Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 1411–1416

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

A rapid and sensitive liquid chromatography–tandem mass spectrometric method for the determination of timosaponin B-II in blood plasma and a study of the pharmacokinetics of saponin in the rat

Fei Cai[a], Lianna Sun[b], Shouhong Gao[a], Yang Yang[a], Qi Yang[a], Wansheng Chen[a,*]

[a] Department of Pharmacy, Changzheng Hospital, Second Military Medical University, No. 415 Fengyang Road, Shanghai 200003, PR China
[b] Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China

ARTICLE INFO

Article history:
Received 27 July 2008
Received in revised form
17 September 2008
Accepted 18 September 2008
Available online 30 September 2008

Keywords:
Timosaponin B-II
Saponin
Pharmacokinetics
Rat plasma
LC–MS/MS

ABSTRACT

A rapid, sensitive and selective liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the determination of Timosaponin B-II (TB-II), a pharmacologically active constituent isolated from *Anemarrhena asphodeloides*. This method was used to examine the pharmacokinetics and bioavailability of TB-II in rats using ginsenoside Re as an internal standard. After simple protein precipitation of the plasma samples with acetonitrile, the analytes were separated on an ODS column (150 mm × 2.1 mm i.d., 5 μm) with the mobile phase of acetonitrile–water (35:65, v/v) containing 0.05% formic acid and detected by electrospray ionization mass spectrometry in the negative multiple reaction monitoring (MRM) mode with a chromatographic run time of 3.0 min. The calibration curves were linear over the range of 5–15,000 ng/ml and the lower limit of quantification (LLOQ) was 5 ng/ml in rat plasma. In this range, relative standard deviations (R.S.D.) were <7.4% for intra-day precision and <9.0% for inter-day precision. The accuracy was within the range of 97.7–107.3%. The method was successfully applied to assess the pharmacokinetics and oral bioavailability of TB-II after intravenous and oral administration in rats, with the oral bioavailability being only 1.1%.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Furostanol glycosides, common members of steroidal saponins, are widely found in plants. Saponins have a broad range of biological activities, and many important traditional medicinal plants are rich in these compounds. Timosaponin B-II (TB-II), (25S)-26-*O*-β-D-glucopyranosyl-22-hydroxy-5β-furost-3β,26-diol-3-*O*-β-D-glucopyranosyl(1 → 2)-β-D-galactopyranoside (Fig. 1), is a typical furostanol saponin originally isolated from the rhizome of *Anemarrhena asphodeloides* (*Liliaceae*), which has long been included in Chinese traditional medical recipes for treatment of inflammatory diseases. It was reported that timosaponins from *A. asphodeloides* suppressed the superoxide generation in human neutrophils [1,2]. Studies showed that timosaponins exerted remarkable inhibiting effects on platelet aggregation in human blood [3,4]. Other experimental results displayed that timosaponins could protect vascular cells by inhibiting proliferation and enhancing apoptosis of vascular smooth muscle cells *via* NO-dependent pathway [5]. In addition, our previous study also showed that timosaponins prevented ovariectomy-induced bone loss in rats by the promotion of bone formation [6]. Previous results also demonstrated that timosaponins could improve the learning and memory abilities in memory-deficit rat models [7–9]. Recently, we investigated the effects of TB-II on focal cerebral ischemia-induced dysfunction of learning and memory in rats [10]; and found that the anti-inflammatory mechanism of TB-II was, at least in part, attributable to TB-II-caused increase of anti-inflammatory cytokine interleukin-10 and its receptors in cerebral infracted rats [10].

