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Determination of Tenacissoside A in rat plasma by liquid chromatography–tandem mass spectrometry method and its application to pharmacokinetic study

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ABSTRACT

A sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for the first time for the estimation of Tenacissoside A in the rats' plasma, which is the major active constituent in *Marsdenia tenacissima*. Tenacissoside A was extracted from the rats' plasma by using liquid–liquid extraction (LLE), medroxyprogesterone acetate was used as the internal standard. An Alltech C18 column (250 mm × 4.6 mm, 5 μm) was used to provide chromatographic separation by detection with mass spectrometry operating in selected ion monitoring (SIM) mode. The method was validated over the concentration range of 1–250 ng/mL for Tenacissoside A. The precisions within and between-batch (CV%) were both less than 15% and accuracy ranged from 90 to 102%. The lower limit of quantification was 1 ng/mL and extraction recovery was 88.3% on average. The validated method was used to study the pharmacokinetic profile of Tenacissoside A in rat after administration.

1. Introduction

The stem of *Marsdenia tenacissima* (Roxb.) Wight et Arm. (*Asclepiadaceae*), known as "Tong-guang-teng" in Chinese folk medicine, has long been used as a remedy to treat asthma, cancer, trachitis, tonsillitis, pharyngitis, cystitis, and pneumonia in China [1]. Chemical investigations into *M. tenacissima* resulted in discovery of many kinds of bioactive components associated with effects on human health, such as phenolic acid, steroidal glycosides, alkaloids and flavone [2–4]. Among these complex chemical constituents, the polyoxypregnane glycosides have been regarded as the most important because of its anti-inflammatory and anti-cancer effects [5]. In clinic, Xiao-ai-ping preparations including tablets, injections and syrup which are all the aqueous extractions of the plants were effective to gastric carcinoma and liver cancer [6,7].

Tenacissoside A (TA) is a steroidal glycoside (structure shown in Fig. 1). It is a major active constituent in *M. tenacissima* and has been reported to suppress the multiplication of Lovo cells and MKN28 cells of colon glands [8,9].

Many reports indicated that steroids in *M. tenacissima* could be determined by gas chromatography (GC) [10] and high-performance liquid chromatography (HPLC) [11]. However, TA is a kind of polar, nonvolatile, and heat-labile compounds, it is not optimal to be detected by GC. So HPLC with different detection systems was employed to analyse this compound. The ultraviolet (UV) detector is the most popular detection system in the developing of the analysis method, while in our experiment, TA had poor absorption above 210 nm and it was difficult to detect with UV detector. So, it is much more important to choose the suitable detection system in this experiment. Though the evaporative light scattering detector (ELSD) is suitable to detect TA in the stem of *M. tenacissima* and has been used to develop the fingerprint of *M. tenacissima* and quantify TA in the Xiao-ai-ping preparations [12], it is not sensitive enough to detect TA in pharmacokinetic study with the lower limits of ng/mL. In this paper, we developed a sensitive and rapid LC–ESI-MS/MS method for determination of Tenacissoside A in rats' plasma for the first time, and application to a pharmacokinetic study.

2. Experimental

2.1. Chemicals, reagents and animals

Tenacissoside A was isolated from the stem of *M. tenacissima* by the author (purity > 98%). The internal standard (IS) medroxyprogesterone acetate (Fig. 1) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Methanol (Merck, Germany)

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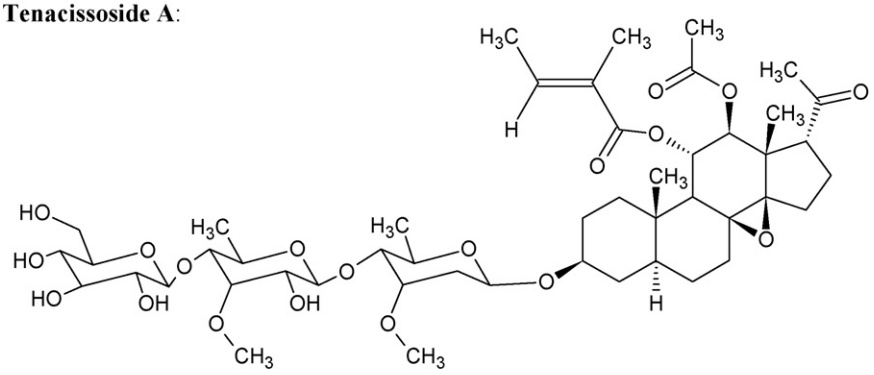
Tenacissoside A:



