*/*z* 100 and 1200. Agilent MassHunter B4.0 software was used for the control of equipment, data acquisition and analysis.

HPLC-MS/MS

For the pharmacokinetic study of eight saponins, an Agilent 1290 high-performance liquid chromatography triple quadrupole mass spectrometer (HPLC-MS/MS) was used, which consisted of a degasser, a G7120A binary pump, a G7167B autosampler and a G7116B thermostated column compartment. An Agilent 6470 triple quadrupole mass spectrometer equipped with standard AJS electrospray ionization (ESI Jet stream – positive ions) was coupled to the HPLC system. Chromatographic separation was performed on the Waters Xbridge C_{18} column (3.5 mm, 3.0 × 100 mm) at 30 °C. The mobile phase system was composed of (A) 0.1 % (*V*/*V*) aqueous formic acid and (B) acetonitrile. The following gradient elution was used: 0–4 min, 5–95 % B and 4–8 min, 95 % B. The flow rate was 0.4 mL min^{-1} and the analysis time was 8 min. An aliquot of 2 μL of the solution was injected into the HPLC-MS/MS system for analysis. The flow outlet was directed to the mass spectrometer. The mass scan mode was positive dynamic MRM (fragmentor 110 V, skimmer 60 V), and each pair of precursor and product transitions of the analytes was auto-optimized by HPLC-MS/MS. The precursor-to-product ion pairs were *m*/*z* 585.1→303.1 for neomangiferin, *m*/*z* 423.1→303.1 for mangiferin, *m*/*z* 937.5→775.5 for timosaponin E1, *m*/*z* 937.5→775.5 for timosaponin E, *m*/*z* 921.5→759.5 for timosaponin B-II, *m*/*z* 903.5→741.5 for timosaponin B-III, *m*/*z* 741.5→579.5 for timosaponin A-III, and *m*/*z* 579.5→417.3 for timosaponin A-I. The following MS/MS working parameters were used after optimization: drying gas temperature 350 °C, drying gas flow rate 10 L min^{-1}, nebulizer gas pressure 275.8 kPa, sheath gas temperature 350 °C, sheath gas flow rate 11 L min^{-1} and capillary voltage 3.5 kV.

HPLC-MS/MS validation

Validation of the HPLC-MS/MS method was performed with respect to specificity, accuracy, precision, the limit of detection (*LOD*), the limit of quantification (*LOQ*), linearity and range, robustness and stability according to the International Conference on Harmonisation (ICH) guidelines (26).

Method specificity was investigated by comparing eight different rat plasma samples after oral administration of saponin extract, with blank plasma.

For each analyte, the linearity range was tested by spiking the saponin and IS into blank plasma samples in the concentration range of 2.5 to 25 ng mL^{-1}, and five independent runs were conducted.

For each saponin, *LOD* and *LOQ* were calculated using calibration curve data following the equations: *LOD* = 3.3 × SD/slope and *LOQ* = 10 × SD/slope, where SD is the standard deviation of the regression line.

The intra- and inter-day precision and accuracy of the method were determined by using QC samples at low, medium and high concentration for three consecutive days. Rat plasma standard solutions including 8 saponins were used. For each concentration, five replicates were prepared. The concentration of each saponin was calculated by using a calibration curve on the same testing day. The accuracy was evaluated as the mean deviation from the theoretical value and expressed as a percentual relative error.

Three sets of samples (A–C) at low, medium and high concentrations were prepared for the evaluation of extraction recovery (RE) and matrix effect (ME), and each concentration was prepared in five replicates. Set A was prepared by diluting the working standard solutions with methanol, set B was composed of blank plasma and working standard solutions spiked before protein precipitation, in set C the spiking of the working solutions in blank plasma was performed after protein precipitation. RE and ME were evaluated by comparing the ratio of peak area of three sets of samples as follows: RE (%) = B/A × 100 and ME (%) = C/A × 100, where A, B and is the mean peak area ratio of saponin to IS of the respective set. In short, RE of saponins was determined by comparing the peak areas obtained from blank plasma spiked with analytes before the extraction with those from samples to which analytes were added after the extraction, whereas ME was assessed by comparing the blank plasma samples mixed with each saponin after the extraction process with the corresponding standard solution.

Robustness was evaluated by deliberate changes in chromatographic conditions such as the mobile phase flow rate (± 0.1 mL min^{-1}), column temperature (± 2 °C), gas temperature (± 10 °C) and drying gas flow rate (± 1 mL min^{-1}).

Stability was evaluated by analysing three QC samples at a low, medium and high level under different storage conditions. Short-term stability was measured by exposing the QC samples to room temperature for 24 h. Long-term stability was tested at –40 °C for 30 days and freeze/thaw stability after three freeze-thaw cycles of the plasma samples.

Plasma samples and pharmacokinetic study

Six SD rats were intragastrically administered *Rhizoma Anemarrhenae* saponin extract at a dose equivalent to 10 g crude drug kg^{-1} rat. After drug administration, rat blood samples were collected into heparinized tubes *via* the caudal vein at 1, 2, 4 h and mixed finally. After centrifugation at 4000 rpm for 10 min, plasma samples were collected and a 100-μL aliquot of plasma sample was removed to a 1.5-mL polypropylene tube, 200 μL acetonitrile added and vortexed for 30 s. This mixture was centrifuged at 12000 rpm for 10 min. Then, the supernatant was transferred into an injection vial and a 2-μL aliquot was injected into the HPLC-Q-TOF/MS for identification of saponins in rat plasma.

Plasma samples for pharmacokinetic analyses were prepared as follows. Six male SD rats were given the saponin extract of *Rhizoma Anemarrhenae* (equivalent to 3 g crude drug kg^{-1} rat) *per os*. Blood samples (0.3 mL) were collected through the caudal vein into heparinized tubes, before drug administration (time zero), and at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after drug administration. Plasma samples were centrifuged at 4000 rpm for

10 min, then collected and frozen at –40 °C until analysis by HPLC-MS/MS. To a 100-μL aliquot of plasma sample, 180 μL of the IS solution and 20 μL acetonitrile were added and vortexed for 30 s in a 1.5-mL polypropylene tube. The obtained mixture was then centrifuged at 12000 rpm for 10 min. After the supernatant was transferred into an injection vial, a 2-μL aliquot was injected into the HPLC-MS/MS system for the determination of eight saponins.

