Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 949–953

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Liquid chromatography–tandem mass spectrometry assay for the quantitation of plagiochin E and its main metabolite plagiochin E glucuronides in rat plasma

Jie Xing, Beibei Lv, Chunfeng Xie, Jianbo Qu, Hongxiang Lou*

School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, PR China

ARTICLE INFO

Article history:
Received 18 January 2008
Received in revised form 2 March 2008
Accepted 3 March 2008
Available online 12 March 2008

Keywords:
Plagiochin E
LC–MS/MS
Pharmacokinetics
Conjugated metabolites
Rat plasma

ABSTRACT

Plagiochin E, a macrocyclic bisbibenzyl isolated from liverwort *Marchantia polymorpha*, was found to have antifungal activity. To evaluate the pharmacokinetics of plagiochin E in rats, a sensitive and specific liquid chromatography/tandem mass spectrometric (LC–MS/MS) method was developed and validated for the quantitation of plagiochin E and its total conjugated metabolites in rat plasma. For detection, a Sciex API 4000 LC–MS/MS with a TurboIonSpray ionization (ESI) inlet in the negative ion-multiple reaction monitoring (MRM) mode was used. The plasma samples were pretreated by a simple liquid–liquid extraction with ethyl acetate. The concentration of plagiochin E parent form was determined directly and the concentration of plagiochin E conjugated metabolites was assayed in the form of plagiochin E after treatment with β-glucuronidase/sulfatase. The statistical evaluation for this method reveals excellent linearity, accuracy and precision for the range of concentrations 0.5–1000.0 ng/mL. The method had a lower limit of quantification (LLOQ) of 0.5 ng/mL for plagiochin E in 50 μL of plasma. The method was successfully applied to the characterization of the pharmacokinetic profile of plagiochin E in rats after an oral and an intravenous administration.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Liverworts have been used as components in various remedies of folk medicine in the Far East, China and Japan [1–3]. Extracts from liverworts were applied for the therapeutic purpose as diuretics, antitumor, antibacterial, and antifungal agents [4]. Bisbibenzyls isolated from liverworts were found to have a wide range of biological properties such as antibacterial, antifungal and 5-lipoxylgenase inhibitory activities [5,6]. It was reported that the antibacterial effect of *Marchantia polymorpha* extracts was associated with its main macrocyclic bisbibenzyl component, marchantin A [7]. In recent years, bioactive bisbibenzyls in liverworts have attracted much attention for their unique structures and various biological activities [2,4]. A number of bisbibenzyls have been obtained from natural or synthetic resources, allowing a clearer understanding of structure–activity relationships and biological mechanisms [8,9]. Plagiochin E (PE, Fig. 1) is a macrocyclic bisbibenzyl isolated from the liverwort *M. polymorpha*. PE showed statistical significant *in vitro* antifungal activity which was performed by the bioautography and broth microdilution techniques [10]. PE was also effective to reverse the fungal resistance in *Candida albicans* [11]. For the further development of pure PE as a clinical useful drug, information about the biological effects of ingested PE and its pharmacokinetics are required.

Several analytical techniques have been reported for qualitative determination of PE or its analogues [12–14]. An HPLC-DAD method was used to screen the bibenzyl compounds in *Dendrobium* species [12]. A rapid liquid chromatography–tandem mass spectrometry method has been developed in our lab to enhance the throughput of bisbibenzyl discovery [13,14]. However, no analytical methods have been validated for the quantitative assay of PE or its related analogues in biological fluids to date.