Medroxyprogesterone acetate:

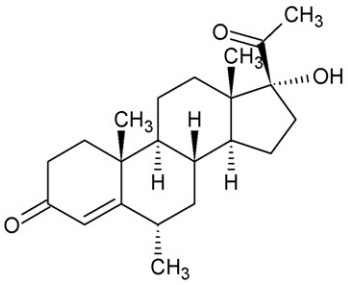


Fig. 1. Chemical structures of Tenacissoside A (TA) and medroxyprogesterone acetate (IS).

was HPLC grade, dichloromethane was commercially available and of analytical grade. Male Sprague–Dawley rats (body weight of 200 ± 20 g) were provided by the Animal Ethics Committee of China Pharmaceutical University. The animals were cared for according as the regulations of the Animal Committee under a constant temperature at 22 ± 1 °C, 12 h light/12 h dark cycle and 10–15 air changes per hour.

2.2. Instrument and analytical conditions

LC–MS/MS analysis was performed using Waters 2695 high-performance liquid chromatography with a photodiode array (PDA) detector together with an automatic liquid chromatographic sampler and an auto-injection system coupled to a Micromass Quattro Ultima tandem quadrupole mass spectrometry (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source using an orthogonal Z-spray electrospray interface (ESI). The LC coupled was carried out on an Alltech C18 column (250 mm × 4.6 mm, 5 μm). The column and autosampler tray temperatures were set at 35 and 4 °C, respectively. A mobile phase composed of methanol–water (87:13, v/v) with a flow rate of 0.3 mL/min was used.

2.3. Mass spectrometric conditions

The effluent from the HPLC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. During the analysis, the ESI parameters were as follows: capillary voltage was 3.0 kV for the positive mode; source temperature was 120 °C; desolvation temperature was 400 °C; cone gas flow was 20 L/h and desolvation gas flow was 500 L/h. The cone voltage of TA was 60 V and the collision energy was 40 eV, and IS was found to be 25 V and 15 eV, respectively. All LC–MS/MS data were processed by the MassLynx version 4.0 NT Quattro data acquisition software.

2.4. Preparation of standard solutions

Stock solution of TA of 1 mg/mL was prepared by dissolving an appropriate amount of the chemical reference substance in methanol. This solution was found to be stable for at least 4 weeks when stored at 4 °C. A series of working solutions were prepared by subsequent dilution of methanol to reach concentration ranges of 1.0–250 ng/mL for TA. A working solution of 0.1 μg/mL for the IS medroxyprogesterone acetate was also prepared in the same solvent and stored at 4 °C.

2.5. Preparation of samples and quality control samples

Vials containing frozen plasma samples were placed in 37 °C water to thaw. 200 μL of plasma and 10 μL of IS working solution were vortex-mixed for 30 s and extracted with dichloromethane (2 mL) by vortex-mixing for 3 min; the tubes were then centrifuged at 3000 rpm for 10 min. The organic phase (1.9 mL) was transferred into another glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL methanol, and centrifuged at 12,000 rpm for 5 min. The supernatant was pipetted to an autosampler vial, and 20 μL was injected into the column for analysis.

Quality control (QC) samples (2, 10, and 100 ng/mL) were made by spiking blank rat plasma with appropriate standard solutions to the required plasma concentrations, followed by the same operation listed above.

2.6. Method validation

2.6.1. Selectivity

Six pre-dose plasma samples from different rats were used to evaluate the specificity. Blank samples were extracted and analyzed by LC–MS/MS assay for potential interfering peaks within the range of the retention time of TA and IS.

2.6.2. Linearity of calibration curves and lower limit of quantification

Five sets of calibration curves of TA were constructed by plotting the peak-area ratios of TA/IS versus plasma additive concentrations. The linearity was detected by calculating the correlation coefficient (r^2) of the curves by means of weighted least-squared linear regression method. The lower limit of quantification (LLOQ) for TA was determined as the plasma concentration of TA giving a signal-to-noise ratio of 10:1.