The pharmacokinetic parameters were calculated using DAS version 3.0 (BioGuider Co., China). A non-compartmental model was used to calculate the following parameters: maximum plasma concentration (C_{max}) and time to attain C_{max} (T_{max}) were obtained directly from the concentration-time curves. The elimination rate constant (k) was calculated by linear regression of the final linear part of the plasma concentration-time curve. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k$. The area under the concentration-time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule. Mean residence time (MRT) was calculated from the formula $MRT = AUMC_{0-\infty}/AUC_{0-\infty}$, where $AUMC_{0-\infty}$ indicates the area under the first part of the plasma concentration-time curve. All the data are presented as the mean ± SD.

RESULTS AND DISCUSSION

Chemical composition of Rhizoma Anemarrhenae saponin extract

The HPLC-Q-TOF/MS chromatogram of *Rhizoma Anemarrhenae* saponin extract is shown in Fig. 1. By comparing three batches of raw *Rhizoma Anemarrhenae* with chemical components reported in the literature (10, 20, 27), 25 common peaks were confirmed (Table I), according to their retention time, accurate molecular mass and molecular formula.

The content of 8 saponins in the *Rhizoma Anemarrhenae* extract was determined by HPLC-MS/MS: neomangiferin 11.86 mg mL^{-1}, mangiferin 14.69 mg mL^{-1}, timosaponin E1 9.56 mg mL^{-1}, timosaponin E 3.15 mg mL^{-1}, timosaponin B-II 40.85 mg mL^{-1}, timosaponin B-III 2.32 mg mL^{-1}, timosaponin A-III 11.03 mg mL^{-1}, timosaponin A-I 3.26 mg mL^{-1}.

Components in rat plasma after oral administration

By comparing retention time and the mass-to-charge ratio of ions with the identified components of *Anemarrhena asphodeloides*, eight major components were identified in rat plasma following oral administration of *Rhizoma Anemarrhenae* saponin extract. These were: neomangiferin, mangiferin, timosaponin E1, timosaponin E, timosaponin B-II, timosaponin B-III, timosaponin A-III and timosaponin A-I. Their molecular structures and mass ion spectra are shown in Figs. 2 and 3. They were also found in the chromatogram of the *Rhizoma Anemarrhenae* saponin extract in Fig. 1. A subsequent pharmacokinetic study on the eight active saponins was performed.

Development and validation of HPLC-MS/MS

Method development. – To achieve the best chromatographic separation, different mobile phase compositions, as well as flow rates, chromatographic columns and column tem-

peratures were investigated. Finally, an acetonitrile-water system containing 0.1 % (*V*/*V*) formic acid was selected as the mobile phase, as it achieved the best baseline. Better separation and peak symmetry were achieved on a Waters Xbridge C_{18} column (3.5 mm, 3.0 × 100 mm) than with other tested columns. By comprehensively considering the retention times and separation, the column temperature was set at 30 °C.

For the pharmacokinetic study, eight saponin standards were first tested in positive and negative modes. Finally, the positive mode was chosen, as all these compounds demonstrated stronger intensity in this mode than in the negative mode. An Agilent

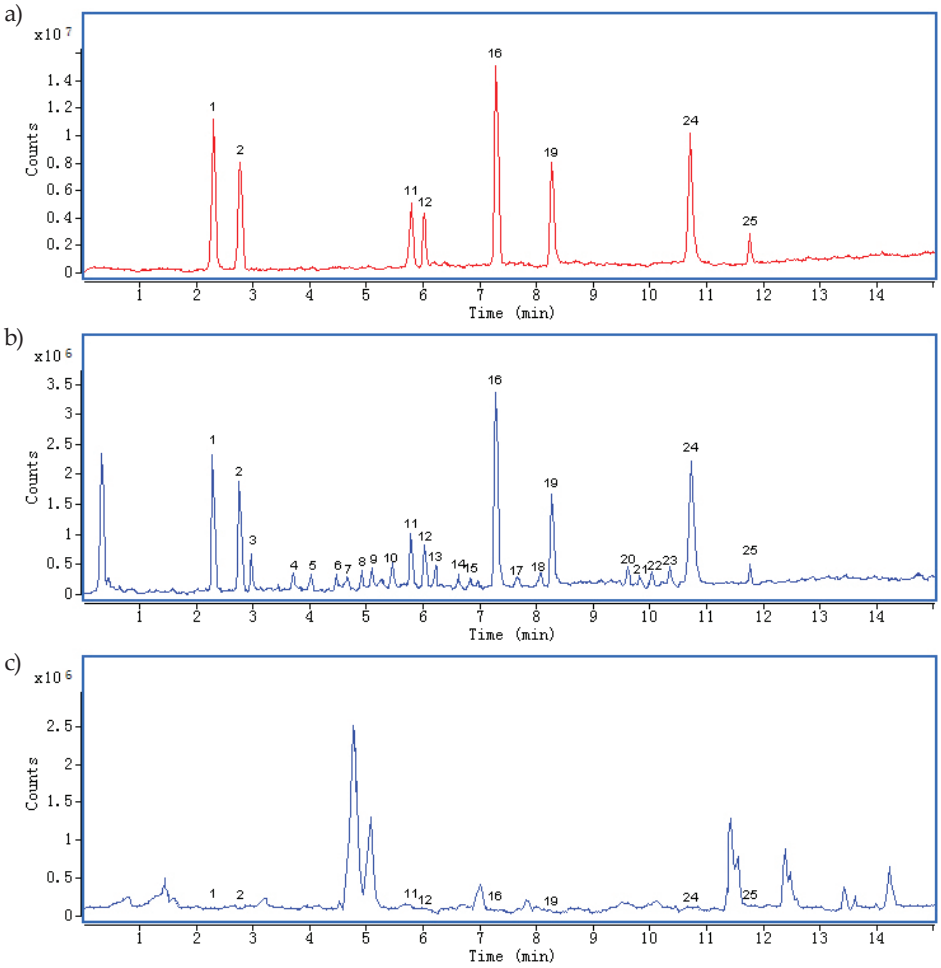

Fig. 1. HPLC-Q-TOF/MS chromatogram of 25 compounds identified in *Rhizoma Anemarrhenae* extract and eight saponins in rat plasma after oral administration. a) Eight saponins mixed standards; b) *Rhizoma Anemarrhenae* saponins extract, c) plasma sample after oral administration of *Rhizoma Anemarrhenae* extract. The compound names for each peak are shown in Table I.