In view of the great clinical potential of TB-II, it is very important to understand its pharmacokinetic and bioavailability characteristics; to develop a validated analytical method suitable for TB-II determination in relevant biological samples is now an urgent task. Up to now only two studies have been retrieved in literature that identified TB-II in rat serum and urine by liquid chromatography-mass spectrometry [11,12], and there has been no report for the determination of TB-II in biological matrix. In this paper, we aimed to establish a rapid, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of TB-II in rat plasma. The method was successfully applied to the pharmacokinetic and bioavailabil-

* Corresponding author. Tel.: +86 21 25074122; fax: +86 21 33100038.
E-mail addresses: chenwanshengsmmu@yahoo.com.cn, chenws@vnet.citiz.net (W. Chen).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.09.032

ity study of TB-II after intravenous and oral administration in rats.

2. Experimental

2.1. Chemicals and reagents

TB-II (99.1% purity) was isolated and purified from the rhizome of *A. asphodeloides*, and its structure was confirmed by IR, MS and NMR spectroscopy. The internal standard (IS) Ginsenoside Re (Fig. 1, 98.8% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Tedia (Fairfield, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other chemicals were of analytical grade.

2.2. Liquid chromatography–tandem mass spectrometry

The analyses were performed on an Agilent 1200 series high-performance liquid chromatograph (HPLC) and an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, MA, USA). The mobile phase was acetonitrile–water (35:65, v/v) containing 0.05% formic acid. A DIKMA Inertsil ODS-3 column (150 mm × 2.1 mm i.d., 5 μm) with a DIKMA C_{18} guard column (10 mm × 2.1 mm i.d., 5 μm) was used for liquid chromatographic separation. The column was equilibrated and eluted under isocratic conditions with a flow rate of 0.25 ml/min, maintained at 25 °C. The sample injection volume was 10 μl and the run time was 3.0 min. Quantification was performed in negative multiple reaction monitoring (MRM) of the transitions m/z 919 → 757 for TB-II and m/z 945 → 475 for the IS. The detection parameters were optimized as follows: drying gas temperature, 350 °C; drying gas flow, 10 l/min; nebulizer pressure, 40 psi; capillary voltage, 4000 V; fragmentor voltage, 300 V for TB-II and 350 V for the IS; collision energy, 53 eV for TB-II and 51 eV for the IS.

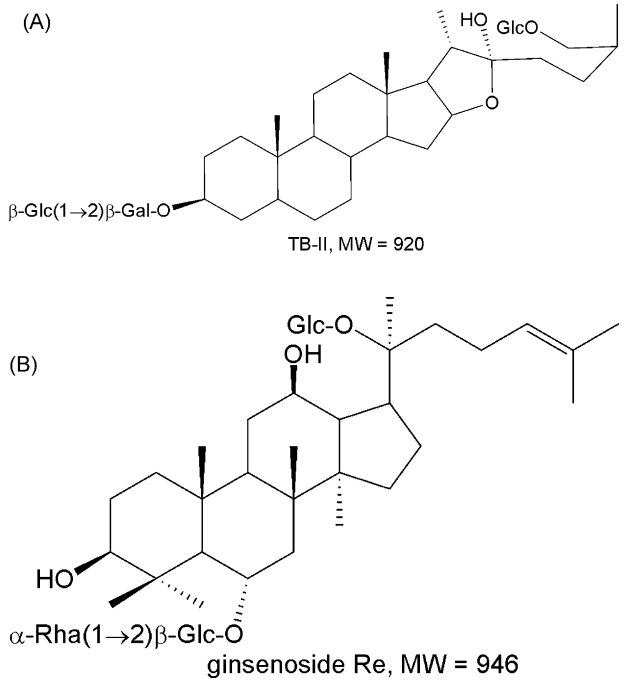

Fig. 1. Chemical structures of TB-II and ginsenoside Re (internal standard). "Glc" means glucose. "Gal" means galactose. "Rha" means rhamnose.