2.6.3. Recovery and matrix effect

To determine the recovery of TA with the liquid–liquid extraction method, plasma samples were spiked with TA at three concentration levels as QC samples. The recovery was assessed by comparing the peak-area ratios (TA/IS) obtained from spiked plasma samples to the peak-area ratios (TA/IS) spiked in deproteinized plasma samples with methanol.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence ionization of the analyte) was examined by comparing the peak areas of the analytes and IS between two different sets of samples. In set 1, analyte standards were dissolved in the reconstitution solvent and analyzed at concentrations of 2, 10 and 100 ng/mL for the analyses and 0.1 μg/mL for the IS. These analyses were repeated five times at each concentration. In set 2, blank plasma samples obtained from five rats were extracted and then spiked with the same concentrations of analytes and IS in the reconstitution solvent. Deviation of the mean peak areas of set 2 versus set 1 would indicate the possibility of ionization suppression or enhancement for analyses and IS; this is called an 'absolute' matrix effect.

2.6.4. Precision and accuracy

To calculate intra-day and inter-day precision, QC samples with actual concentrations of 2, 10 and 100 ng/mL were reanalyzed for five times on a single day and once on 5 consecutive days, respectively. The determined concentrations, which were obtained from a calibration curve prepared on the same day, were used to evaluate the method accuracy and precision. The accuracy was determined by the relative error (RE%), which was calculated by the equation: (mean of determined concentration − actual concentration)/actual concentration × 100%, and the precision was evaluated by the coefficient of variation (CV%).

2.6.5. Stability

Within-run stability was tested by reanalyzing QC samples at three different concentration levels kept under the autosampler conditions (4 °C) till the end in every routine analysis. The amount of QC samples was at least 5% of the total samples. The long-term stability and the effect of three freeze–thaw cycles were also studied by analyzing QC samples which were stored at −20 °C for a whole month and then followed by three freeze–thaw cycles, each of which contained a storage at −20 °C for 24 h and thaw at 37 °C.

2.7. Pharmacokinetic study

The rats were acclimatized in the facilities for 5 days, and then fasted, had free access to water for 12 h prior to experiment. The dosages of TA were 0.1 mg/kg for intravenous route and 1 mg/kg for intragastric administration. The dosing solution for intravenous route, prepared by dissolving the TA powder in isotonic saline containing 1% ethanol, was delivered using a 1 mL syringe into a rat's femoral vein. The dosage preparation for intragastric approach was made by dispersing the TA powder in water containing 1% ethanol, and mixing well. All preparations were prepared immediately before drug administration.

About 0.5 mL volume of whole blood samples via the post-orbital venous plexus vein were collected in heparinized centrifuge tubes at 0.05, 0.12, 0.18, 0.25, 0.33, 0.5, 1, 1.5, 2, 3, 5 and 7 h for intragastric (i.g.) groups and intravenous (i.v.) group after drug administration. Plasma was transferred and stored at −20 °C till analysis after the centrifugation.

To determine the pharmacokinetic parameters of TA, the pivotal pharmacokinetic parameters were calculated using DAS software (Ver. 1.0, Medical College of Wannan, China). Bioavailability is calculated according to the following equation: $F(\%) = (\text{AUC i.g.}) \times (\text{Dose i.v.})/(\text{AUC i.v.}) \times (\text{Dose i.g.}) \times 100$ [13].

3. Results and discussion

3.1. Selection of IS

It is necessary to use an IS to get high accuracy when a mass spectrometer is used as an HPLC detector. Medroxyprogesterone acetate was chosen in the end because of its similarity of structure (the structure of four rings, Fig. 1), retention time and ionization with the analyte and the less endogenous interferences at product ion of $[M+Na]^+$ at m/z 409.2.

3.2. Chromatography conditions

Elution of TA needed high percentage (at least 80%) of organic solvent in mobile phase, but using high proportion of organic solvent (88% or so), the analytes could not be totally separated from the endogenous plasma components. 87% methanol was the finally optimized ratio. Under the current chromatographic conditions, run-time was set at 5.5 min. To optimize chromatographic conditions and shorten chromatographic cycle time, different methanol ratios have been tried. The retention time of TA was short (3.4 min), thus suitable for the high throughout sample determination in a pharmacokinetic study.