Table I. Components identified in Rhizoma Anemarrhenae *by HPLC-Q-TOF/MS*

No.	t_R (min)	Compound	Formula	Selected ion	Expected *m/z*	Detected *m/z*	Error (ppm)
1	2.32	Neomangiferin[a]	$C_{25}H_{28}O_{16}$	$[M+H]^+$	585.1456	585.1468	2.19
2	2.78	Mangiferin[a]	$C_{19}H_{18}O_{11}$	$[M+H]^+$	423.0927	423.0919	–1.92
3	2.98	Isomangiferin	$C_{19}H_{18}O_{11}$	$[M+H]^+$	423.0927	423.0931	0.78
4	3.72	Timosaponin B-V	$C_{57}H_{96}O_{29}$	$[M+H]^+$	1245.6116	1245.6137	1.69
5	4.04	Timosaponin B-VI	$C_{58}H_{98}O_{29}$	$[M+H]^+$	1259.6272	1259.6293	1.66
6	4.48	Timosaponin H1	$C_{56}H_{92}O_{28}$	$[M+H]^+$	1213.5853	1213.585	–0.24
7	4.67	Timosaponin I1	$C_{56}H_{94}O_{28}$	$[M+H]^+$	1215.601	1215.6046	2.97
8	4.49	Timosaponin B-IV(C57)	$C_{57}H_{94}O_{28}$	$[M+H]^+$	1227.601	1227.5987	–1.87
9	5.1	Timosaponin I2	$C_{57}H_{96}O_{28}$	$[M+H]^+$	1229.6166	1229.6193	2.18
10	5.47	Timosaponin H2	$C_{57}H_{94}O_{28}$	$[M+H]^+$	1227.601	1227.6026	1.35
11	5.79	Timosaponin E1[a]	$C_{45}H_{76}O_{20}$	$[M+H]^+$	937.5008	937.5016	0.83
12	6.04	Timosaponin E[a]	$C_{45}H_{76}O_{20}$	$[M+H]^+$	937.5008	937.5025	1.78
13	6.23	Timosaponin N	$C_{45}H_{76}O_{20}$	$[M+H]^+$	937.5008	937.5021	1.35
14	6.63	Timosaponin E2	$C_{46}H_{78}O_{20}$	$[M+H]^+$	951.5165	951.5183	1.96
15	6.84	Macrostemonoside K	$C_{46}H_{78}O_{20}$	$[M+H]^+$	983.4885	983.4911	2.58
16	7.29	Timosaponin B-II[a]	$C_{45}H_{76}O_{19}$	$[M+H]^+$	921.5059	921.5066	0.81
17	7.67	Timosaponin D	$C_{45}H_{74}O_{19}$	$[M+H]^+$	919.4903	919.4929	2.92
18	8.07	Timosaponin B-I	$C_{46}H_{78}O_{19}$	$[M+H]^+$	935.5216	935.5227	1.23
19	8.28	Timosaponin B-III[a]	$C_{45}H_{74}O_{18}$	$[M+H]^+$	903.4953	903.4964	1.18
20	9.62	Timosaponin F	$C_{39}H_{64}O_{15}$	$[M+H]^+$	773.4323	773.4311	–1.61
21	9.83	Anemarrhenasaponin I	$C_{39}H_{66}O_{14}$	$[M+H]^+$	759.4531	759.4522	–1.21
22	10.04	Anemarrhenasaponin Ia	$C_{40}H_{68}O_{14}$	$[M+H]^+$	773.4687	773.4703	2.03
23	10.35	Timosaponin G	$C_{39}H_{64}O_{14}$	$[M+H]^+$	757.4374	757.4372	–0.35
24	10.74	Timosaponin AIII[a]	$C_{39}H_{64}O_{13}$	$[M+H]^+$	741.4425	741.4431	0.74
25	11.77	Timosaponin A-I[a]	$C_{33}H_{54}O_8$	$[M+H]^+$	579.3897	579.389	–1.18

[a] Compound identified in rat plasma after oral administration.

Automatic Optimizer was used to optimize the MS/MS method to achieve the highest responses. Finally, the precursor transitions $[M+H]^+$ were monitored.

Validation. – By analysing 8 different blank plasma samples, no interference of endogenous substances was observed for any of the saponins, indicating the good specificity of this method. Typical MRM chromatograms of blank plasma, blank plasma spiked with the

