Original Article
Pharmacokinetic and bioavailability study of tenacissoside G in rat plasma by UPLC-MS/MS

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Abstract: Tenacissoside G, as a major C21 steroidal glycoside, is abundant in Marsdenia tenacissima. In this work, a sensitive and selective UPLC-MS/MS method for determination of tenacissoside G in rat plasma is developed. After addition of tenacissoside I as an internal standard (IS), protein precipitation by acetonitrile-methanol (9:1, v/v) was used to prepare samples. Chromatographic separation was achieved on a UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with 0.1% formic acid and methanol as the mobile phase with gradient elution. An electrospray ionization source was applied and operated in positive ion mode; multiple reactions monitoring (MRM) mode was used for quantification using target fragment ions m/z 815.3→755.4 for tenacissoside G, and m/z 837.3→85.0 for IS. Calibration plots were linear throughout the range 2-2000 ng/mL for tenacissoside G in rat plasma. Mean recoveries of tenacissoside G in rat plasma ranged from 84.2% to 92.3%. RSD of intra-day and inter-day precision were both < 12%. The accuracy of the method was between 97.9% and 107.8%. The method was successfully applied to pharmacokinetic study of tenacissoside G after either oral or intravenous administration. For the first time, the absolute bioavailability of tenacissoside G was reported as high as 29.2%.

Keywords: Tenacissoside G, UPLC-MS/MS, pharmacokinetics, rat plasma

Introduction

Marsdenia tenacissima (Roxb.) Wight et Arn. is a medicinal herb distributed in the southwest of China. Its stem commonly known as "Tong-Guan-Teng" in Chinese folk medicine has been used to treat cancer and inflammation in China [1]. Previous studies have shown that Marsdenia tenacissima extract possesses anticancer activity against many types of cancer cells like lymphoma, esophageal carcinoma and leukemia [2-4] and enhances the effects of chemotherapeutic drugs [5-7]. In clinic, Xiao-ai-ping preparations including injections, tablets and syrup which are the aqueous extractions of the herb have been proved to be effective to various cancers [8, 9]. Chemical investigations into Marsdenia tenacissima have resulted in the discovery of numerous kinds of bioactive components, among which C21 steroidal glycosides are regarded as the main bioactive compo-

nents [10]. Tenacissoside G (**Figure 1A**), as a major C21 steroidal glycoside, is abundant in Marsdenia tenacissima and is therefore considered a promising anti-cancer candidate for drug development.

Quantitative and pharmacokinetical studies on constituents of Traditional Chinese Medicine in plasma are required to offer suitable references in clinical application. However, there exist few reports on the analytical methods quantifying tenacissoside G and no papers on its pharmacokinetic profile. Liquid chromatography-evaporative light-scattering detection (LC-ELSD) was used in methods previously reported for measurement of tenacissoside G which was time-consuming, poorly sensitive and laborious [11, 12].

As a result of recent advances in analysis techniques, ultra-performance liquid chromatogra-

A

B
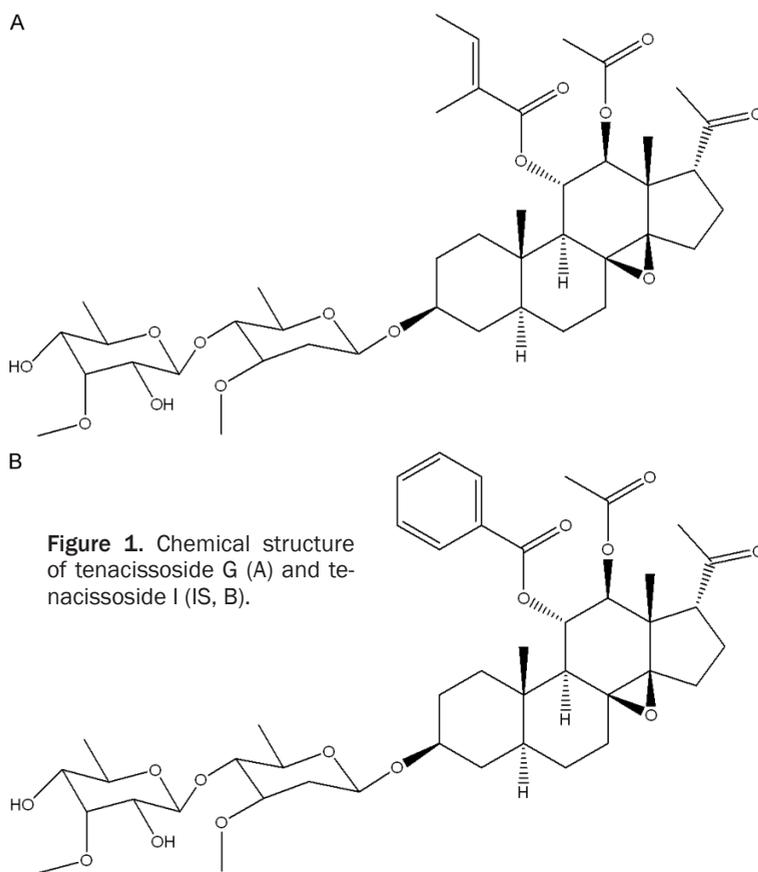
Figure 1. Chemical structure of tenacissoside G (A) and tenacissoside I (IS, B).