3.3. Mass spectrometric detection condition

Both ESI and APCI with positive or negative ionization have been tested for the determination and the results revealed that both the TA and medroxyprogesterone acetate (IS) were more sensitive in positive ionization mode. Since ESI was suitable to analysis the polar compounds while APCI was good at the nonpolar compounds. According to the structure of TA with the polar group, ESI was the best choice. The advantages of ESI were demonstrated in this experiment by much less matrix influence and better sensitivity.

The responses of TA to ESI were evaluated by recording the mass spectra scanned from m/z 200 to 1000 in both positive and negative ionization modes with this mobile phase condition. The positive mode yielded strong signals of $[M+Na]^+$ for TA (m/z 977.7); on the contrary, no obvious signals were obtained in negative mode under the same signal intensity $[M+Na]^+$ of IS (m/z 409.2) was also predominant under such conditions. Considering the sensitivity, we finally chose $[M+Na]^+$ of TA and IS as the precursor ion, and fragmented by helium gas collision. For TA, the most abundant product ion was m/z 917.6 and for IS was m/z 349.2. These product ions were extracted for quantification (Fig. 2).

3.4. Choice of the extraction method

We explored the feasibility of utilizing liquid–liquid extraction for TA from plasma samples. The deproteinization is much more important. Because if deproteinization was not thorough, the impurity in sample liquid might block the LC–MS. Compared with the

