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Sensitive and selective liquid chromatography–electrospray ionization mass spectrometry analysis of ginkgolide B in dog plasma

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Abstract

As an important active constituent of *Ginkgo biloba extract*, ginkoglide B is a highly selective and competitive PAF receptor antagonist which has been widely used in clinical applications. A novel high-performance liquid chromatography–electrospray ionization mass spectromentry (LC–ESI-MS) method was developed for the determination of ginkgolide B in dog plasma. After liquid/liquid extraction with ether and high-performance liquid chromatography (HPLC) gradient separation with 0.01% of ammonia water (v/v)—methanol as the mobile phase, the deprotonized anions $[M–H]^{-1}$ at m/z 423 of ginkoglide B, and $[M–H]^{-1}$ at m/z 492 of internal standard (IS) glibenclamide were analyzed by LC–ESI-MS in selected ion monitoring (SIM) mode. Chromatographic separation was achieved in less than 9 min and calibration curve was linear over a concentration range of 0.1–20 ng/ml. The described assay method was successfully applied to the pre-clinical pharmacokinetic study of ginkoglide B. After intragastric administration of ginkgolide B to beagle dogs, C_{max} and T_{max} of ginkgolide B were 43.8 ± 6.24 ng/ml and 0.5 h, respectively, and the elimination half-life ($t_{1/2}$) was 2.85 ± 0.54 h.

Keywords: Ginkgolide B; LC–ESI-MS; Pharmacokinetics

1. Introduction

Ginkgo biloba (Ginkgoaceae) is an ancient Chinese tree, which has been cultivated and held sacred for its health-promoting properties [1]. Over the last decade, substantial scientific evidence has been accumulated which suggest that concentrated and partially purified extracts of *G. biloba* leaves afford protection against some kinds of neural and vascular damage and treat cognitive deficits and other age-associated impairments [2,3]. Ginkgolides are a unique group of diterpenes that exist naturally in the leaves of the *G. biloba* tree. The Institute Henri Beaufour uses the nomenclature BN-52020, BN-52021 and BN-52022 to refer to ginkgolides A–C, respectively; ginkgolide B is by far the most potent PAF receptor antagonists [9]. Ginkgolides J (BN-52024) and M (BN-52023) have been identified more recently. Structurally, these five ginkgolides differ only in the number and position of hydroxyl group [10]. And there are many the possible clinical applications of ginkgolide B. In particular, ginkgolide B has been shown to protect against neural damage in a variety of circumstances. In addition, it has been show to have beneficial effects on circulatory and inflammatory conditions [1] (Fig. 1).

Compared to the extensive literatures on phytochemical and clinical investigations, there were few data concerning the pharmacokinetics of *G. biloba* components. Biber succeeded in this task using gas chromatography/mass spectrometry (GC/MS) after derivatisation of ginkgolides [4]. Li and Wong estimated ginkgolides concentrations in animal plasma using bioassay based on ginkgolides' capacity to inhibit the binding of platelet activating factor (PAF) to its receptor [5]. Yang et al. used GC/MS to study the pharmacokinetics of ginkgolides in human volunteers [8]. Recently, Mauri et al. used liquid chromatography/atmospheric pressure chemical ionization-ion trap mass spectrometry (LC/APCI-IT MS) to

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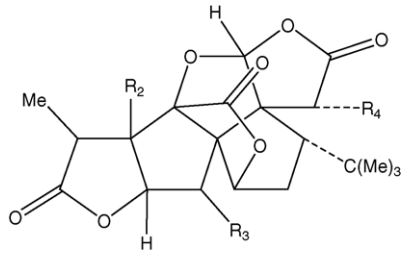

R_1	R_2	R_3	R_4	
H	H	OH	OH	Ginkgolide A(GA)
H	OH	OH	OH	Ginkgolide B(GB)
OH	OH	OH	OH	Ginkgolide C(GC)

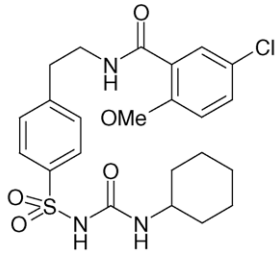

Fig. 1. The chemical structures of: (a) the compounds studied and (b) glibenclamide (IS).

study the pharmacokinetics of terpene lactones both in animals and human [6,7].

But the previous methods have many limitations due to the complicated sample processing procedures or low sensitivity. This paper describes a relatively simple and rapid liquid chromatography–mass spectrometry method to determine ginkgolide B in dog plasma. The method with a lowest quantification limit of 0.1 ng/ml offered higher sensitivity as compared with existing methods. It was successfully applied to a pharmacokinetics study of ginkgolide B in dogs.

2. Experimental

2.1. Chemicals and reagents

The reference standard of ginkgolide B, and internal standard glibenclamide were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Ether was obtained from Nanjing Chemistry Company (Nanjing, China). Ultrapure water was generated by passing distilled water through Quantum EX ultrapure organex cartridge (Cat. No. QTUM000EX).