(Bedford, MA, USA). Rat blank plasma samples were supplied by drug-free rats (Laboratory Animal Center of Wenzhou Medical University).

Instrumentation and conditions

A UPLC-MS/MS system with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), equipped with an electrospray ionization (ESI) interface, was used to analyze the compounds. The UPLC system was comprised of a Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN). Masslynx 4.1 software (Waters Corp.) was used for data acquisition and instrument control.

Tenacissoside G and tenacissoside I (IS) were separated using a UPLC BEH HILIC column (2.1 mm × 100 mm, 1.7 µm, Waters, USA) maintained at 40°C. The initial mobile phase consisted of acetonitrile and water (containing 0.1% formic acid) with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 2 µL. Elution was in a linear gradient, where the acetonitrile content increased from 20% to 80% between 0 and 1.0 min. The acetonitrile content was maintained at 80% for 1.0 min, then droped to 20% within 0.5 min. The total run time of the analytes was 3 min.

phy coupled with tandem mass spectrometry (UPLC/MS/MS) is recognized as an efficient analytical tool for the quantitative determination of the active compound of herbal drug in biological samples with improved sensitivity, selectivity, and specificity [13-18]. Therefore, in the present study, a simple, precise, and accurate UPLC-MS/MS method for the quantitation of tenacissoside G was established and successfully applied to study the pharmacokinetics in rats. To our best knowledge, this is the first method ever reported for the quantitative analysis of tenacissoside G in biological samples using UPLC-MS/MS.

Experimental

Chemicals and reagents

Tenacissoside G (purity > 98%) and tenacissoside I (IS, purity > 98%) were purchased from the Chengdu Mansite Pharmaceutical CO. LTD. (Chengdu, China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q purification system

Mass spectrometric detection was performed on a triple-quadrupole mass spectrometer equipped with an ESI interface in positive mode. Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (50 L/h). Ion monitoring conditions were defined as capillary voltage of 2.5 kV, source temperature of 150°C, and desolvation temperature of 500°C. Multiple reaction monitoring (MRM) modes of m/z 815.3→755.4 for tenacissoside G, and m/z 837.3→85.0 for IS were utilized to conduct quantitative analysis.

Calibration standards and quality control samples

The stock solutions of tenacissoside G (1.0 mg/mL) and tenacissoside I (IS) (1.0 mg/mL) were prepared in methanol-water (50:50). The 0.5 μg/mL working standard solution of the IS was prepared from the IS stock solution by dilution with methanol; working solutions for calibration and controls were prepared from stock solutions similarly, using methanol diluent. All of the solutions were stored at 4°C and were brought to room temperature before use.

Tenacissoside G calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were offset to range between 2-2000 ng/mL for tenacissoside G in rat plasma at 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL, each by adding 10 μL of the appropriate working solution to 100 μL of blank rat plasma, followed by short vortex mixing. Quality-control (QC) samples were prepared in the same manner as the calibration standards, in three different plasma concentrations (4, 800, and 1600 ng/mL). The calibration standards and QC samples protein precipitation by acetonitrile-methanol (9:1, v/v) before UPLC-MS/MS analysis.

