Journal of Chromatography B, 879 (2011) 1033–1037

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous quantification of tracheloside and trachelogenin in rat plasma using liquid chromatography/tandem mass spectrometry

Li Li[a,b], Fanhua Meng[a], Jifen Guo[a,*], Lu Sun[b], Nengjiang Yu[a], Yimin Zhao[a]

[a] Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Beijing 100850, China
[b] Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China

ARTICLE INFO

Article history:
Received 20 August 2010
Accepted 12 February 2011
Available online 16 March 2011

Keywords:
Lignan
Tracheloside
Trachelogenin
Tandem mass spectrometry
Quantification
Pharmacokinetics

ABSTRACT

We developed and validated a quantitative method for simultaneously determining the concentrations of tracheloside and trachelogenin in rat plasma. Plasma samples were prepared by liquid–liquid extraction with ethyl acetate. Isocratic chromatographic separation was performed on a reversed-phase Diamonsil C_{18} column (4.6 × 200 mm, 5 μm). The mobile phase consisted of methanol and 10 mM aqueous ammonium formate (80:20, v/v). Analyte detection was achieved by positive electrospray ionization (ESI) tandem mass spectrometry. Calibration was performed by internal standardization with glipizide, and regression curves ranging from 0.625 to 625 ng/mL were constructed for both the analytes. The intra- and inter-day precision values were below 8%, and accuracy ranged from −5.33% to 2.53% in all quality control samples. In this study, the validated method was successfully applied to determine the pharmacokinetic profile of tracheloside and trachelogenin in rat plasma after oral and intravenous administration of trachelospermi total lignans.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Tracheloside and trachelogenin are the major components of the total lignan extract obtained from the stems of *Trachelospermum jasminoides*, which is a traditional Chinese medicine [1]. *T. jasminoides* has been shown to exhibit anti-inflammatory and anti-cancer properties, among others [2–5]. In advanced pharmacological studies, the total lignan extract from *T. jasminoides* showed significant anti-inflammatory and analgesic effects [6]. To further understand the biological effects and pharmacokinetic properties of the total lignan extract, it is necessary to develop a specific bioanalytical assay for the evaluation of the major effective constituents of the extract, tracheloside and trachelogenin.

Lignans are found in many medicinal plants and possess a variety of biological properties [7–13]. Techniques used to analyze lignans in biological samples include high-performance liquid chromatography (HPLC) coupled with UV [14], spectrofluorimetry [15], and gas chromatography/mass spectrometry (GC/MS) [16,17]. Liquid chromatography/mass spectrometry (LC/MS) [18,19] and tandem mass spectrometry (LC/MS/MS) [20–22] methods have also been used for this purpose. Recently, Boldizsár et al. confirmed that the butyrolactone-type lignans (tracheloside, trachelogenin, arctiin and arctigenin) possess the similar fragmentation behavior by GC/MS and LC/MS/MS [23]. However, no method for the determination of tracheloside and trachelogenin in biological samples has been described. In this study, we developed a selective and sensitive LC/MS/MS method to simultaneously determine the concentrations of tracheloside and trachelogenin in rat plasma after oral and intravenous administration of trachelospermi total lignans. This method has been completely validated for sensitivity, selectivity, accuracy and precision, matrix effects, recovery, and stability. The validated method will be useful to study the absorption and bioavailability of the total lignan extract derived from *T. jasminoides*.

2. Materials and methods

2.1. Materials

Tracheloside and trachelogenin were isolated from the stems of *T. jasminoides* in our laboratory, and their structures were confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. The purities of the compounds were greater than 98%, as determined by reverse phase-HPLC (RP-HPLC). An internal standard (IS), glipizide, was obtained from Beijing Institute of Pharmaceutical Research. Trachelospermi total lignans tablets were obtained from Beijing Institute of Pharmacology and Toxicology. Methanol (HPLC grade) was purchased from Fisher Scientific. Ethyl acetate was purchased from Burdic & Jackson. All other chemicals and solvents used were of analytical grade and were purchased from Beijing Chemical Reagent Company (Beijing, China). Milli-Q

* Corresponding author. Tel.: +86 10 66931614; fax: +86 10 68211656.
E-mail address: guojifen@sohu.com (J. Guo).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.02.021

Table 1
Precision and accuracy of quality control (QC) samples in rat plasma ($n = 3$ days, 6 replicates per day).