In the present work, a sensitive and simple method using LC–MS/MS was developed for quantitation of PE and its conjugated metabolites in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Plagiochin E (PE, purity > 99.3%) and dihydroresveratrol (purity > 99.5%, as internal standard, IS, Fig. 1) were obtained in

* Corresponding author. Tel.: +86 531 88382019; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H. Lou).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.03.001

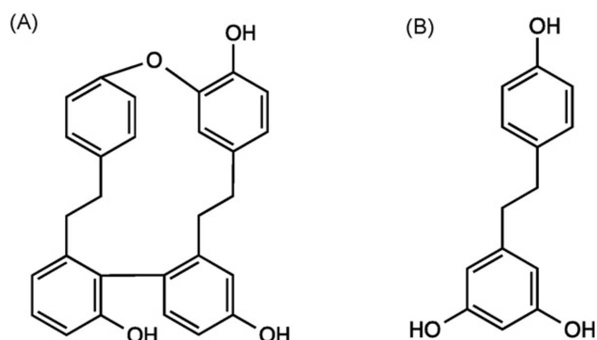

Fig. 1. Chemical structures of plagiochin E (A) and dihydroresveratrol (internal standard, B).

our lab and their structures have been confirmed by HR-MS, ^{1}H NMR, ^{13}C NMR and 2D NMR spectroscopy. β-Glucuronidase (with arylsulfatase activity, partial purified, G-4259) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Fisher Chemicals (Fairlawn, NJ, USA). Ethyl acetate was of analytical grade from Beifang Chemicals Ltd. (Tianjin, China). Other chemicals used were of analytical reagent grade and purchased from commercial sources. Water was deionized, filtered and purified (resistivity > 18 MΩ cm) on a Milli-Q reagent grade water system (Millipore Corporation, Bedford, MA, USA).

2.2. Instrumentation

Liquid chromatography–tandem mass spectrometry assay for the quantitation of PE and its conjugated metabolites was performed on an Agilent 1100 system (PaloAlto, CA, USA) equipped with an autosampler, a vacuum degasser unit, and a binary pump. The HPLC system was coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a TurboIonSpray ionization (ESI) interface. Data were collected and analyzed by the Analyst 1.3 data acquisition and processing software (Applied Biosystems/MDS Sciex).

The conjugated metabolites of PE was identified using a ThermoFinnigan LTQ-Orbitrap XL mass spectrometer (ThermoFinnigan, Bremen, Germany) equipped with an electrospray ionization interface. An Accela HPLC system (ThermoElectron) was equipped with an autosampler, a vacuum degasser unit, and a quaternary pump.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved on a Luna ODS C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA) with a 4.0 mm × 3.0 mm i.d. SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA). The chromatography was performed at 20 °C. The mobile phase consisted of acetonitrile–water (85:15, v/v), delivered at a flow rate of 0.8 mL/min for quantitation assays. The mobile phase consisting of acetonitrile–water (40:60, v/v) was used at a flow rate of 0.5 mL/min for PE metabolite determination.

The API4000 mass spectrometer was operated in negative ion mode with an ion spray voltage of −3.5 kV, backpressures for collision gas of 3 psi, curtain gas of 12 psi, nebulizer gas of 50 psi and heater gas of 45 psi. The heater gas temperature was set at 500 °C. Ultrapure nitrogen was used as nebulizer, heater, curtain, and collision-activated dissociation (CAD) gas. The fragmentation transitions for the multiple reaction monitoring (MRM) were m/z 423.4–210.2 for PE, and m/z 229.2–123.1 for the IS, with a dwell time of 200 ms per transition.

The LTQ-Orbitrap XL mass spectrometer employing negative ionization was calibrated using the manufacturer's calibration standards mixture allowing for mass accuracies <10 ppm in external calibration mode. The scan was collected in the orbitrap at R > 60,000. The ionization voltage was −3.2 kV, the capillary temperature was set at 300 °C. Nitrogen was used as both the sheath gas at a flow rate of 0.75 L/min and auxiliary gas at a flow rate of 0.15 L/min. The $[M-H]^-$ precursor ion of PE (m/z 423.0) and its conjugated metabolites (m/z 599.0) was dissociated in the HCD mode using the normalized collision energy at 80%.

2.4. Preparation of calibration standards and quality control samples

Stock solutions of PE (400 μg/mL) and IS (100 μg/mL) were prepared in acetonitrile. Working solutions of PE (0.5, 1, 2.5, 10, 50, 200, 500 and 1000 ng/mL) and IS (1 μg/mL) were prepared by diluting the stock solutions with acetonitrile. QC working solutions (1, 50 and 800 ng/mL) were similarly prepared. All solutions were stored at 4 °C.