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 10 μL of the IS working solution (0.5 μg/mL) was added to 100 μL of the collected plasma sample in a 1.5 mL centrifuge tube, followed by the addition of 200 μL of acetonitrile-methanol (9:1, v/v). The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 g for 10 min, the supernatant (2 μL) was injected into the UPLC-MS/MS system for analysis.

Method validation

Rigorous tests for selectivity, linearity, accuracy, precision, recovery, and stability, according to the guidelines set by the United States Food and Drug Administration (FDA) [19] and European Medicines Agency (EMA) [20], were conducted in order to thoroughly validate the proposed bioanalytical method. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples.

The selectivity of the method was evaluated by analyzing blank rat plasma, blank plasma-spiked tenacissoside G and IS, and a rat plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of tenacissoside G-to-IS were plotted against analyte concentrations. Resultant standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration $(1/x)$ in the concentration range of 2-2000 ng/mL. The LLOQ was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rat plasma was extracted and spiked with the analyte at 4, 800, and 1600 ng/mL concentrations (n = 6). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, this peak area ratio is defined as the matrix effect. The matrix effect of the IS was evaluated at a concentration of 50 ng/mL in a similar manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (4, 800, and 1600 ng/mL) over three days of validation testing. The precision is expressed as RSD.

The recovery of tenacissoside G was evaluated by comparing the peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts (n = 6). The recovery of the IS was determined in the same way.

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy [21].

Stability values of tenacissoside G in rat plasma were evaluated by analyzing three replicates of plasma samples at concentrations of 4 or 1600 ng/mL which were all exposed to different conditions. These results were compared with the freshly-prepared plasma samples. Short-term stability was determined after the exposure of the spiked samples to room
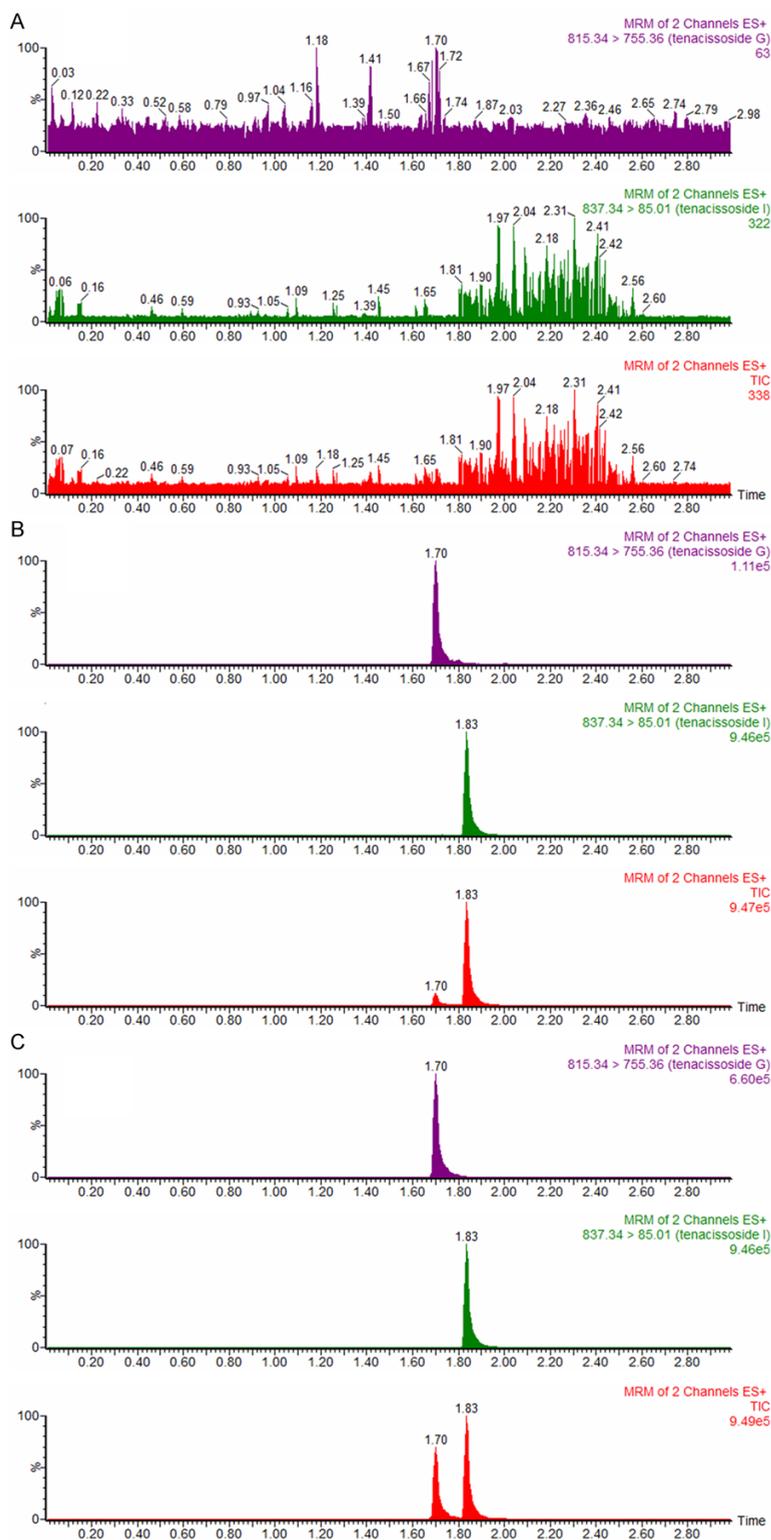
Figure 2. Representative UPLC-MS/MS chromatograms of tenacissoside G and tenacissoside I (IS). A. Blank plasma; B. Blank plasma spiked with tenacissoside G (2 ng/mL) and IS (50 ng/mL); C. A rat plasma sample 4 h after intravenous administration of single dosage 2 mg/kg Tenacissoside G.