Analyte	Added C (ng/mL)	Found C (ng/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
Tracheloside	1.25	1.28	4.72	0.84	2.53
	12.5	12.6	7.94	3.07	0.58
	625	636	5.56	7.18	1.82
Trachelogenin	1.25	1.27	5.55	2.70	1.56
	12.5	12.6	5.88	1.84	0.84
	625	592	4.13	6.35	−5.33

(Millipore, Bedford, MA, USA) deionized water was used throughout the study.

2.2. Instrumentation

An Agilent 1100 system (Palo Alto, CA, USA) consisting of a vacuum degasser, a quaternary pump, and an autosampler was used for solvent and sample delivery. An AB MDS Sciex API 3000 triple-quadrupole mass spectrometer (Concord, Ontario, Canada) equipped with a Turbo IonSpray ESI source was used for mass analysis and detection. Analyst 1.4 software (AB MDS Sciex) was used for data acquisition.

2.3. Liquid chromatographic conditions

Chromatographic separation was achieved on a Diamonsil $C_{18}(2)$ column (200 × 4.6 mm; internal diameter (i.d.), 5 μm; Dikma, Beijing, China) and a C_{18} guard column (4 × 3.0 mm; i.d., 5 μm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 24 °C. The mobile phase was an isocratic solvent system consisting of methanol and 10 mM aqueous ammonium formate (80:20, v/v), at a flow rate of 0.5 mL/min.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. The tuning parameters were carefully optimized for tracheloside and trachelogenin. Ultrapure nitrogen was used as a nebulizer, curtain, and collision-activated dissociation (CAD) gas at 8, 11, and 4 instrument units, respectively. The Turbo IonSpray voltage and temperature were set to 4500 V and 400 °C, respectively. Quantification was performed by multiple reaction monitoring (MRM) of the transitions of m/z 568 → m/z 389 and 371 for tracheloside, m/z 406.6 → m/z 247 and 151 for trachelogenin, and m/z 446 → m/z 321 for glipizide (IS). Each transition was monitored with a 150 ms dwell time. The optimized collision energies (CE) of 19, 32, and 19 eV were selected for tracheloside, trachelogenin, and IS, respectively.

2.5. Preparation of standard and quality control samples

Stock solutions of the analytes were prepared at 1 mg/mL in methanol:water (1:1, v/v) and stored at −20 °C. A series of standard working solutions for tracheloside and trachelogenin were prepared by diluting stock solutions with methanol:water (1:1, v/v) to concentrations in the range of 2.5–2500 ng/mL. The IS solution was brought to a final concentration of 200 ng/mL in methanol:water (1:1, v/v). All working solutions were stored at 4 °C and were brought to room temperature before use. Calibration standards and quality control samples were prepared by spiking 50 μL of the working solutions and 50 μL of IS into 200 μL of drug-free rat plasma. Matrix-matched tracheloside and trachelogenin calibration standards were prepared in plasma at concentrations of 0.625, 1.25, 2.5, 6.25, 12.5, 25, 125, and 625 ng/mL. The tracheloside and trachelogenin quality control (QC) samples were prepared in plasma at concentrations of 1.25, 12.5, and 625 ng/mL.

2.6. Sample preparation

Plasma samples, calibration standards, and QC samples were extracted by using the liquid–liquid extraction technique. First, 50 μL of the IS working solution, 100 μL of methanol:water (1:1, v/v) mixture, and 50 μL of ascorbic acid (20 mg/mL) were added to 200 μL of rat plasma, and then 2 mL of ethyl acetate was added to the mixture. The mixture was vortexed for 3 min and centrifuged at 3000 r/min for 10 min. The supernatant organic layer was transferred to another tube and evaporated under a gentle stream of air at 40 °C until dry. The residue was reconstituted in 150 μL mobile phase. A 30 μL aliquot of the solution was injected into the LC/MS/MS system for analysis. Plasma samples at concentrations above the calibration range were diluted with blank plasma.

2.7. Method validation

During the pre-study validation, the calibration curves were defined in 3 runs on the basis of triplicate assays of the spiked plasma samples, and on the same day, QC samples from 3 concentrations (see Table 1) were determined in replicates ($n = 6$) to determine the method accuracy and precision. Calibration curves were constructed using a $1/x^2$ weighted linear regression of the peak:area ratios of analyte:IS versus the plasma concentration of the analyte. During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate, and the unknown samples. Extraction recovery was measured by comparing the response of the analytes, spiked before and after sample preparation.