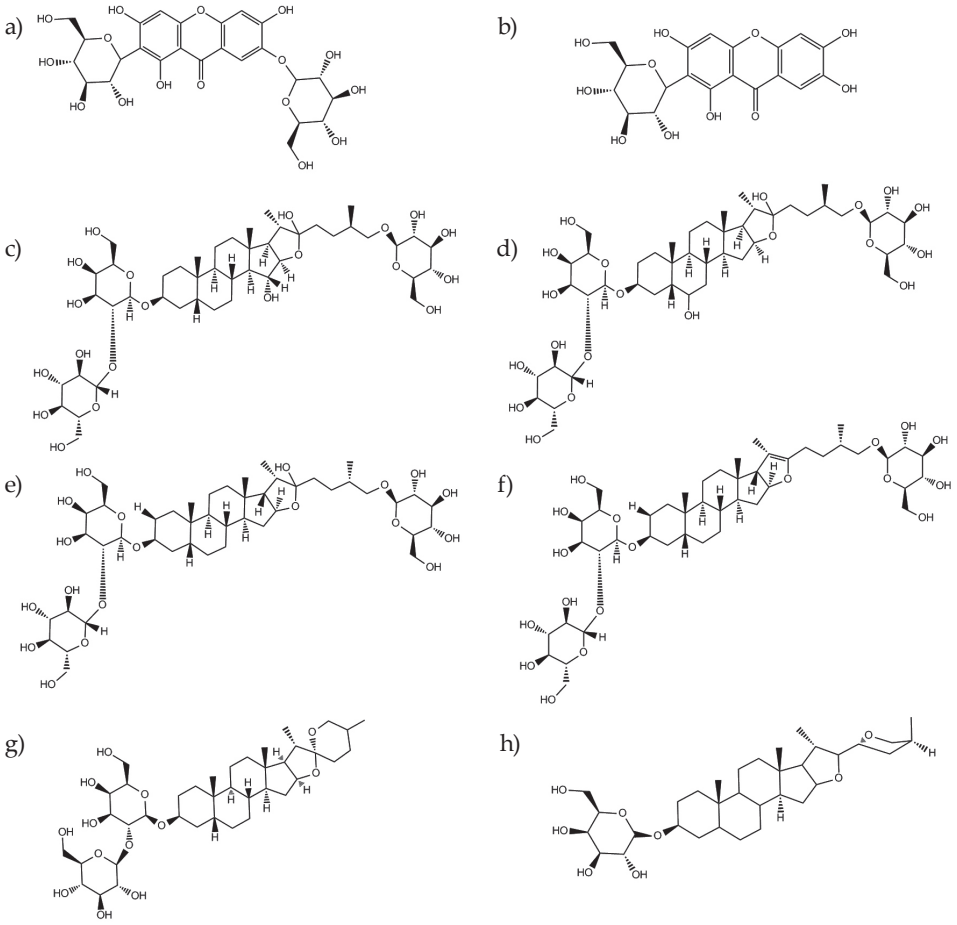

Fig. 2. Molecular structures of eight saponins detected in rat plasma: a) neomangiferin, b) mangiferin, c) timosaponin E1, d) timosaponin E, e) timosaponin B-II, f) timosaponin B-III, g) timosaponin AIII, h) timosaponin A-I.

IS and a plasma sample from a rat administered by *Rhizoma Anemarrhenae* saponin extract are shown in Fig. 4.

As shown in Table II, the peak area ratios of each saponin to the IS were plotted against used concentrations. The coefficient of determination (R^2) for each of these calibration curves was higher than 0.995, indicating a good linear detector response over the investigated ranges. The *LODs* of the eight saponins ranged 0.81–1.62 ng mL^{-1}, while the *LOQs* ranged from 2.46–4.92 ng mL^{-1}, which indicated good sensitivity in rat plasma.

As shown in Table III, intra- and inter-day precision values were all within 1 and 10 %, as well as inaccuracy values through the respective relative error. e_r for the eight saponins ranged from –9.5 to 9.0 %, indicating acceptable accuracy of the method.

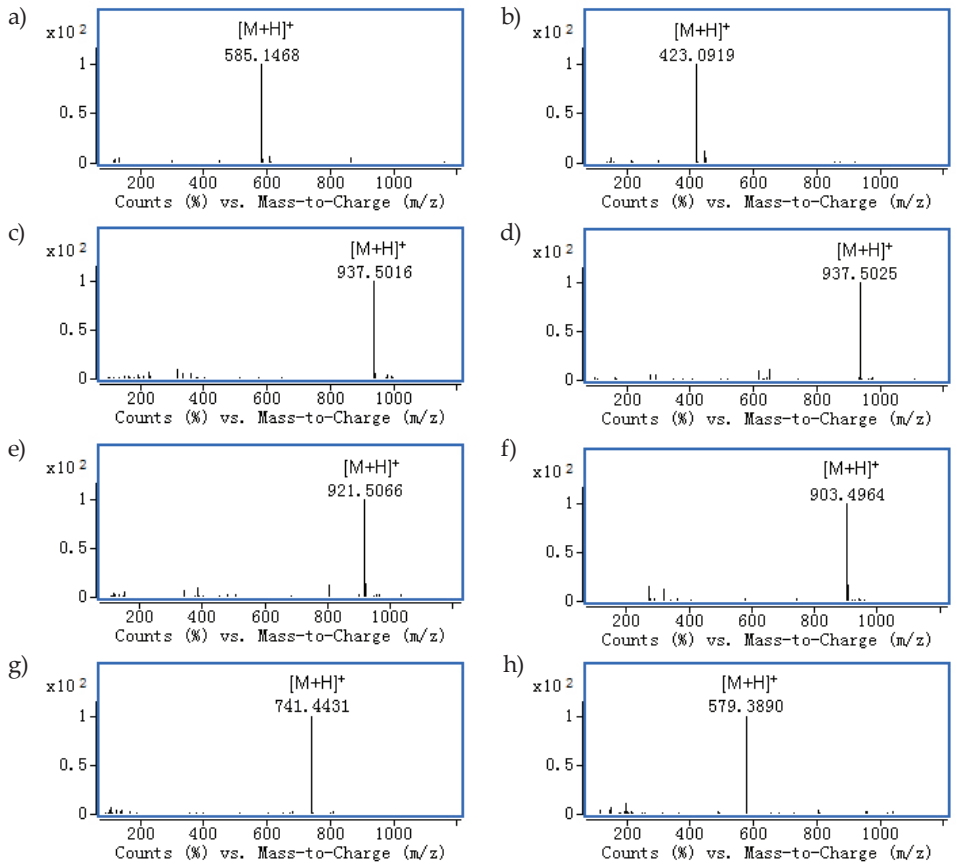

Fig. 3. Mass spectra of eight saponins: a) neomangiferin, b) mangiferin, c) timosaponin E1, d) timosaponin E, e) timosaponin B-II, f) timosaponin B-III, g) timosaponin AIII, h) timosaponin A-I.