HPLC autosampler at room temperature for 24 h. Freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25°C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at -20°C for 20 days. The stability of the IS (50 ng/mL) was evaluated similarly [22, 23].

Pharmacokinetic study

Male Sprague-Dawley rats (200-220 g) were obtained from the Laboratory Animal Center of Wenzhou Medical University to study the pharmacokinetics of tenacissoside G. All twelve rats were housed at the Laboratory Animal Center of Wenzhou Medical University. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University, and were in accordance with the Guide for the Care and Use of Laboratory Animals. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.0333, 0.15, 0.5, 1, 1.5, 2, 3, 4, 6, 8 h after oral (10 mg/kg) or intravenous (2 mg/kg) administration of tenacissoside G. Tenacissoside G (40 mg) was dissolved in 4 mL saline with little 0.1% HCl, about 3 mL for oral admnistration and 1 mL for intravenous admnistration. The samples were immediately centrifuged at 3000 *g* for 10 min. The plasma as-obtained (100 μL) was stored at -20°C until analysis.

temperature for 2 h, and the ready-to-inject samples (after protein precipitation), in the

Plasma tenacissoside G concentration versus time data for each rat was analyzed by DAS
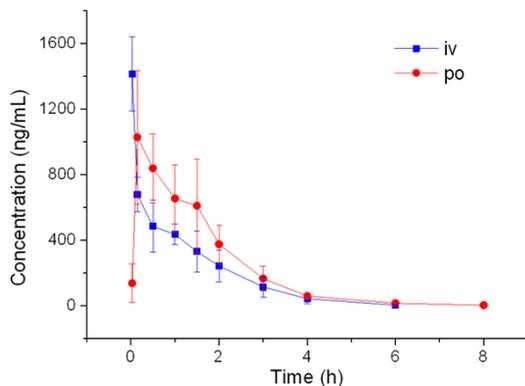
Figure 3. Mean plasma concentration time profile after oral (10 mg/kg) and intravenous (2 mg/kg) administration of tenacissoside G in rats.

(Drug and statistics) software (Version 2.0, Wenzhou Medical University). The maximum plasma concentration (C_{max}) was observed directly from the concentration-time curve. The area under the plasma concentration-time curve (AUC) was estimated by the trapezoidal rule. The plasma clearance (CL), apparent volume of distribution (V), and the half-life ($t_{1/2}$) were estimated using non-compartmental calculations performed with DAS software. The absolute bioavailability (F_{abs}) is the dose-corrected area under curve (AUC) non-intravenous divided by AUC intravenous. The formula for calculating F for a drug administered by the oral route (po) is given below.

$$F_{abs} = 100 \times \frac{AUC_{po} \times Dose_{iv}}{AUC_{iv} \times Dose_{po}}$$

Results and discussion

Selectivity and matrix effect

Figure 2 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with tenacissoside G and IS, and a plasma sample. There were no interfering endogenous substances observed at the retention time of the tenacissoside G and IS.