2.3. Preparation of standards and quality control (QC) samples

Stock solution of TB-II (1 mg/ml) was prepared by dissolving the compound in acetonitrile–water (80:20, v/v). IS stock solution (100 μg/ml) was prepared similarly. Pooled rat plasma containing no TB-II was used as the blank plasma to prepare calibration standards and QC samples for this study. TB-II standard working solutions at 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μg/ml were prepared by a serial dilution of the stock solution with acetonitrile–water (50:50, v/v). The calibration standards were prepared by a 1:20 dilution of the corresponding TB-II standard working solutions with the blank plasma to give nominal concentrations of 5, 15, 50, 150, 500, 1500, 5000 and 15,000 ng/ml. TB-II QC samples were independently prepared using a different stock solution to obtain plasma concentrations of 15, 500 and 12,000 ng/ml. All stock solutions, working solutions and plasma samples were stored at −20 °C until use.

2.4. Sample preparation

A simple and rapid protein precipitation method was used for the preparation of plasma samples. 200 μl of acetonitrile containing the IS (150 ng/ml) was added to 100 μl of plasma sample. The mixture was vortexed for 2 min and centrifuged at 13,000 × g for 10 min. Then 150 μl of the supernatant was added to 300 μl of water. After mixing, the solution was transferred to 1.5 ml autosampler vial and a 10 μl aliquot was injected into the LC–MS/MS system for analysis.

2.5. Method validation

Full validation was performed to evaluate the performance of the method based on the recommendations published by FDA [13]. Calibration standards at eight concentrations of TB-II were prepared and assayed. The calibration curves were generated by plotting the peak area ratios of TB-II to the IS versus the concentrations of TB-II with $1/x^2$ weighted least-squares linear regression analysis. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that for each back-calculated standard concentration must be within±15% deviation from the spiked value (±20% at the LLOQ).

When analyzing the supernatant from a plasma sample using protein precipitation, salts and endogenous components are present and they can cause ion suppression or enhancement that will result in greater variation than that of solid-phase and liquid–liquid extracts [14]. Matrix effects, including both absolute and relative matrix effects, and extraction recoveries of TB-II were assessed in quintuple by comparing the peak areas of the analytes between three different sets of standards at three QC concentrations (15, 500 and 12,000 ng/ml) [15]. The same evaluation was performed for the IS. The first set (set 1) was prepared in the mobile phase. The second set (set 2) was prepared in plasma extracts originating from five different rats and spiked after extraction. The third set (set 3) was prepared in plasma from the same five different rats as in set 2, but the plasma samples were spiked before extraction. The absolute matrix effect was assessed by comparing the mean peak areas of the analyte in set 2 to that of the neat standards in set 1. The variability in the peak areas of the analyte in set 2 expressed as relative standard deviation (R.S.D., %) was considered as a measure of the relative matrix effect. The recoveries of TB-II and the IS were determined by calculating the ratios of the mean peak areas of the regularly prepared plasma samples in set 3 to that of the spiked post-extraction samples in set 2.

Intra-day and inter-day validation studies for precision and accuracy were performed at three QC levels (15, 500 and 12,000 ng/ml). To determine intra-day precision and accuracy, the