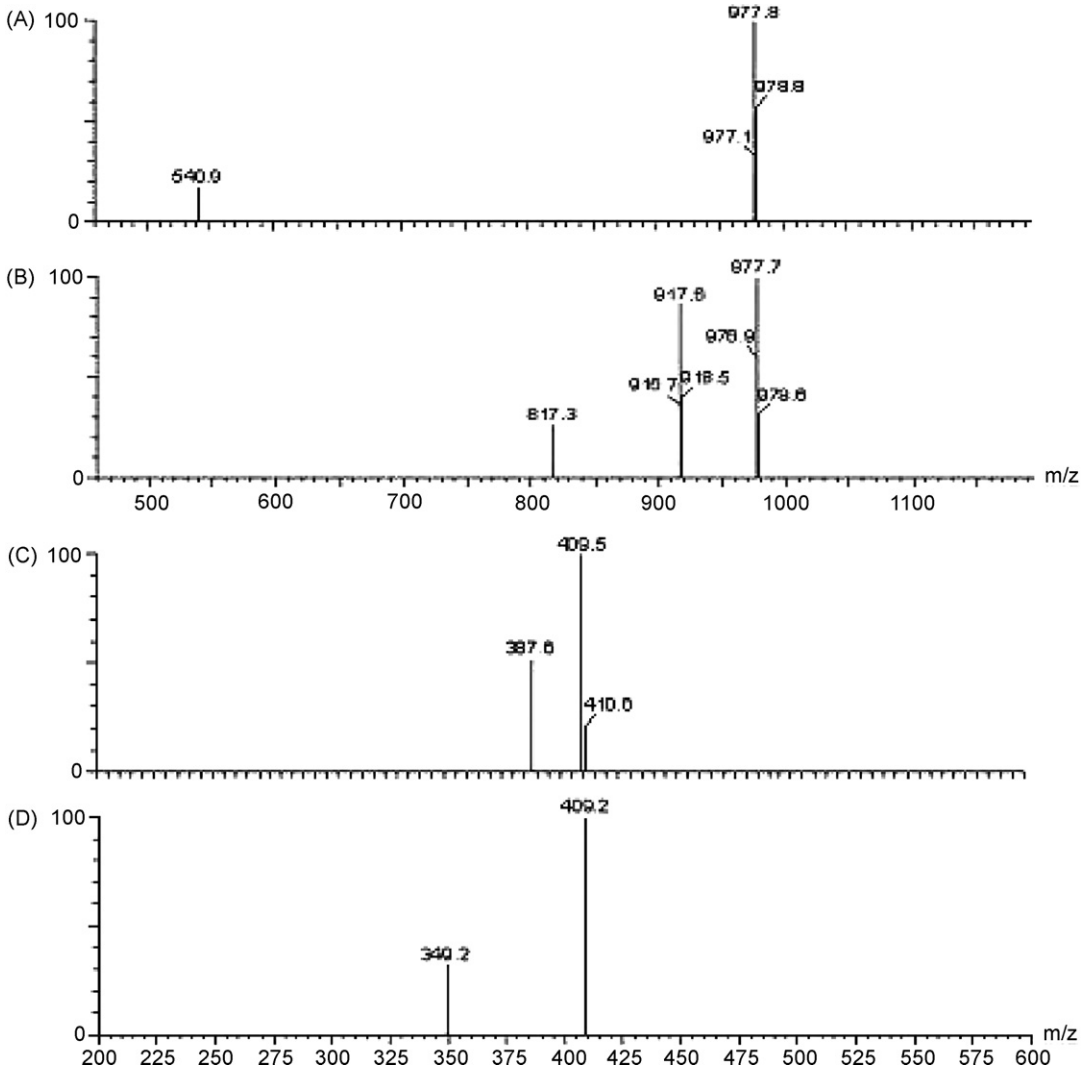


Fig. 2. Mass spectrum TA and IS: (A) full scan mass spectrum of TA; (B) product ion spectrum of $[M+Na]^+$ of TA at *m/z* 977.7; (C) full scan mass spectrum of IS; (D) product ion spectrum of $[M+Na]^+$ of IS at *m/z* 409.2.

more recent and popular technique of solid-phase extraction (SPE), the separation of liquid–liquid extraction (LLE) was through the usage of specific reagents. This resulted in less potential interfering compounds. Furthermore, liquid–liquid extraction was easy and economical.

Different types of solvents with different pH conditions were tested to extract the analyte and IS. TA could be extracted with dichloromethane, diethyl ether, ethyl acetate. While the result of neutralized condition with dichloromethane showed the best recovery. Moreover, IS, which had a similar structure to TA, even had better recovery under current conditions.

3.5. Method validation

3.5.1. Selectivity

In the SIM mode of positive ions, blank plasma yielded relatively clean chromatograms without significant interfering peaks at the retention time of TA and IS. A typical chromatogram of an extract from a dosed rat's plasma containing TA and IS gave peaks not presented in the chromatogram of blank sample (Fig. 3.). The representative peaks had the same *m/z* values as those from standard samples. The retention times of TA and IS were about 3.36 and 4.83 min, respectively.

3.5.2. Linearity of calibration curves and lower limits of quantification

Five sets of the calibration curves showed good linearity from 1 to 250 ng/mL for TA in rat plasma. The following mean regression equation was derived from these calibration curves: $y = 6.5x + 1.18$ ($r^2 = 0.9997$). The lower limit of quantification for TA in plasma, defined at a signal-to-noise ratio of 10:1, was 1 ng/mL.

3.5.3. Recovery and matrix effect

A single-step liquid–liquid extraction with dichloromethane proved to be simple, rapid and successful with an average recovery rate greater than 85% for the analyte under all tested concentrations, indicating the liquid–liquid extraction efficiency was acceptable. The results in detail were shown in Table 1.

For TA and IS, the matrix effects were 95.31% and 93.93%, respectively. These results suggested that negligible matrix effect occurred

Table 1
Recovery of TA from spiked rat plasma ($n = 5$).

Spiked plasma concentration (ng/mL)	Recovery mean ± SD (%)	CV (%)
2	90.2 ± 1.8	2.0
10	87.0 ± 2.5	2.9
100	88.5 ± 3.0	3.4