The matrix effect for tenacissoside G at concentrations of 4, 800, and 1600 ng/mL were measured between 106.4% and 110.4% (*n* = 6). The matrix effect for IS (50 ng/mL) was 103.6% (*n* = 6). As a result, matrix effect from plasma is considered negligible in this method.

Calibration curve and sensitivity

Linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 2-2000 ng/mL for tenacissoside G in rat plasma. The equation utilized to express the calibration curve is: *y = 0.000899285*x+0.00179665*, *r* = 0.9965, where *y* represents the ratios of tenacissoside G peak area to that of IS, and *x* represents the plasma concentration. The LLOQ for the determination of tenacissoside G in plasma was 2 ng/mL. The precision and accuracy at LLOQ were 13.4% and 112.5%, respectively. The LOD, defined as a signal/noise ratio of 3, was 0.7 ng/mL for tenacissoside G in rat plasma.

Precision, accuracy and recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three days of validation tests. Intra-day precision was 5% or less, and inter-day precision was 12% or less at each QC level. The accuracy of the method ranged from 97.9% to 107.8% at each QC level. Mean recoveries of tenacissoside G were higher than 86.1%. Absolute recoveries were between 83.2% and 84.2%. The recovery of the IS (50 ng/mL) was 87.6%.

Carry-over

None of the analytes showed any significant peak (≥ 20% of the LLOQ and 5% of the IS) in blank samples injected after the ULOQ samples. Adding 0.5 extra minutes to the end of the gradient elution effectively washed the system between samples, thereby eliminating carry-over [21].

Stability

Results from the auto-sampler showed that the analyte was stable under room temperature, freeze-thaw, and long-term (20 days) conditions, confirmed because the bias in concentrations were within ± 12% of their nominal values. To this effect, the established method is suitable for pharmacokinetic study.

Application

The method was applied to a pharmacokinetic study in rats. The mean plasma concentration-

Table 1. Primary pharmacokinetic parameters after oral and intravenous administration of tenacissoside G in rats (n = 6)

Parameters	Unit	Mean	SD	Mean	SD
		po 10 mg/kg		iv 2 mg/kg	
$AUC_{(0-t)}$	ng/mL*h	1811.7	514.7	1238.9	312.3
$AUC_{(0-\infty)}$	ng/mL *h	1817.3	508.6	1246.4	318.7
$t_{1/2}$	h	0.9	0.3	0.7	0.2
CL	L/h/kg	5.9	1.8	1.7	0.5
V	L/kg	7.9	5.3	1.7	0.4
C_{max}	ng/mL	1044.7	382.5	1414.7	225.5
Absolute bioavailability/F_{abs}		29.2%			

time curve after oral (10 mg/kg) or intravenous (2 mg/kg) administration of tenacissoside G is shown in **Figure 3**. Primary pharmacokinetic parameters, based on non-compartment model analysis, are summarized in **Table 1**.

The pharmacokinetic profile of tenacissoside G in rats was characterized for the first time in this study, and the absolute bioavailability of tenacissoside G was reported as high as 29.2%, it helps to build a better understanding of the pharmacological features of tenacissoside G. No current UPLC-MS/MS method exists for the determination of tenacissoside G to characterize pharmacokinetic properties.

Conclusion

In present study, a simple, precise, and accurate UPLC-MS/MS method for the quantitation of tenacissoside G in rat plasma was established, utilizing 100 µL of plasma with an LLOQ of 2 ng/mL. The UPLC-MS/MS method was successfully applied to a pharmacokinetic study of tenacissoside G after both oral and intravenous administration. Notably, the pharmacokinetic profile of tenacissoside G in rats was characterized for the first time. The absolute bioavailability of tenacissoside G was identified at 29.2% for first time, as well.

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Disclosure of conflict of interest

None.

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