Calibration standards and QC samples were prepared by spiking 50 μL of working solutions and 20 μL of IS (1 μg/mL) with 50 μL of drug-free plasma and 100 μL of phosphate buffer (pH 5.0, 0.05 M). This mixture was acidified with 50 μL of phosphate buffer (pH 2.5, 0.05 M) and extracted with 3 mL of ethyl acetate by shaking for 10 min. The organic phase, obtained by centrifugation at 3000 × g for 10 min, was evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μL of the mobile phase and a 10 μL aliquot of the solution was injected onto the LC–MS/MS system for analysis. Matrix matched calibration standards were obtained with concentrations of 0.5, 1, 2.5, 10, 50, 200, 500 and 1000 ng/mL of PE in plasma. QC samples were obtained with three concentration levels of 1, 50 and 800 ng/mL of PE in plasma.

2.5. Sample preparation

The concentration of PE conjugated metabolites in plasma was determined in the form of PE after β-glucuronidase/sulfatase treatment. For enzymatic analysis, 50 μL of plasma was mixed with 100 μL of β-glucuronidase/sulfatase (4000 U/mL in pH 5 phosphate buffer), and incubated at 37 °C for 16 h. After hydrolysis, the plasma was acidified with 50 μL of phosphate buffer (pH 2.5) after adding 20 μL of IS (1 μg/mL) and 50 μL of acetonitrile. This mixture was partitioned with 3 mL of ethyl acetate and then treated as above. For the assay of PE parent drug, 50 μL of plasma was subjected to the process described above except for the addition of 100 μL pH 5.0 buffer instead of β-glucuronidase/sulfatase.

2.6. Stability studies

The stability studies were carried out to evaluate PE stability under the conditions used in this work, which included the storage of plasma at room temperature on the bench-top for at least 4 h, processed samples in the autosampler tray up to 24 h, plasma samples at −20 °C for at least 4 weeks and plasma samples over at least three freeze/thaw cycles.

2.7. Pharmacokinetic study

Male Wistar rats (200–230 g) were supplied by Lab Animal Center of Shandong University (Grade II, Certificate No. SYXK 2003-0004). PE was given orally to rats (n = 6) at a dose of 80 mg/kg and the intravenous bolus injection was given to other rats (n = 6) via the tail vein at a dose of 25 mg/kg. PE was dissolved in a mixture

containing DMSO:1,2-propanediol:water (0.2:8:2, v/v) for injection, and suspended in a mixture containing DMSO:ethanol:water (0.2:6:4, v/v) for oral administration. Blood samples of 250 μL were withdrawn from one jugular vein before dosing and at 0.017, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h after i.v. dosing, and at 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h after p.o. dosing. Blood samples were collected into heparinized tubes and plasma was obtained by centrifugation at 3000 × g for 10 min. All plasma samples were stored at −20 °C and assayed within 1 month.

2.8. Data analysis

The peak plasma concentration (C_{max}) and the time to peak concentration (t_{max}) were obtained from experimental observations. The other pharmacokinetic parameters were analyzed by a non-compartmental model with the aid of the program TOPFIT (version 2.0, Thomae GmbH, Germany). The area under the plasma concentration–time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule to the last point. The mean residence time (MRT) was obtained by dividing the area under the first moment–time curve ($AUMC_{inf}$) by the area under the curve (AUC_{inf}). Total body clearance (CL/*F*) was calculated as dose/AUC_{inf}. The bioavailability (*F*) of PE was calculated using $AUC_{0-t(p.o.)} \times 25/AUC_{0-t(i.v.)} \times 80 \times 100\%$.

3. Results and discussions

3.1. Method development

The mobile phase composed of acetonitrile and water was selected to obtain improved MS response. Due to the similarity of physical–chemical properties and impossibility of interconversion *in vivo*, dihydroresveratrol was selected as IS in this experiment.