Table IV shows that the absolute recovery for the eight saponins and IS ranged from 85.0 to 95.0 %, suggesting that the recoveries of the eight saponins and IS were consistent. In addition, no significant differences of extract recovery were found among the three concentrations levels of 10, 50, 250 ng mL^{-1} for timosaponin B-II and 5, 25, 100 ng mL^{-1} for the other seven saponins, indicating that the recoveries of the eight saponins and IS were not concentration-dependent. Furthermore, the matrix effect of the analytes ranged from 0 to 10 %.

The small deliberate changes of chromatographic conditions did not affect accuracy (recovery ranged 99.1–101.5 %), and hence the method was found robust (Table V).

The stability of saponins throughout processing and storage was comprehensively assessed for three different concentration levels, and five independent runs were performed for each concentration. The $\pm e_r$ values for eight saponins were less than 10 %, indi-

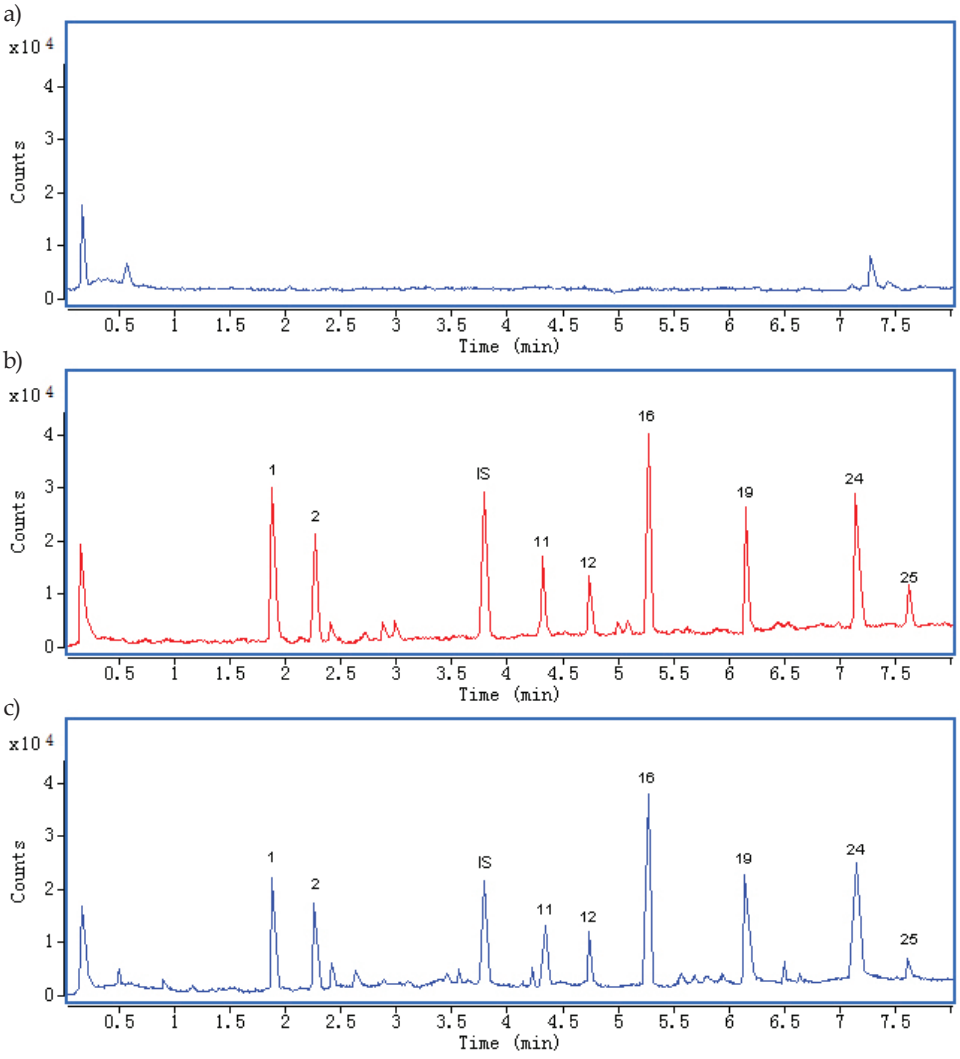

Fig. 4. HPLC-MS/MS chromatogram of eight saponins: a) blank plasma, b) eight saponins spiked in rat plasma, c) plasma sample after oral administration of *Rhizoma Anemarrhenae* extract. The compound names for each peak are shown in Table I.

cating that the analytes were stable in rat plasma under the following storage conditions: short-term stability at room temperature for 24 h, long-term stability at –40 °C for 30 days, and after three freeze-thaw cycles. All data are shown in Table VI.

The HPLC-MS/MS chromatographic parameters obtained under final conditions are summarized in Table VII.

Table II. Regression equation and correlation coefficient (R) *for saponins in rat plasma* (n = 5)

Analyte	Linear range (ng mL^{-1})	Standard curve	R	*LOD* (ng mL^{-1})
Neomangiferin	2.5–250	$y = 2.562x - 1.268$	0.9963	0.81
Mangiferin	2.5–250	$y = 3.051x - 1.451$	0.9978	0.84
TimosaponinE1	2.5–250	$y = 2.152x - 3.302$	0.9947	0.82
Timosaponin E	2.5–250	$y = 2.145x - 3.558$	0.9931	0.83
Timosaponin BII	5–500	$y = 2.618x - 3.049$	0.9949	1.62
TimosaponinBIII	2.5–250	$y = 1.683x - 1.852$	0.9986	0.82
TimosaponinAIII	2.5–250	$y = 2.017x - 3.041$	0.9928	0.81
Timosaponin A-I	2.5–250	$y = 1.211x - 1.817$	0.9961	0.83