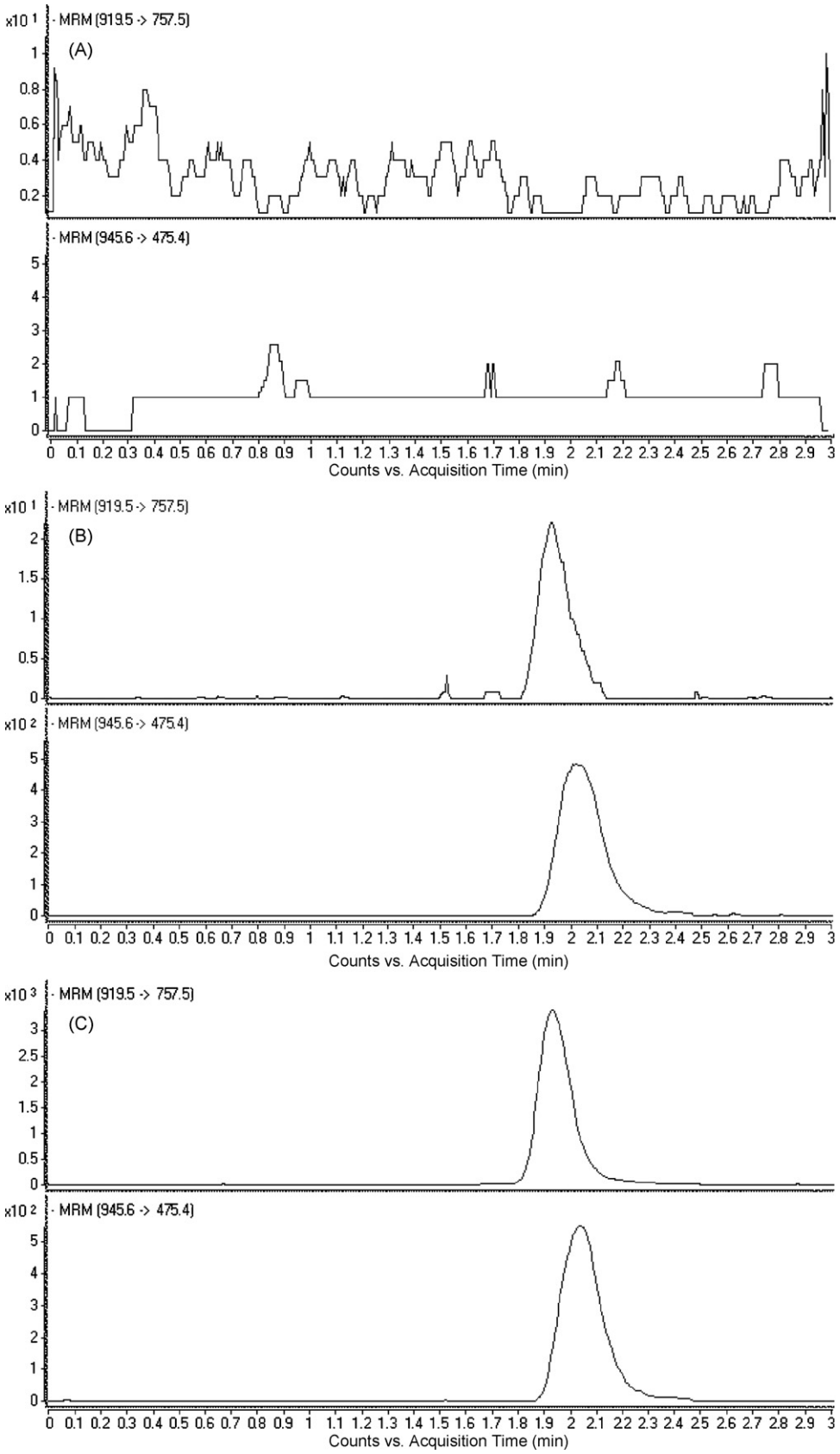

Fig. 2. Representative MRM chromatograms: (A) blank plasma sample; (B) blank plasma sample spiked with TB-II (5 ng/ml) and IS (150 ng/ml); (C) plasma sample collected from a rat 2 h after oral administration of TB-II at a dose of 180 mg/kg (concentration 803.6 ng/ml). The retention times of TB-II and IS were 1.9 and 2.0 min, respectively.

assay was carried out on QC samples at different time points during the same day. Inter-day precision and accuracy were determined by assaying the QC samples over 5 consecutive days. The precision was expressed as R.S.D. (%) and the accuracy was determined as percentage between the mean measured concentrations and the spiked concentrations.