When PE and IS were injected directly into the mass spectrometer along with the mobile phase in the negative mode, deprotonated molecules $[M-H]^-$ of PE and IS were observed in abundance. Each of the precursor ions was subjected to collision-induced dissociation to determine the resulting product ions. Interface independent instrument parameters were optimized during the infusion of a solution of PE and IS.

The sample clean-up involved addition of acetonitrile, phosphate buffer and extraction organic solvent. The processes of protein precipitation and extraction gave a high and stable recovery of PE and IS.

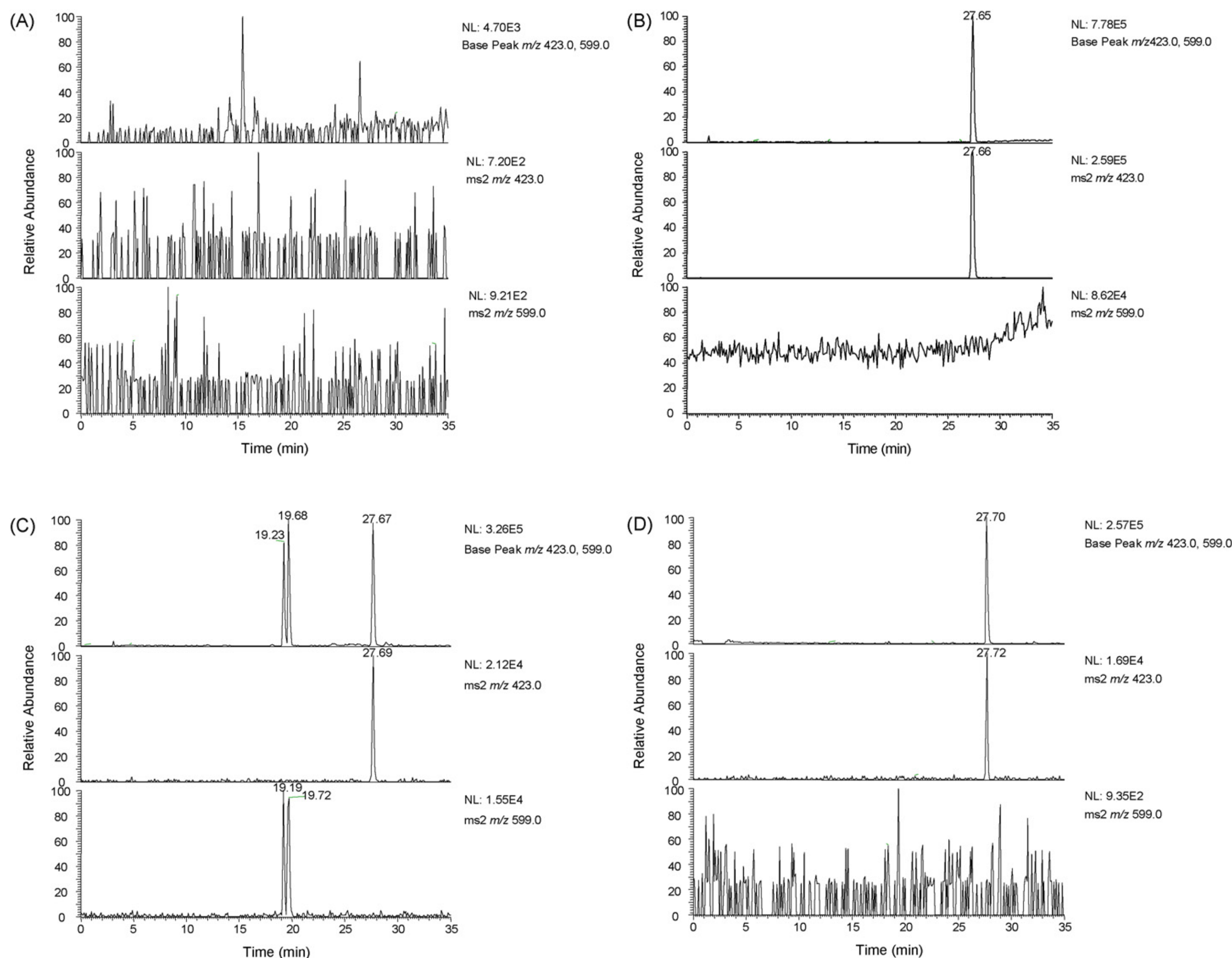

Fig. 2. Representative chromatograms of (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with plagiochin E (PE); (C) a rat plasma sample at 1.0 h after an oral dose of PE (80 mg/kg) and (D) a rat plasma sample at 1.0 h pretreated by enzyme hydrolysis after an oral dose of PE (80 mg/kg). PE: *m*/*z* 423.0; PE metabolites: *m*/*z* 599.0.

3.2. Separation and relative retention time

Under optimized HPLC and MS conditions, the total run time was 4.0 min. PE and dihydroresveratrol (IS) were detected at the retention times of 3.35 and 2.15 min, respectively. Blank rat plasma from six lots showed no significant interfering peaks at the retention times of PE and IS. The ion suppression effect was evaluated by comparing the peak areas of PE (six QC samples of three concentration levels) and the IS (1 μg/mL) obtained from blank plasma extracts spiked with reference solutions with those of reference solutions at same concentration levels in acetonitrile, which was used as the reconstitution solution. For PE, the mean peak areas from the six QC samples had relative error of −9.2%, when compared with those of QC working solutions. For the IS, the relative error was 9.0%. These observations indicated that endogenous substances did not significantly influenced the ionization of PE and IS.

Carryover effect was tested by injecting the high concentration standard (4000 ng/mL for PE and IS) in system followed by acetonitrile injection. No analytes were detected in the acetonitrile injection, which indicated the absence of carryover effect.

3.3. Linearity