LOQ = 3×LOD

Table III. Intra-day and inter-day precision and accuracy for saponins in rat plasma (n = 5)

Analyte	Expected concentration (ng mL^{-1})	Intra-day			Inter-day		
		Measured (ng mL^{-1})	RSD (%)	e_r (%)	Measured (ng mL^{-1})	RSD (%)	e_r (%)
Neomangiferin	5.000	4.857	2.6	–2.9	4.945	9.0	–1.1
	25.001	26.609	9.1	6.4	26.776	9.8	7.1
	100.005	90.455	4.8	–9.5	91.200	1.4	–8.8
Mangiferin	5.001	5.220	6.5	4.4	4.699	7.4	–6.0
	25.003	25.199	4.9	0.8	27.244	2.0	9.0
	100.010	93.051	8.4	–7.0	107.599	7.4	7.6
Timosaponin E1	5.001	4.779	2.7	–4.4	4.838	3.1	–3.3
	25.004	26.698	6.4	6.8	26.711	6.6	6.8
	100.015	106.370	2.7	6.4	93.855	2.1	–6.2
Timosaponin E	5.000	4.931	1.4	–1.4	5.147	6.7	2.9
	25.001	22.886	3.8	–8.5	24.829	8.7	–0.7
	100.005	104.126	8.2	4.1	107.069	9.4	7.1
Timosaponin B-II	10.001	9.536	3.0	–4.6	10.447	2.6	4.5
	50.004	52.468	9.1	4.9	47.874	8.3	–4.3
	250.020	230.805	7.3	–7.7	240.708	8.2	–3.7
Timosaponin B-III	5.002	4.600	3.7	–8.0	4.944	9.1	–1.2
	25.008	23.048	6.2	–7.8	27.070	7.2	8.2
	100.015	105.203	5.9	5.2	103.712	2.6	3.7
Timosaponin AIII	5.003	5.151	7.2	3.0	5.030	2.0	0.6
	25.013	27.001	9.1	8.0	24.114	6.5	–3.6
	100.020	96.621	4.4	–3.4	106.723	5.0	6.7
Timosaponin A-I	5.001	4.869	1.7	–2.6	4.620	1.9	–7.6
	25.005	25.885	5.8	3.5	24.054	9.7	–3.8
	100.025	105.242	2.8	5.2	103.962	6.1	3.9

Table IV. Matrix effect and extraction recovery of saponins and IS in rat plasma (n = 5)

Analyte	Concentration (ng mL^{-1})	Matrix effect		Extraction recovery	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
Neomangiferin	5.000	4.2	2.9	88.1	5.4
	25.001	3.1	3.2	94.9	4.5
	100.005	1.7	7.4	92.0	5.2
Mangiferin	5.001	5.7	3.3	91.7	2.4
	25.003	5.4	2.2	94.6	5.4
	100.010	2.9	7.4	94.8	6.6
Timosaponin E1	5.001	7.6	5.0	85.2	2.0
	25.004	9.0	6.2	90.2	6.2
	100.015	7.6	2.1	92.6	1.5
Timosaponin E	5.000	3.1	1.3	86.6	1.4
	25.001	1.8	7.4	94.0	4.5
	100.005	0.8	1.9	89.2	5.6
Timosaponin B-II	10.001	7.2	9.7	87.5	8.4
	50.004	4.8	7.7	92.1	2.5
	250.020	4.2	2.0	85.3	9.3
Timosaponin B-III	5.002	6.9	3.3	87.2	6.2
	25.008	8.2	6.6	92.7	6.2
	100.015	9.5	3.0	93.4	7.4
Timosaponin AIII	5.003	7.1	9.7	90.2	6.1
	25.013	1.9	8.0	94.3	6.3
	100.020	0.4	1.7	85.5	7.5
Timosaponin A-I	5.001	3.1	2.5	85.2	6.9
	25.005	4.8	2.4	94.8	1.5
	100.025	1.2	9.2	88.4	4.1
Internal standard	60.002	4.2	2.9	88.1	5.4

Table V. Method robustness for saponins in rat plasma

Chromatographic conditions	Recovery (%)							
	Neo-mangi-ferin	Mangi-ferin	Timosa-ponin E1	Timosa-ponin E	Timosa-ponin BII	Timosa-ponin BIII	Timosa-ponin AIII	Timosa-ponin A-I
Mobile phase flow rate								
0.3 mL min^{-1}	100.9	99.4	100.1	100.4	100.3	99.5	100.9	99.4
0.5 mL min^{-1}	100.8	100	99.1	100.1	101.1	100.3	100.8	100.0
Column temperature								
28 °C	100.7	101.0	99.1	100.6	99.5	100.5	100.7	101.0
32 °C	99.1	100.9	99.5	101.4	99.7	100.8	99.1	100.9
Drying gas temperature								
340 °C	100.9	101.5	100.3	99.3	99.1	99.6	100.9	101.5
360 °C	101.2	99.5	100.7	99.8	100.4	101.5	101.2	99.5
Drying gas flow rate								
9 L min^{-1}	100.7	101.0	99.1	100.6	99.5	100.5	100.7	101.0
11 L min^{-1}	99.1	100.9	99.5	101.4	99.7	100.8	99.1	100.9

Table VI. Stability of eight saponins in rat plasma (n = 5)