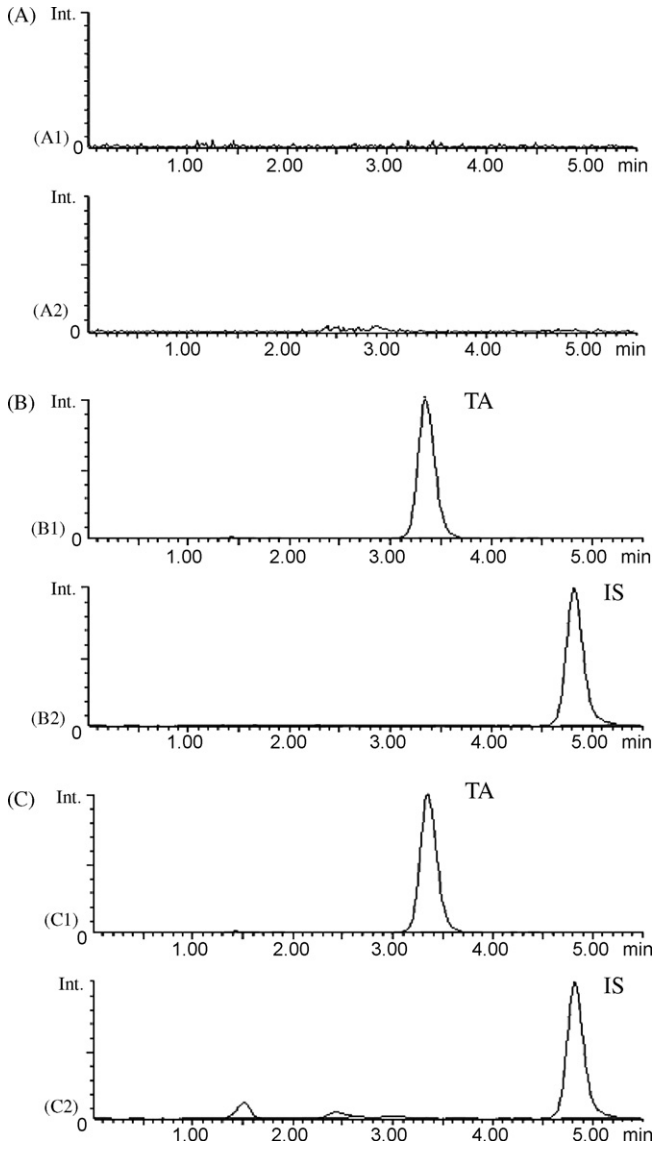


Fig. 3. Representative SIM chromatograms of TA and IS in rat plasma: (A) blank plasma sample; (B) plasma sample spiked with TA and IS; (C) plasma sample 1 h after i.g. administration of TA (1 mg/kg).

in this method, indicating that the extracts were "clean" with no co-eluting "unseen" compounds that could influence the ionization of the analytes.

3.5.4. Precision and accuracy

The accuracy and precision of the method for rat plasma were summarized in Table 2. The intra-day and inter-day precision were analyzed by injecting replicates ($n = 5$) of QC samples containing known concentrations of 2, 10, 100 ng/mL of TA. The precision of the method was described as CV%. The intra-day CV% were always below 4.3% and the inter-day CV% were within 6.3%. The accuracy

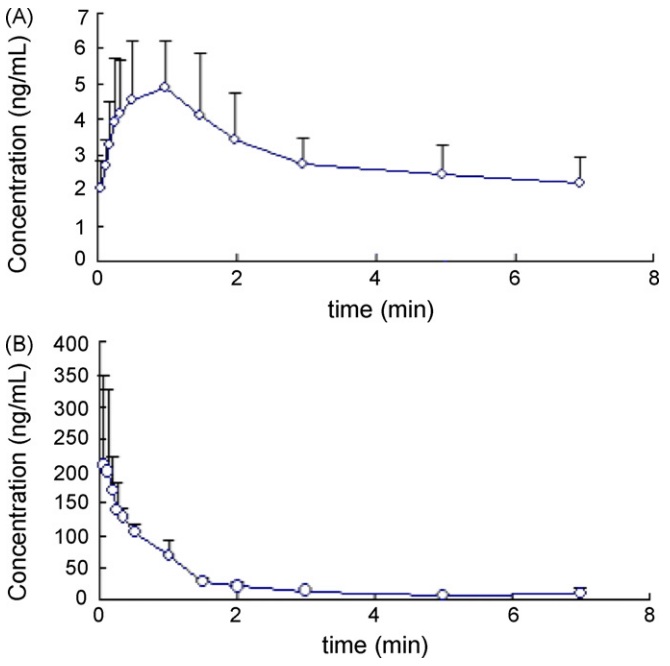


Fig. 4. Mean plasma concentration–time curves of TA after administration ($n = 6$): (A) i.g. administration (1 mg/kg); (B) i.v. administration (0.1 mg/kg).

of the method was within 90–102% of their nominal concentration. The results of the precision and accuracy of the proposed method were acceptable.