The calibration curve was linear over the concentration range of 0.5–1000.0 ng/mL of PE in rat plasma with correlation coefficients $r > 0.99$ and consistent slope values when evaluated by weighed ($1/x^2$) least squares linear regression. Residuals were randomly distributed when plotted against concentration. A typical equation of the calibration curve was as follows: $y = 1.45 \times 10^{-5}\, x - 7.41 \times 10^{-6}$, $r = 0.9970$, where, y represents the peak area ratios of PE to that of IS, and x represents the plasma concentrations of PE. The lower limit of quantification (LLOQ) of PE was established at 0.5 ng/mL. The LLOQ was accepted with a relative standard deviation of less than 15% and signal to noise ratio of 8:1 for the analysis for PE.

3.4. Accuracy and precision

The accuracy and precision of the method were assessed by determining LLOQ (lower limit of quantification, 0.5 ng/mL), QC (1, 50 and 800 ng/mL) and ULOQ (upper limit of quantification, 1000 ng/mL) samples using six replicated preparations of plasma samples at five concentration levels, respectively. Intra-day accuracy and precision were evaluated on the same day. To assess the inter-day accuracy and precision, the intra-day assays were repeated on three different days. Table 1 shows the results of calibration accuracy and the intra-day and inter-day precision in the 3-day validation study. The method was found to be highly accurate with deviation <10.9% from the nominal values and highly precise with intra-day precision <10.0% and inter-day precision <11.5% at each concentration of QC, LLOQ and ULOQ sample tested.

Table 1
Intra-day and inter-day precision and accuracy for plagiochin E in rat plasma ($n = 3$ days, six replicates per day)

Added concentration (ng/mL)	Accuracy (%CV)		Precision (%CV)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.5	6.0	7.1	9.1	8.0
1	10.9	6.2	10.0	6.1
50	5.3	4.1	3.3	3.6
800	8.1	10.4	6.5	11.5
1000	2.5	3.8	4.9	4.7

3.5. Recovery

The extraction recoveries of PE and IS were determined at three QC levels by comparing the peak area of each analyte in plasma samples that had been spiked with the analytes prior to extraction with those for samples to which the analytes had been added post-extraction. The mean recovery was 83.7 ± 2.6% and 66.0 ± 7.2% for PE and IS, respectively. These results suggested that there was no relevant difference in extraction recovery at different concentration levels for both PE and IS.