Analytes	Concentration (ng mL^{-1})	Frozen at –40 °C for 30 days		Room temperature for 24 h		Freeze-thaw cycles (3x)	
		Measured (ng mL^{-1})	e_r (%)	Measured (ng mL^{-1})	e_r (%)	Measured (ng mL^{-1})	e_r (%)
Neomangiferin	5.000	4.824	–3.5	4.674	–6.5	5.263	5.3
	25.001	24.395	–2.4	26.041	4.2	23.200	–7.2
	100.005	106.791	6.8	98.045	–2.0	90.464	–9.5
Mangiferin	5.001	5.471	9.4	4.571	–8.6	5.378	7.6
	25.003	24.557	–1.8	25.387	1.5	23.014	–8.0
	100.010	106.742	6.7	102.267	2.3	109.088	9.1
Timosaponin E1	5.001	4.792	–4.2	4.852	–3.0	5.353	7.0
	25.004	23.300	–6.8	26.745	7.0	23.876	–4.5
	100.015	93.866	–6.1	98.127	–1.9	100.946	0.9
Timosaponin E	5.000	5.363	7.3	5.384	7.7	4.676	–6.5
	25.001	25.210	0.8	23.333	–6.7	27.034	8.1
	100.005	97.655	–2.3	94.291	–5.7	107.734	7.7
Timosaponin B-II	10.001	10.290	2.9	9.368	–6.3	9.262	–7.4
	50.004	49.209	–1.6	51.374	2.7	47.028	–6.0
	250.020	253.615	1.4	227.978	–8.8	266.853	6.7
Timosaponin B-III	5.002	4.678	–6.5	5.474	9.4	4.622	–7.6
	25.008	26.937	7.7	25.378	1.5	25.692	2.7
	100.015	102.201	2.2	104.269	4.3	99.680	–0.3
Timosaponin AIII	5.003	4.998	–0.1	4.789	–4.3	4.674	–6.6
	25.013	25.257	1.0	26.039	4.1	25.338	1.3
	100.020	95.411	–4.6	91.862	–8.2	90.821	–9.2
Timosaponin A-I	5.001	4.529	–9.4	4.598	–8.1	4.520	–9.6
	25.005	26.859	7.4	25.488	1.9	22.996	–8.0
	100.025	103.335	3.3	90.883	–9.1	107.383	7.4

Pharmacokinetic study after intragastric administration of Rhizoma Anemarrhenae *saponin extract*

Since eight saponins were detected in rat plasma after oral administration of *Rhizoma Anemarrhenae* saponin extract, their pharmacokinetic profiles *in vivo* were further investigated by the use of the HPLC-MS/MS method. The pharmacokinetic profile of timosaponin AIII was determined up to 48 h after oral administration, whereas timosaponin E′ profile was determined up to 24 h due to the drop of the plasma concentration below *LOQ* 24 h after oral administration. The other six saponins were all determined up to 36 h after oral administration. The mean plasma concentrations in the concentration-time curves are shown in Fig. 5. The mean pharmacokinetic parameter values were calculated by using DAS 3.0 based on the non-compartmental pharmacokinetic model and are summarized in Table VIII.

As shown in Fig. 5 and Table VIII, T_{max} values of the eight saponins ranged from 2 to 8 h, with timosaponin E1 with the lowest (T_{max} 2.12 h) and timosaponin AIII the highest

Table VII. The chromatographic parameters of the optimized UHPLC-MS/MS method

Parameter	Neomangiferin	Mangiferin	Timosaponin E1	Timosaponin E	Timosaponin BII	Timosaponin BIII	Timosaponin AIII	Timosaponin A-I
Retention time (t_R,min)	1.87	2.26	4.31	4.74	5.27	6.14	7.14	7.63
Capacity factor (k')	0.87	1.09	2.12	2.49	2.79	3.12	3.69	3.92
Tailing factor (T_f)	1.05	1.02	0.97	1.04	1.00	1.04	0.98	0.99
Number of theoretical plates (N)[a]	6012	5508	8432	6487	8075	17007	15773	25469
Peak asymmetry factor (A_s)	1.09	1.04	0.95	1.07	1.00	1.07	0.97	0.98
Chromatographic resolution (R_s)	2.32	1.59	2.06	1.97	1.63	1.96	1.66	1.72
Peak repeatability (RSD, %)	2.5	3.1	2.7	3.1	4.7	3.7	4.9	3.6

Table VIII. Pharmacokinetic parameters for saponins in male SD rats after oral administration of Rhizoma Anemarrhenae *extract*[a]

Parameters	Neomangiferin	Mangiferin	Timosaponin E1	Timosaponin E	Timosaponin B-II	Timosaponin B-III	Timosaponin AIII	Timosaponin A-I
T_{max} (h)	3.85 ± 1.16	4.06 ± 0.53	2.12 ± 0.52	4.24 ± 1.01	4.38 ± 1.37	4.27 ± 0.29	7.85 ± 2.11	4.22 ± 1.74
C_{max} (ng mL^{-1})	136.44 ± 15.89	156.9 ± 14.11	118.9 ± 11.23	63.11 ± 6.29	469.7 ± 52.13	48.11 ± 5.26	130.9 ± 10.44	98.10 ± 8.71
$t_{1/2}$ (h)	6.94 ± 1.24	6.71 ± 1.03	5.12 ± 0.58	4.06 ± 0.82	5.88 ± 1.46	5.99 ± 1.71	9.77 ± 1.61	6.38 ± 1.16
AUC_{0-T} (ng mL^{-1} h)	1330 ± 64.78	1049 ± 79.37	863 ± 66.80	400.1 ± 35.11	2501 ± 152.8	729.9 ± 36.43	1716 ± 106.5	1251 ± 52.62
$AUC_{0-\infty}$ (ng mL^{-1} h)	1376 ± 68.04	1166 ± 99.24	897 ± 54.49	407.6 ± 73.15	2535 ± 172.2	760.5 ± 72.04	1779 ± 117.6	1341 ± 44.40
$AUMC_{0-T}$ (ng mL^{-1} h^2)	11007 ± 364	9013 ± 1911	7609 ± 717	2406 ± 235	14647 ± 1374	8420 ± 790	23282 ± 1963	10260 ± 1038
$AUMC_{0-\infty}$ (ng mL^{-1} h^2)	12457 ± 245	12004 ± 3804	9108 ± 485	2616 ± 331	16286 ± 1420	9884 ± 954	27213 ± 2851	11276 ± 1536
CL (L h^{-1} kg^{-1})	14.57 ± 0.69	16.70 ± 1.04	20.10 ± 1.24	28.58 ± 2.69	7.91 ± 0.44	22.32 ± 1.73	11.28 ± 0.68	17.26 ± 1.33

[a] Mean ± SD, n = 6.