2.8. Animal studies

Sprague–Dawley (SD) rats (male and female, 180–200 g) were supplied by Beijing Vital River Lab Animal Technology Co., Ltd. One day before drug administration, the rats were fasted overnight but were provided water *ad libitum*. The trachelospermi total lignans were dissolved in physiological saline or corn oil and administered to the rats by oral gavage (200 mg/kg) or were dissolved in physiological saline and injected via the tail vein (15 mg/kg). Blood samples were withdrawn from the postocular ophthalmic vein at 10 min, 20 min, 40 min, 1.0 h, 1.5 h, 2.0 h, 4.0 h, 8.0 h, 12 h, and 30 h after oral administration or at 5 min, 10 min, 20 min, 40 min, 1.0 h, 1.5 h, 2.0 h, 4.0 h, 8.0 h, 12 h, 24 h, and 48 h after intravenous administration. Plasma was separated from each sample by centrifugation at 3000 r/min for 10 min and stored at −20 °C until analysis.

3. Results and discussion

3.1. LC/MS/MS optimization

To quantify the analytes using the MRM mode, the full scan and product ion spectra of the 2 analytes and IS were investi-

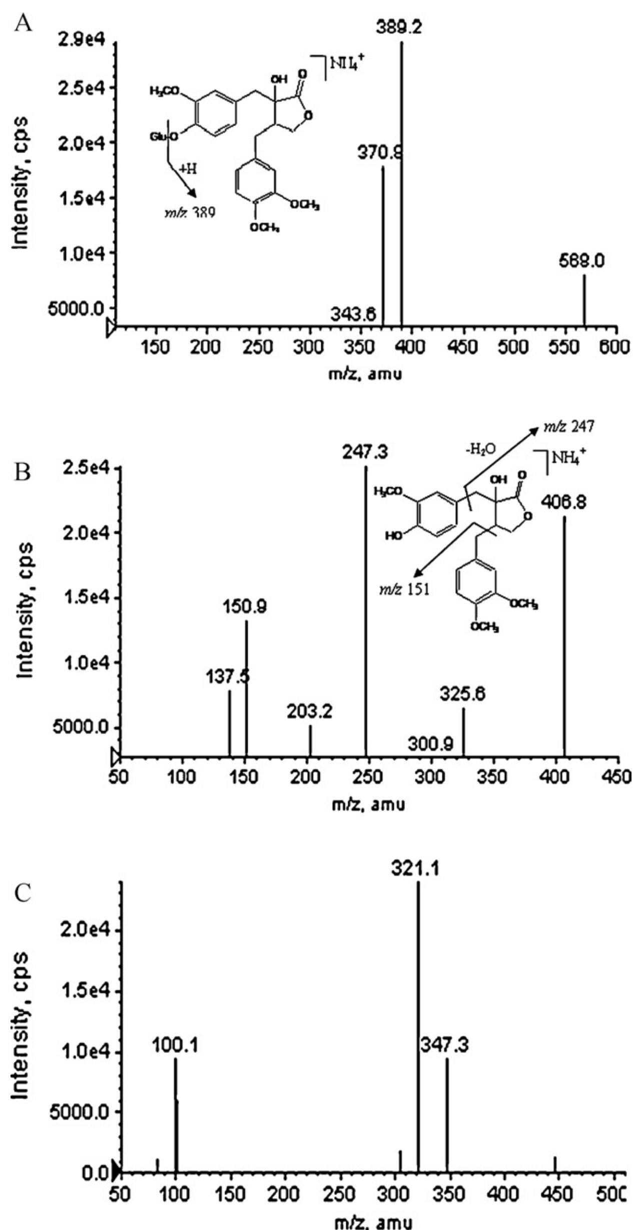

Fig. 1. Product ion spectra of tracheloside (A), trachelogenin (B) and IS (C).

gated. Positive ESI was found to be more sensitive than negative ESI. In the positive ESI, the analytes predominantly formed ammonium adduct ions $[M+NH_4]^+$ in the full-scan spectrum, which has the same fragment ion as the protonated molecular ion $[M+H]^+$. Higher sensitivity could be achieved by the summation of the characteristic fragment intensities. The transitions monitored were m/z 568 → m/z 389 and 371 for tracheloside and m/z 406.6 → m/z 247 and 151 for trachelogenin. The instrument was tuned to yield maximum product ion for each compound. Fig. 1 displays the product ion spectra of the 2 analytes and IS.