3.6. Stability

Stability of PE was assessed by leaving the QC samples of three different concentrations under several conditions and all the QC samples were analyzed in triplicate. There was no significant difference (%CV < 15%) between the responses of standards at time zero and after storage of plasma at room temperature on the bench-top for at least 4 h in terms of %CV (8.5%) for PE. Processed samples were stable up to 24 h in the autosampler tray with the %CV value of 7.3%. Plasma samples were stable at −20 °C for at least 4 weeks with no significant loss (<10.1%). Plasma samples were stable over at least three freeze/thaw cycles in terms of %CV (7.0%).

Stability of PE glucuronides was evaluated indirectly by leaving QC rat plasma samples (containing PE and PE glucuronides; rat samples diluted with proper volume of blank plasma; their calculated total PE concentrations: 1, 50, 800 ng/mL) under the conditions above (except in the autosampler tray), and the QC samples were subjected to the extraction process of determining total PE. All QC plasma samples were analyzed in triplicate, and there was no significant difference (%CV < 15%) between the calculated concentrations of total PE at time zero and after storage processes.

3.7. Application to rat plasma sample analysis

After PE was administrated orally/intravenously to rats, PE and two metabolites were detected in plasma. Representative chromatograms of a blank plasma, a spiked blank plasma with PE, and a rat plasma at 1 h after oral administration of PE to rats are presented in Fig. 2. Comparing the LC retention time and full scan multistage mass spectra of the reference substance, PE (t_R 27.7 min) was iden-

Table 2
The main pharmacokinetic parameters of plagiochin E (PE) and total plagiochin E (TPE) after an oral and an intravenous administration to six rats (mean ± S.D.)

Parameters	i.v.		p.o.	
	PE	TPE	PE	TPE
AUC_{0-t} (ng h/mL)	3022.3 ± 238.7	9398.8 ± 1985.8	1021.4 ± 257.5	8999.5 ± 4086.7
C_{max} (ng/mL)	N.A.	N.A.	313.5 ± 147.3	2788.7 ± 366.8
t_{max} (h)	N.A.	N.A.	0.33	0.5 ± 0.2
MRT (h)	2.3 ± 0.5	5.1 ± 1.3	7.0 ± 2.2	6.8 ± 1.4
CL (L/(min kg))	0.1 ± 0.01	0.05 ± 0.01	1.4 ± 0.3	0.2 ± 0.06
F (%)			10.6 ± 2.7	29.9 ± 13.6

N.A., not applicable.

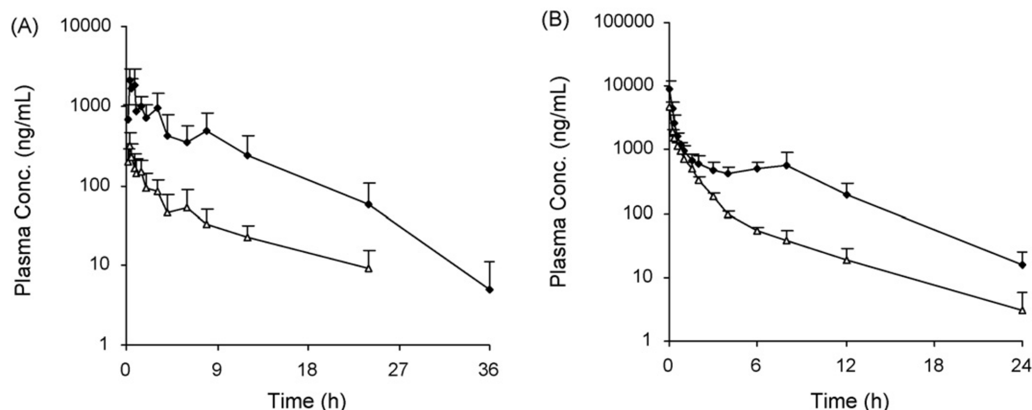

Fig. 3. Mean (+S.D.) plasma concentration–time profiles of plagiochin E (△) and total plagiochin E (♦) in rats ($n = 6$) following an oral (A, 80 mg/kg) and an intravenous (B, 25 mg/kg) dose of plagiochin E.