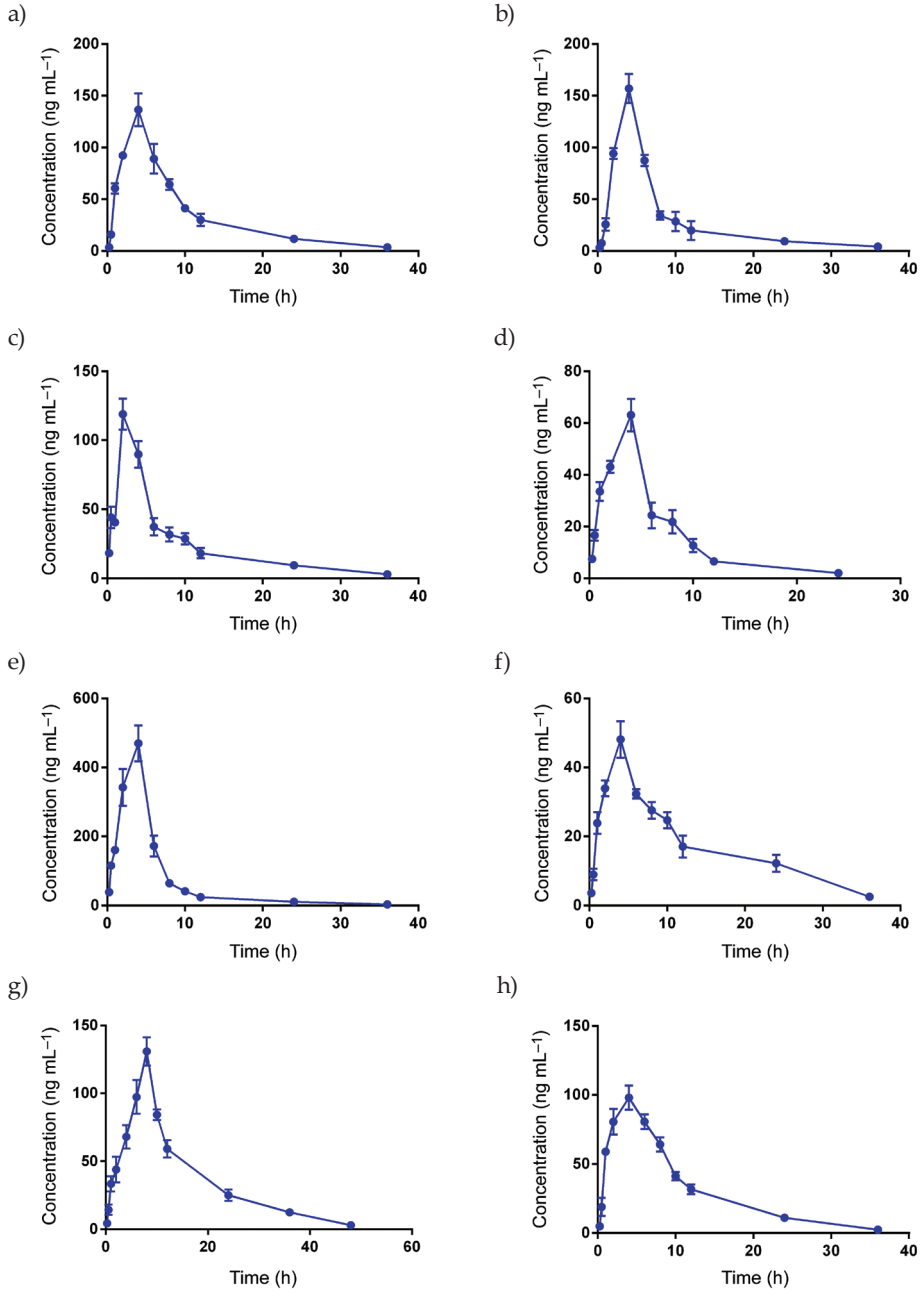

Fig. 5. Plasma concentration-time curves of eight saponins in rats after oral administration of *Rhizoma Anemarrhenae* saponin extract: a) neomangiferin, b) mangiferin, c) timosaponin E1, d) timosaponin E, e) timosaponin B-II, f) timosaponin B-III, g) timosaponin AIII, h) timosaponin A-I. Mean ± SD, $n = 6$.

(T_{max} 7.85 h) value. The T_{max} values of the other six saponins were approximately 4 h. The elimination half-life ($t_{1/2}$) of the eight saponins ranged from 4.06 to 9.77 h, with timosaponin AIII showing the highest value (9.77 h), indicating a relatively long body residence time and slow excretion. The plasma concentrations of the eight saponins were very low, even for timosaponin B-II which dominated in the *Rhizoma Anemarrhenae* saponin extract, indicating a relatively low oral bioavailability for these saponins.

CONCLUSIONS

In this paper, 25 compounds were identified in raw *Rhizoma Anemarrhenae* extract, and eight saponins were further detected in rat plasma after oral administration of the extract by HPLC-Q-TOF/MS. Then, a sensitive, specific and accurate HPLC-MS/MS method was developed, validated and effectively employed in quantitative analysis of eight saponins in the extract (neomangiferin, mangiferin, timosaponin, timosaponin, timosaponin B-II, timosaponin B-III, timosaponin A-III and timosaponin A-I) and in their pharmacokinetic study after oral administration to rats.

Pharmacokinetic parameter, T_{max}, of the eight saponins ranged from 2 to 8 h; elimination half-life ($t_{1/2}$) ranged from 4 to 10 h, indicating slow excretion. Concentrations of the saponins in plasma were all very low, indicating a relatively low oral bioavailability.

This is the first systematic study investigating the pharmacokinetic characteristics of *Rhizoma Anemarrhenae* saponins, therefore, we believe that the results from this research could provide meaningful guidance for future clinical research in pharmacodynamics, pharmacology and toxicology of this plant.

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