The stability of TB-II was studied under various conditions [13]. The mean values and standard deviations of the ratios between the mean measured concentrations and the spiked concentrations were used for stability evaluation. Freeze-thaw stability was assessed over three cycles. QC samples were thawed at room temperature and refrozen at −20 °C over three cycles and assayed. Short-term stability was assessed by analyzing QC samples kept at room temperature for 4 h to cover the sample preparation. Long-term stability was tested by assaying QC samples after storage at −20 °C for 2 weeks. Post-preparative stability was assessed by analyzing the processed samples kept in the autosampler for 12 h.

2.6. Animals and application of the method

Twelve male Sprague–Dawley rats (220 ± 10 g) were supplied by the Animal Center of the Chinese Academy of Science in Shanghai (Shanghai, China). They were housed in controlled conditions (temperature 23 ± 1 °C; humidity 55 ± 5%; 12 h light/dark cycle) and received a standard rat chow and tap water *ad libitum* for a week prior to experiments. All animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Second Military Medical University.

The animals were fasted overnight prior to dosing and for 4 h post-dose, with water provided *ad libitum*. For intravenous (i.v., 1.8 mg/kg) administration, TB-II dissolved in injection water was delivered using a 1 ml syringe into the caudal vein of rats. For oral (180 mg/kg) administration, a single dose of TB-II in sterile water was delivered by oral gavage using a ball-tipped oral gavage needle and a 2.5 ml syringe. About 0.25 ml blood samples via the postorbital venous plexus veins were collected in heparinized tubes at 2, 5, 10, 20, 30 and 45 min, 1, 1.5, 2, 3, 4, 6 and 10 h after i.v. administration and at 2, 5, 10 and 20 min, 1, 1.5, 2, 3, 4, 6, 8, 10 and 16 h after oral administration. Heparinized blood was centrifuged at 3000 × *g* at room temperature for 5 min to obtain plasma, which was stored at −20 °C until analysis.

Data fitting and pharmacokinetic parameter calculations were carried out using the DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society) in which the non-compartmental model was chosen. C_{max} and T_{max} values were obtained directly from the observed concentration versus time data. All results were expressed as arithmetic mean ± standard deviation (S.D.). Bioavailability was calculated according to the following equation:

$$\text{bioavailability}\,(F\%) = \frac{\text{AUC}_{0\to\infty}(\text{p.o.}) \times \text{dose(i.v.)}}{\text{AUC}_{0\to\infty}(\text{i.v.}) \times \text{dose(p.o.)}} \times 100.$$

3. Results and discussion

3.1. LC–MS/MS optimization

TB-II gave a strong mass response in negative electrospray ionization (ESI) mode. Although the full scan spectra of TB-II showed a fairly strong intensity of $[M+Na]^+$ in the positive mode, the product ion was difficult to find. By negative ESI, TB-II and the IS formed predominantly deprotonated molecular ions $[M-H]^-$ at *m*/*z* 919 and *m*/*z* 945 in full scan mass spectra. After optimization of fragmentor voltage and collision energy, the predominant product ion of TB-II was monitored at *m*/*z* 757 $[M-H-C_6H_{10}O_5]^-$(suggesting a loss of glucose) and that of the IS was monitored at *m*/*z* 475 $[M-H-C_6H_{10}O_4-2C_6H_{10}O_5]^-$(suggesting a loss of rhamnose and two glucose) in the third quadrupole. So the most sensitive mass transitions *m*/*z* 919 → 757 for TB-II and *m*/*z* 945 → 475 for the IS were selected for detection.

With respect to the mobile phase, it was recommended that the analysis of furostanol saponins such as TB-II by HPLC–MS was performed using aqueous acetonitrile mobile phase and avoiding methanol due to the interconversion of the C-22 hydroxy and C-22 methoxy forms [16,17]. Different mobile phases containing varying percentages of acetonitrile were evaluated to improve HPLC separation and enhance sensitivity in MS. To maintain symmetric peak shapes whilst being consistent with good ionization for the analyte and the IS, the addition of formic acid in different concentrations (0, 0.02, 0.05, 0.1 and 0.5%) were tested. The best signal was achieved using acetonitrile–water (35:65, v/v) containing 0.05% formic acid.