Chromatographic separation of tracheloside and trachelogenin is essential to avoid interference. Chromatographic conditions were optimized to reduce analytical cycle time and to obtain good peak shape and resolution. Four C_{18} columns with 5 μm particle size [viz., Nucleosil (50 × 2.0 mm), Gemini (150 × 2.0 mm), Pinnacle II (150 × 2.1 mm), and Diamonsil (200 × 4.6 mm)] were tried to achieve the best efficiency and peak shape. When using the fist three C_{18} columns, the significant ion suppression was observed or the sensitivity was too low. Therefore, a 200 × 4.6 mm Diamonsil C_{18} (2) column was selected to achieve efficient chromatographic separation of the 2 analytes and the endogenous plasma components by eliminating the matrix effect from the endogenous substances. Different mobile phases were evaluated to improve HPLC separation and to enhance MS sensitivity. Methanol was selected as the organic modifier because its ability to ionize tracheloside and trachelogenin is superior to that of acetonitrile. The addition of 10 mM ammonium formate to the mobile phase was found to stabilize the production of ammonium adduct ion $[M+NH_4]^+$ and the symmetry of the chromatographic peak, and it further improved sensitivity over ammonium acetate and *N*-butylamine. An isocratic system with a mobile phase consisting of methanol and 10 mM aqueous ammonium formate (80:20, v/v) was optimal for the analytes with respect to peak shape and mass spectral response. Under these chromatographic conditions, the retention time of tracheloside, trachelogenin, and IS were 4.19, 4.86, and 5.77 min (Fig. 2), respectively. The method was satisfactorily selective for endogenous plasma compounds.

3.2. Internal standard selection

To achieve acceptable performance, especially in LC/MS/MS, an IS that mimics the analyte during the sample extraction, chromatographic elution, and mass spectrometric detection should be selected. An appropriate IS will compensate for potential inconsistent responses due to matrix effects and will not cause interference to the analyte and vice versa. In most cases, using a homolog or structural analog of the analyte as IS is preferable for quantification in complex matrices by LC/MS/MS analysis. However, all structural analogs available at our laboratory are present in the total lignan extract; hence, their use may generate interference during analyte determination. We synthesized acetylated trachelogenin to use as an IS, but it degraded during the extraction procedure. Therefore, we screened other compounds like pelargidenon 1449, rutoside, tricin, glyclopyramide, luteolin, hirsutin, and glipizide for potential use as an IS. Pelargidenon 1449 had a long retention time, and rutoside, tricin, glyclopyramide, luteolin and hirsutin had poor peak shape. Glipizide was selected due to its high extraction recovery, suitable retention time, and optimal analyte correction.

3.3. Sample preparation

Sample preparation is a critical step for accurate and reliable LC/MS/MS assays. Currently, the most widely used methodologies to prepare biological samples are protein precipitation (PPT), solid-phase extraction (SPE), and liquid–liquid extraction (LLE). In this study, we used the LLE procedure, which provided satisfactory extraction efficiency. This method is more stable, reproducible, and specific than the PPT and SPE methods. A number of organic solvents such as ethyl acetate, ethyl ether, N-hexane/dichloromethane/isopropanol, and ethyl acetate/isopropanol were evaluated for use in the LLE method. The capability of N-hexane/dichloromethane/isopropanol in the extraction of analytes is poor; the extraction recovery of ethyl ether and ethyl acetate/isopropanol is lower than ethyl acetate. The results showed that ethyl acetate was preferable for increased extraction recovery for both the analytes and decreased chemical noise in the sample.

3.4. Assay specificity

Method specificity was determined by comparing the MRM chromatograms of blank samples with those of spiked samples. No interference was detected from endogenous substances within the analytes and IS (Fig. 2).