tified. The metabolites peaks (t_R 19.2 min, M1; t_R 19.7 min, M2) had a similar deprotonated ion at m/z 599 and a product ion at m/z 423 (loss of a glucuronic acid). Treatment of plasma samples with β-glucuronidase/sulfatase before LC/MS/MS analysis led to the disappearance of metabolites M1 and M2. The results above showed that M1 and M2 were the glucuronides of PE, which was also proved by the elemental composition data.

The LC–MS/MS method in this study was successfully applied to the quantitation of PE in rat plasma. The mean (+S.D.) ($n = 6$) plasma concentration versus time profiles for PE and total PE after an oral (80 mg/kg) and an intravenous (25 mg/kg) dose are depicted in Fig. 3. The pharmacokinetic parameters are presented in Table 2.

4. Conclusions

The present optimized LC–MS/MS method was validated to guarantee a reliable quantitation of plagiochin E in rat plasma. The analysis requires 0.1 mL of plasma which is an advantage in pharmacokinetic studies. This LC–MS/MS method has an LLOQ of 0.5 ng/mL for plagiochin E and the analysis time is 4 min per sample. The results of validation show that the method is reproducible and accurate. The assay procedure is simple and relatively short allowing sufficient sample throughput to be applied to pharmacokinetic studies of plagiochin E.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30672531), Special Foundation for State Major Basic Research Program of China (973 program, No. 2006CB708511) and Shandong Provincial Research Foundation (6GG1102023).

References

[1] W. Herz, G.W. Kirby, R.E. Moore, W. Steglick, C. Tamm, Progress in the Chemistry of Organic Natural Products, Springer, New York, 1995.
[2] Y. Asakawa, M. Toyota, M. Tori, T. Hashimoto, Spectroscopy 14 (2000) 149–175.
[3] T. Hashimoto, T. Yoshida, Y. Kan, S. Takaoka, M. Tori, Y. Asakawa, Tetrahedron Lett. 35 (1994) 909–910.
[4] Y. Asakawa, Phytochemistry 56 (2001) 297–312.
[5] E. Kámory, G.M. Keserü, B. Papp, Planta Med. 61 (1995) 387–388.
[6] C. Niu, J.B. Qu, H.X. Lou, Chem. Biodivers. 3 (2006) 34–40.
[7] G.M. Keserü, M. Nogradi, Nat. Prod. Rep. 12 (1995) 69–75.
[8] L. Harinantenaina, D.N. Quang, N. Takeshi, T. Hashimoto, C. Kohchi, G. Soma, Y. Asakawa, J. Nat. Prod. 68 (2005) 1779–1781.
[9] G.M. Keserü, M. Nógrádi, J. Mol. Recognit. 9 (1996) 133–138.
[10] J.B. Qu, C.F. Xie, H.F. Guo, W.T. Yu, H.X. Lou, Phytochemistry 68 (2007) 1767–1774.
[11] P. Leng, X.L. Guo, Y. Yang, H.X. Lou, Chin. Pharm. J. 42 (2007) 349–352.
[12] L. Yang, Z.T. Wang, L.S. Xu, J. Chromatogr. A 1104 (2006) 230–237.
[13] H.F. Guo, J. Xing, C.F. Xie, J.B. Qu, Y.H. Gao, H.X. Lou, Rapid Commun. Mass Spectrom. 21 (2007) 1367–1374.
[14] J. Xing, C.F. Xie, J.B. Qu, H.F. Guo, B.B. Lv, H.X. Lou, Rapid Commun. Mass Spectrom. 21 (2007) 2467–2476.