Choosing the suitable IS is important to achieve acceptable precision and accuracy, especially with LC–MS/MS, where matrix effects can lead to poor analytical performance. Ginsenoside Re was chosen as the IS because it is a structurally similar saponin to TB-II. They were found to have similar retention times (Fig. 2), similar recoveries (see Section 3.3.2) and similar ionization properties.

3.2. Sample preparation

At the earlier stage of the method development, several liquid-liquid extraction (LLE) methods were investigated. It was found that the recoveries of TB-II were below 30% when ethyl acetate, diethyl ether, cyclohexane, *tert*-butyl methyl ether or *n*-butanol was used as the extraction solvent. The high hydrophilicity of the analyte might have made it difficult to extract from plasma by conventional LLE. Protein precipitation (PPT) often provides higher recovery compared with LLE for the compound of high hydrophilicity. So a simple and rapid PPT protocol was developed that used acetonitrile as deproteinizing solvent. It was found that adding 300 μl water to 150 μl supernatant could yield symmetric peak shape and the least dilution for TB-II.

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was examined by comparing the blank plasma and spiked plasma ($n = 5$). Under the above conditions the retention time was about 1.9 min for TB-II and 2.0 min for the IS. As shown in Fig. 2A, no interfering peaks were observed in the representative chromatogram of a blank plasma sample at the retention times of the analyte and the IS.

3.3.2. Matrix effect and recovery

The mean absolute matrix effect values obtained were 58.7, 62.1 and 59.8% for TB-II at 15, 500 and 12,000 ng/ml, respectively; and was 59.8–63.4% for the IS (Table 1). Values less than 100% indicated there were ionization suppressions for TB-II and the IS

Table 1
Matrix effect for TB-II and ginsenoside Re (IS) ($n = 5$).

Spiked concentration (ng/ml)	TB-II		Ginsenoside Re[a] (IS)	
	Mean ± S.D. (%)	R.S.D. (%)	Mean ± S.D. (%)	R.S.D. (%)
15	58.7 ± 4.5	7.7	59.8 ± 3.8	6.4
500	62.1 ± 4.1	6.6	63.4 ± 4.0	6.3
12,000	59.8 ± 3.7	6.2	60.1 ± 2.5	4.2

[a] The concentration of ginsenoside Re was 150 ng/ml in the acetonitrile to precipitate protein of three QC levels samples.

Table 2
Precision and accuracy for TB-II in rat plasma at different QC levels ($n = 5$).

Spiked concentration (ng/ml)	Intra-day			Inter-day		
	Measured concentration (mean ± S.D., ng/ml)	R.S.D. (%)	Accuracy (%)	Measured concentration (mean ± S.D., ng/ml)	R.S.D. (%)	Accuracy (%)
15	16.1 ± 1.2	7.4	107.3	14.7 ± 1.3	8.8	98.0
500	488.6 ± 14.6	3.0	97.7	527.2 ± 47.3	9.0	105.4
12,000	12,490 ± 395	3.2	104.1	12,397.2 ± 603.9	4.9	103.3

under the present conditions, but their values were similar and kept consistent at three QC concentrations. The relative matrix effects, expressed as R.S.D. (%) were acceptable with <7.7% for TB-II and <6.4% for the IS (Table 1). The above results confirmed that the presence of matrix effects practically had no influence on the determination of TB-II in rat plasma and the present LC–MS/MS method was reliable [15].

The extraction recoveries in rat plasma were excellent for TB-II: 90.0, 91.5 and 90.2% at 15, 500 and 12,000 ng/ml, respectively ($n = 5$), and that for the IS (150 ng/ml) was 93.2% ($n = 5$).