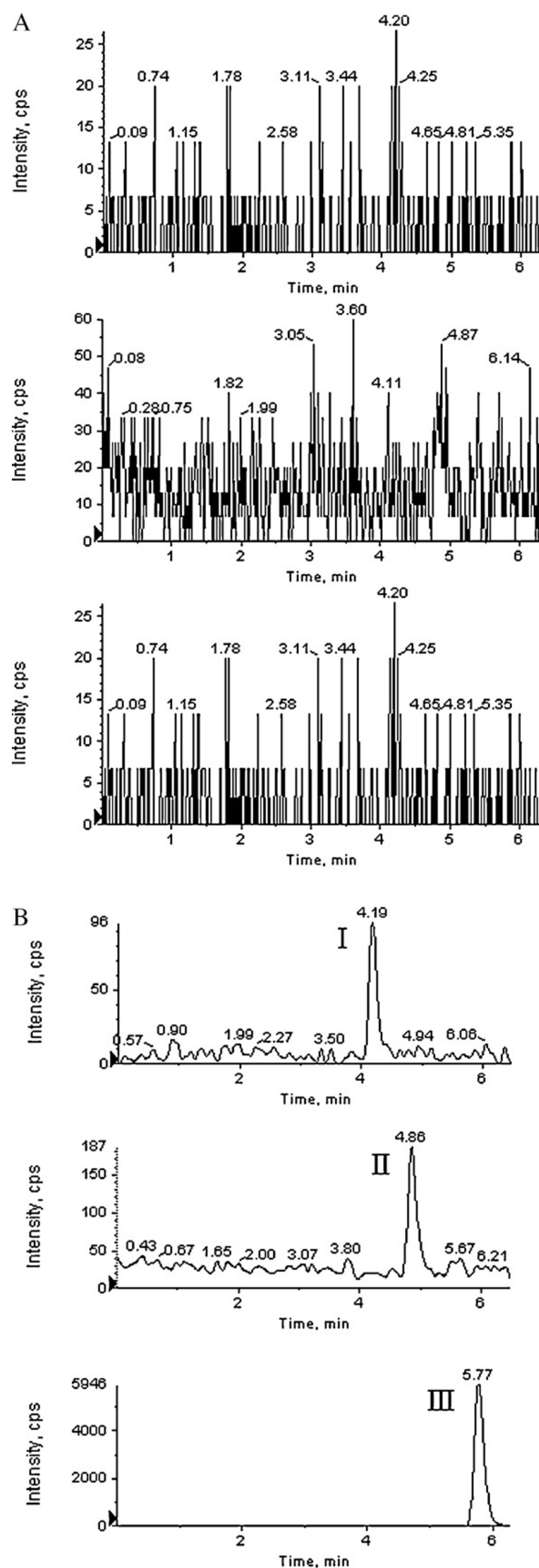

Fig. 2. Representative multiple reaction monitoring (MRM) chromatograms of tracheloside (I), trachelogenin (II), and internal standard (IS) (III) in rat plasma. Blank plasma sample (A), blank plasma sample spiked with tracheloside and trachelogenin at the lower limit of quantification (0.625 ng/mL) and IS (200 ng/ml) (B), and plasma sample obtained from a rat at 40 min after oral administration of 200 mg/kg trachelospermi total lignans tablet (C).

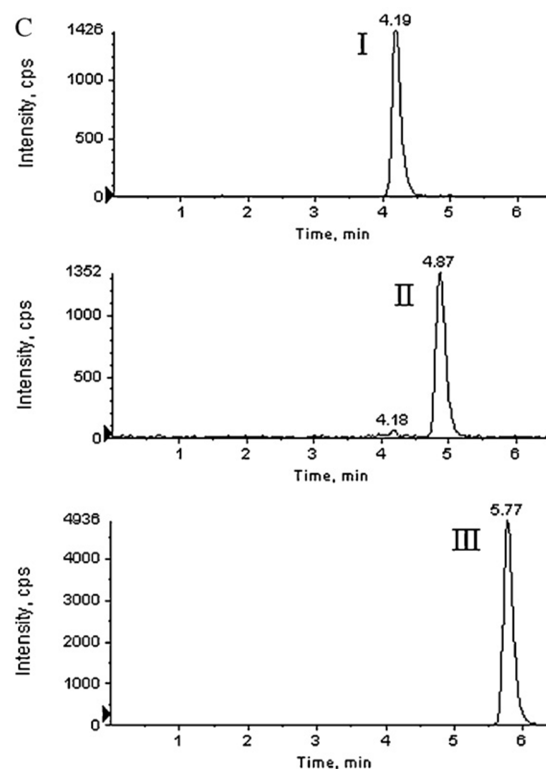

Fig. 2. *(Continued).*

To determine whether a matrix effect is present, the peak area of the compound in the plasma samples spiked after extraction was compared with that of each compound in the mobile phase injected into the system. The results indicated that co-eluting endogenous species did not interfere with the ionization of the analytes and IS. The chosen mobile phase provided adequate separation between the solvent front and the analytes. This could also have reduced the risk of ion suppression.