3.5.5. Stability

The stability of TA during the sample storing and processing procedures was fully evaluated by analyzing QC samples. The concentration variations after one cycle of freezing and thawing were within ±15% of nominal concentrations, indicating no significant substance loss during thawing and freezing. When processed samples were stored in the autosampler at 4 °C, TA and IS showed good stability as the responses varied no more than ±10% at QC concentrations during 24 h. Meanwhile, storing at −20 °C for a whole month followed by three freeze–thaw cycles did not affect its stability markedly. The results in detail were shown in Table 3.

3.6. Pharmacokinetic study

The developed assay method was applied to a pharmacokinetic study after intragastric administration of 1 mg/kg TA to rats. This simple, precise and accurate LC–MS/MS method yielded satisfactory results for determination of Tenacissoside A in rat plasma and was successfully applied to a pharmacokinetic study after intragastric administration of 1 mg/kg TA and intravenous administration of 0.1 mg/kg TA to rats. The mean plasma concentration–time curves were illustrated in Fig. 4. Based on these results the PK parameters were calculated and summarized in Table 4. Moreover, it was the first time to provide the parameter $t_{1/2}$, C_{max}, AUC,

Table 2
Precision and accuracy of the method for the analysis of TA ($n = 5$).

Spiked plasma concentration (ng/mL)	Intra-day			Inter-day		
	Measured concentration (mean ± SD, ng/mL)	CV (%)	RE (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	RE (%)
2	2.03 ± 0.1	3.2	1.5	1.94 ± 0.1	6.33	−2.7
10	9.05 ± 0.2	2.7	−9.5	9.25 ± 0.4	4.95	−7.5
100	94.78 ± 4.1	4.3	−5.2	93.84 ± 5.4	5.78	−6.2

Table 3
Stability of TA in rat plasma ($n = 5$).

Spiked plasma concentration (ng/mL)	Within-run (kept in autosampler for 24 h)			Long-term and freeze–thaw (stored at −20 °C for 1 month and followed by three freeze–thaw cycles)		
	Measured concentration (mean ± SD, ng/mL)	CV (%)	RE (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	RE (%)
2	2.05 ± 0.12	5.7	2.5	1.92 ± 0.13	6.9	−3.9
10	9.33 ± 0.43	4.6	−6.7	9.56 ± 0.50	5.3	−4.4
100	95.56 ± 3.61	3.8	−4.4	96.32 ± 3.31	3.4	−7.3

Table 4
Pharmacokinetic parameters of Tenacissoside A following intravenous (0.1 mg/kg) and intragastric (1 mg/kg) administration, each value represented the mean ± SD ($n = 6$).

Pharmacokinetic parameters	i.g. administration	i.v. administration
C_{max} (ng/mL)	5.5 ± 1.6	293.4 ± 61.6
MRT (h)	3.2 ± 0.2	1.4 ± 0.4
CL (L/(kg h))	0.37 ± 0.15	0.48 ± 0.08
AUC_{0-7} (ng h mL^{-1})	21.2 ± 6.6	208.1 ± 34.1
$AUC_{0-\infty}$ (ng h mL^{-1})	44.1 ± 12.5	214.6 ± 36.5
F (%)	2.6 ± 0.7	

MRT and many other main pharmacokinetic parameters of TA in rats.

4. Conclusion

This paper described a sensitive, specific, accurate and precise HPLC–MS/MS method for the determination of Tenacissoside A in rat plasma. Good linearity was observed in the range 1–250 ng/mL and the lower limit of quantification (1 ng/mL) makes this method suitable for the pharmacokinetic, bioavailability and bioequivalence studies. To the best of our knowledge there is no LC–MS/MS method that determines Tenacissoside A in rats vivo.

Acknowledgement

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