3.3.3. Linearity and lower limit of quantification (LLOQ)

Linear responses were obtained for TB-II ranging from 5 to 15,000 ng/ml. A typical calibration equation was $y = 0.00750x + 0.0101$ ($r = 0.997$), where y represents the peak area ratios of TB-II to the IS and x represents the plasma concentrations of TB-II. The present method offered a LLOQ of 5 ng/ml with R.S.D. of 11.7% and an accuracy of 102.2% in 100 μl plasma sample. The LLOQ was sufficient for the pharmacokinetic studies of TB-II in rats.

3.3.4. Precision and accuracy

Good performance with low deviation and consistent accuracy was observed at three QC levels of 15, 500 and 12,000 ng/ml during the validation. As shown in Table 2, the intra- and inter-day precision were less than 7.4 and 9.0%, respectively, and the accuracy were within the range of 97.7–107.3% during the validation, indicating a precise and accurate method for the determination of TB-II in rat plasma.

3.3.5. Stability

The stability results are presented in Table 3. The data showed that TB-II, at the three concentrations studied, had acceptable stabilities after three freeze-thaw cycles, at room temperature for 4 h, at −20 °C for 2 weeks and in the autosampler at ambient temperature for 12 h after protein precipitation with the values being 98.5–107.9%, 99.3–105.3%, 97.2–99.9% and 103.0–108.0%, respectively.

3.4. Pharmacokinetic study

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of TB-II were determined for 10 h after i.v. administration (1.8 mg/kg, $n = 6$) and for 16 h after oral administration (180 mg/kg, $n = 6$). The pharmacokinetic profiles of TB-II are shown in Fig. 3 and the main pharmacokinetic parameters are given in Table 4. The plasma concentration–time curve of the compound exhibited distinct double peaks after oral administration and this might involve enterohepatic recirculation. The absolute oral bioavailability of TB-II in rats was only 1.1 ± 0.3%. The result suggests that TB-II is poorly absorbed *via* the gastrointestinal segment or may have undergone acid-induced degradation following oral administration to rats. Further studies of absorption, distribution, excretion and metabolism of TB-II are underway in rats and dogs in our laboratory.

Table 3
Stability of TB-II in rat plasma at different QC levels ($n = 5$).

Sample condition	Stability (Mean ± S.D., % of initial)		
	15 ng/ml	500 ng/ml	12,000 ng/ml
Freeze-thaw stability	107.9 ± 3.2	98.5 ± 6.2	100.2 ± 3.3
Short-term stability	105.3 ± 6.5	102.1 ± 2.0	99.3 ± 3.6
Long-term stability	99.9 ± 7.3	97.2 ± 5.5	98.1 ± 5.0
Post-preparative stability	108.0 ± 7.1	104.4 ± 3.3	103.0 ± 2.1

15, 500 and 12,000 ng/ml are initial concentrations.

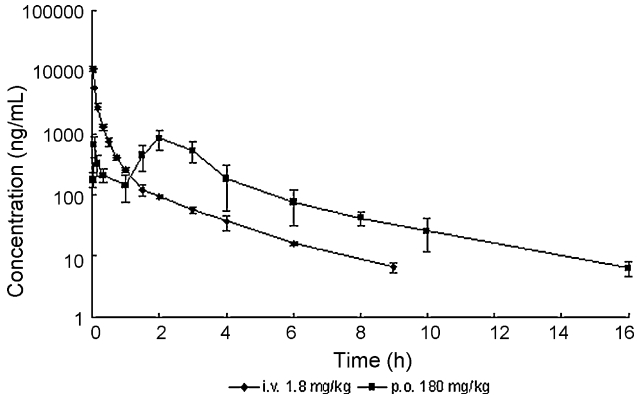

Fig. 3. Mean plasma concentration–time profile of TB-II in rats after intravenous administration of 1.8 mg/kg and oral administration of 180 mg/kg (each point represents mean ± S.D., $n = 6$).

Table 4
The main pharmacokinetic parameters of TB-II after oral and intravenous administrations (mean ± S.D., $n = 6$).