3.5. Linearity of calibration curve and lower limit of quantification

The linear range was 0.625–625 ng/mL for both the analytes in the rat plasma with correlation coefficients of >0.997. The lower limit of quantification (LLOQ), defined as the lowest concentration analyzed with ±15% accuracy and ≤15% precision, was 0.625 ng/mL for both the analytes.

3.6. Precision, accuracy, and extraction recovery

Assay precision and accuracy were determined by using QC samples at 3 concentrations in replicate ($n = 6$) by performing complete analytical runs on the same day and on 3 consecutive days. The data from these QC samples were analyzed by one-way analysis of variance (ANOVA). The intra- and inter-day precisions were less than 8% for each QC level of the 2 analytes. The accuracy, as assessed by calculating the percentage deviation observed in the analysis of QC samples, was within ±6% for each QC level. The results are summarized in Table 1.

Extraction recoveries of the analytes were determined by comparing the peak area of each analyte in pre-extraction analyte-spiked plasma samples with post-extraction analyte-spiked samples. The results indicated that the extraction recoveries of tracheloside were 93.7% (5.2), 99.2% (5.8), and 98.7% (1.4) at the concentrations of 1.25, 12.5, and 625 ng/mL, respectively. For tra-

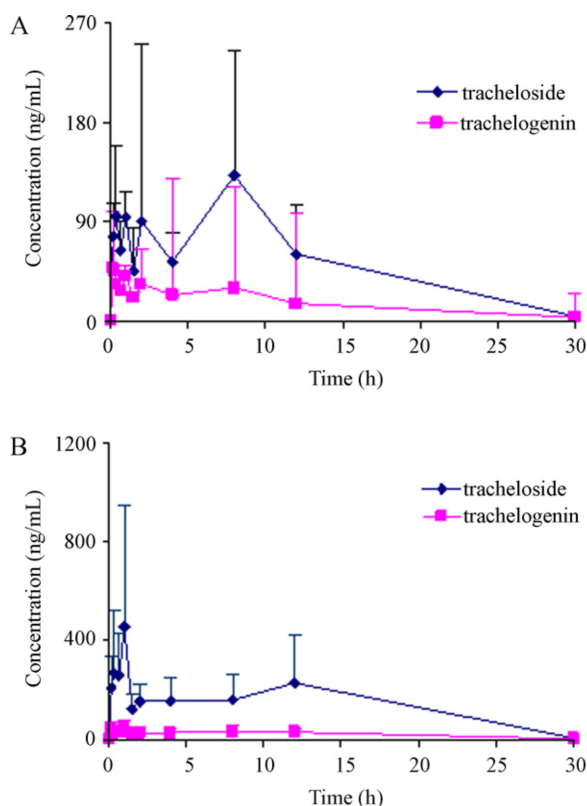

Fig. 3. Mean plasma concentration–time curves of tracheloside and trachelogenin after oral administration of 200 mg/kg trachelospermi total lignans tablet dissolved in physiological saline (A) and corn oil (B) to Sprague–Dawley (SD) rats ($n = 5$).

chelogenin, the extraction recoveries were 89.3% (2.9), 98.8% (7.1), and 88.6% (2.8) at concentrations of 1.25, 12.5, and 625 ng/mL, respectively. The extraction recovery of the IS was 93.7% (5.2).

3.7. Analyte stability

The stability of tracheloside and trachelogenin in rat plasma was investigated under a variety of storage and process conditions. Tracheloside and trachelogenin were found to be unstable in rat plasma; both the analytes decreased in concentration by about 30% after undergoing 3 freeze–thaw (−20 °C-ambient) cycles and decreased by about 60% after 6 h of storage at room temperature. Therefore, it was required to add a stabilizer to the sample. Ascorbic acid and phosphoric acid, which have the same pH, were compared as stabilizers; ascorbic acid was found to provide better stabilization while 15% of the analytes degraded when phosphoric acid was used as the stabilizer. Next, different concentrations (2.0 mg/mL and 20 mg/mL) of ascorbic acid were investigated to obtain the optimal concentration for analyte stabilization in rat plasma. Including ascorbic acid in the sample at 20 mg/mL was found to prevent degradation. However, 25% of the analytes had degraded when a lower concentration of ascorbic acid was used as the stabilizer. The lack of degradation may be due to the reducing and acidic properties of ascorbic acid.