Parameter	Administration mode	
	Oral (180 mg/kg)	Intravenous (1.8 mg/kg)
T_{max} (min)	140.0 ± 31.0	
C_{max} (ng/ml)	816.0 ± 289.1	11,295.4 ± 806.7
$t_{1/2}$ (min)	170.5 ± 76.6	97.5 ± 37.9
MRT (min)	222.7 ± 31.5	42.5 ± 12.5
CL (ml/(min kg))	18.4 ± 6.1	13.3 ± 1.0
AUC_{0-t} (ng min/ml)	131,502 ± 51,673	135,818 ± 10,826
$AUC_{0-\infty}$ (ng min/ml)	145,213 ± 55,597	136,525 ± 11,009
F (%)	1.1 ± 0.3	

4. Conclusions

A rapid, sensitive and selective LC–MS/MS method was developed and validated for the determination of TB-II in rat plasma. The satisfactory selectivity, sensitivity, precision, accuracy and dynamic range made the method suitable for pharmacokinetic study. In addition, the relatively short chromatographic run time (3.0 min) and straightforward sample pretreatment procedure made it easy and fast to perform. To the best of our knowledge, this is the first study demonstrating the quantitative method and pharmacokinetics of TB-II *in vivo* so far.

Acknowledgements

This study was supported by National Natural Science Foundation of China (30672627) and Shuguang Project of Education Council of Shanghai (05SG40).

References

[1] J. Zhang, M. Zhang, K. Sugahara, Y. Sagara, Z. Meng, S. Xu, H. Kodama, Biochem. Biophys. Res. Commun. 259 (1999) 636–639.
[2] Z.Y. Meng, J.Y. Zhang, S.X. Xu, K. Sugahara, Planta Med. 65 (1999) 661–663.
[3] J. Zhang, Z. Meng, M. Zhang, D. Ma, S. Xu, H. Kodama, Clin. Chim. Acta 289 (1999) 79–88.
[4] N. Kaname, J. Zhang, Z. Meng, S. Xu, K. Sugahara, Y. Doi, H. Kodama, Clin. Chim. Acta 295 (2000) 129–140.
[5] S.Z. Xiao, M.E. Xu, Y.K. Ge, G.F. Xiao, Biomed. Environ. Sci. 19 (2006) 185–191.
[6] H. Nian, L.P. Qin, W.S. Chen, Q.Y. Zhang, H.C. Zheng, Y. Wang, Acta Pharmacol. Sin. 27 (2006) 728–734.
[7] S. Ouyang, L.S. Sun, S.L. Guo, X. Liu, J.P. Xu, Di Yi Jun Yi Da Xue Xue Bao 25 (2005) 121–126.
[8] Y.E. Hu, Q.X. Sun, Z.Q. Xia, Chin. Pharmacol. Bull. 19 (2003) 149–151.
[9] Y. Hu, Z. Xia, Q. Sun, A. Orsi, D. Rees, Brain Res. 1060 (2005) 26–39.
[10] T.J. Li, Y. Qiu, P.Y. Yang, Y.C. Rui, W.S. Chen, Neurosci. Lett. 421 (2007) 147–151.
[11] C. Ma, L. Wang, Y. Tang, M. Fan, H. Xiao, C. Huang, Biomed. Chromatogr. 22 (2008) 1066–1083.
[12] C. Ma, M. Fan, Y. Tang, Z. Li, Z. Sun, G. Ye, C. Huang, Biomed. Chromatogr. 22 (2008) 835–850.
[13] USFDA, 2001, http://www.fda.gov/cder/guidance/4252fnl.pdf.
[14] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[15] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[16] B.P. Ma, J.X. Dong, B.J. Wang, X.Z. Yan, Yao Xue Xue Bao 31 (1996) 271–277.
[17] G.C. Kite, E.A. Porter, M.S. Simmonds, J. Chromatogr. A 1148 (2007) 177–183.