The analyte samples with 20 mg/mL ascorbic acid were found to be stable (RE, <12%) after undergoing 3 freeze–thaw (−20 °C-ambient) cycles or after being stored at room temperature for 6 h. The analytes were also shown to be stable after 24 h of storage in reconstitution solutions at room temperature (RE, <5%).

3.8. Application of the method

Plasma samples from the pharmacokinetic study were analyzed, and the tracheloside and trachelogenin concentrations were successfully measured. Fig. 3 shows the mean plasma concentration–time curves of tracheloside and trachelogenin ($n = 5$) after the administration of the tablet. Additional pharmacokinetic data on tracheloside and trachelogenin in rats or other animals can be easily estimated using this LC/MS/MS analysis; the resulting data may help to clarify the pharmacological mechanisms of total lignan extract derived from *T. jasminoides*.

4. Conclusions

In this study, an LC/MS/MS method, which to our knowledge has not been reported previously, was established, optimized, and validated to guarantee reliable determination of tracheloside and trachelogenin concentrations in rat plasma. This method was successfully applied to pharmacokinetic studies of tracheloside and trachelogenin after oral or intravenous administration of the lignan extract to rats. Trachelospermi total lignans tablets were found to dissolve better in corn oil and exhibit better absorption than when dissolved in physiological saline, with higher absolute bioavailability of the former. Individual pharmacokinetic profiles showed dual peaks, indicating the absorption of lignans via hepato-enteric circulation.

Acknowledgement

This work was supported by a grant from the National Natural Science Foundation of the People's Republic of China (No. 81001468).

References

[1] Chinese Pharmacopoeia Commission, Pharmacopoeia of the People's Republie of China Beijing, Chemical Industry Press, 2005, p. 190.
[2] M.-J. Sheu, P.-Y. Chou, H.-C. Cheng, J. Ethnopharmacol. 126 (2009) 332.
[3] M.H. Lee, J.M. Lee, S.H. Jun, Pharm. Pharmacol. 59 (2007) 123.
[4] R.W. Li, G.D. Lin, S.P. Myers, J. Ethnopharmacol. 85 (2003) 61.
[5] X. Tan, H. Chen, R. Liu, Planta Med. 71 (2005) 93.
[6] N. Yu, Y. Zhao, F. Ren, China Patent No.200510093357.X.
[7] M.L.C. Borsato, C.F.F. Grael, G.E.P. Souza, Phytochemistry 55 (2000) 809.
[8] E. Küpeli, N. Erdemoglu, E. Yesilada, J. Ethnopharmacol. 89 (2003) 265.
[9] H. Abou-Gazar, E. Bedir, S. Takamatsu, Phytochemistry 65 (2004) 2499.
[10] N.M. Saarinen, P.E. Penttinen, A.I. Smedsc, J. Steroid Biochem. Mol. Biol. 93 (2005) 209.
[11] W.M. Mazur, J.A. Duke, K. Wahala, Nutr. Biochem. 9 (1998) 193.
[12] J.D. Brooks, L.U. Thompson, J. Steroid Biochem. Mol. Biol. 94 (2005) 461.
[13] A.-S. A. Newairy, H.M. Abdou, Food Chem. Toxicol. 47 (2009) 813.
[14] A.P. Wilkinson, K. Wahala, G. Williamsona, J. Chromatogr. B 777 (2002) 93.
[15] V. Murugaiyah, K.L. Chan, J. Chromatogr. B 852 (2007) 138.
[16] M. Morton, O. Arisaka, A. Miyake, Environ. Toxicol. Pharmacol. 7 (1999) 221.
[17] T. Nurmi, H. Adlercreutz, Anal. Biochem. 274 (1999) 110.
[18] B.-L. Wang, J.-P. Hu, W. Tan, J. Chromatogr. B 865 (2008) 114.
[19] A. Smedsa, K. Hakalab, J. Chromatogr. B 793 (2003) 297.
[20] U. Knust, W.E. Hull, B. Spiegelhalder, Food Chem. Toxicol. 44 (2006) 1038.
[21] A.I. Smeds, K. Hakala, T.T. Hurmerinta, J. Pharm. Biomed. Anal. 41 (2006) 898.
[22] A.I. Smeds, N.M. Saarinen, T.T.P. Hurmerinta, J. Chromatogr. B 813 (2004) 303.
[23] I. Boldizsár, M. Kraszni, F. Tóth, J. Chromatogr. A 1217 (2010) 6281.