2.2. Animal

Eight beagle dogs were offered by the experimental animal feeding center of China Pharmaceutical University and the experiment was approved by the University Committee.

2.3. Liquid chromatograph–mass spectrometry (LC–MS)

The HPLC system consisted of a DGU-14 AM degasser, two Shimadzu 10ADvp Pump, a high pressure mixer, a CTO-10Avp column oven and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with Electrospray Ionization (ESI) probe, Q-array-Octapole-Quadrupole mass analyzer (QoQ system) was used in the study.

The analysis was carried out on an ODS column (Shim-pack, 5 μm, 2.1 mm × 150 mm i.d., Shimadzu, Japan), equipped with an ODS guard column (Security Guard, Phenomenex, USA). A gradient elution mode was adopted using two mobile phases: (A) 0.01% of ammonia water (v/v) and (B) methanol. The flow rate is 0.2 ml/min.

The gradient elution program is indicated in Table 1.

Mass spectrometric conditions were optimized to obtain maximum sensitivity. The final ESI conditions used were as follows: curve dissolution line (CDL) voltage was fixed as that in tuning and probe high voltage was set at 4.5 kV, Q-array voltage of dc −35 V and rf 150 V. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China.) was used as the nebulizing gas at 4.5 l/min. LCMass Solution Version 2.02 worked on Windows 2000. Vacuum in the mass detector was obtained by Turbo molecular pump (Edwards 28, England).

2.4. Preparation of stock and sample solutions

A stock solution of ginkolide B was prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 1 mg/ml. The solution was then serially diluted with methanol to achieve standard working solutions at the concentrations of 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml for ginkgolide B. A 2.4 μg/ml internal standard working solution was prepared by diluting the 24 μg/ml stock solution of glibenclamide with methanol.

Table 1
Gradient elution program for the separation of ginkoglide B

Time (min)	Solvent A (%)	Solvent B (%)	Gradient curve
0.0	40	60	–
1.8	40	60	–
2.5	20	80	Linear
4.0	20	80	–
7.0	60	40	Linear
9.0	40	60	Linear

A = 0.01% of ammonia water (v/v) and B = methanol.

2.5. Sample preparation

Venous blood samples (2.5 ml) were withdrawn into the heparinized tubes, and were centrifuged at 2000 × *g* for 10 min at 4 °C. A 1.0 ml volume of plasma was obtained and stored at −20 °C until analysis.

A 0.5 ml volume of the plasma sample was transferred to a 15 ml plastic test tube together with 10 μl of IS solution. After vortex shaking for 30 s, 5 ml ether was added. The analyte and IS were extracted from plasma by vortexing for 5 min. Then the sample was centrifuged for at 2000 × *g* 10 min the organic layer was quantitatively transferred to a 10 ml glass tube and evaporated to dryness using evaporator at 45 °C. Then, the dried extract was reconstituted in 100 μl solvent (water–methanol, 60:40, v/v) and 10 μl aliquot was injected into chromatographic system.

2.6. Calibration curves and assay validation

The calibration curve consisted of eight concentration points (0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml of ginkgolide B in dog plasma). The calibration curve was prepared by adding 10 μl of IS and varying the concentrations of ginkgolide B to blank dog plasma. Plasma samples were quantified using the ratio of the peak area of ginkgolide B to that of IS. Peak area ratios were plotted against concentrations and ginkgolide B concentrations were calculated using a least squares linear regression. Linear regression analysis was performed using Microsoft Excel 2002.

The precision and accuracy of the assay were obtained by comparing the predicted concentration (obtained from the calibration curve) to the actual concentration of ginkgolide B spiked in blank plasma. The standard deviation (S.D.) and the coefficient of variation [CV = (S.D./mean of the recoveries) × 100%] were calculated over the entire calibration range. The limit of detection (LOD) was considered as the concentration that produced a signal-to-noise (S/N) ratio of 3. The recovery was calculated from the ratio of the peak area of ginkgolide B after extration from plasma to the peak area of an equivalent amount of the standard solution. The precision and accuracy were determined by back calculation of spiked plasma samples at three concentrations with respect to a calibration graph prepared each day. The precision was express as the inter-day and intra-day coefficient of variation (%). The accuracy was calculated as the mean deviation of each concentration from the theoretical value.

2.7. Freeze and thaw stablity

The freeze and thaw stablity study samples were obtained by adding the standard solution in the blank dog plasma at the concentrations of 20, 5 and 1 ng/ml. These samples were frozen at −20 °C for 7 days, then thawed at room temperature. After they completely thawed, the samples been refrozen for 24 h under the same conditions. This freeze–thaw cycle been repeated for three times before these samples been analysis using the method described above.

3. Results and discussion

3.1. Method development

To develop a sensitive LC–MS-based method for quantifying ginkolide B in dog plasma, ESI and APCI sources were evaluated. The ESI source produced greater sensitivity and exhibited less interference for ginkolide B than those of APCI source, compared to the reference mentioned previously [6]. Negative MS spectra for ginkoglide B and glibenclamide were dominated by the $[M–H]^{-1}$ ions. Therefore, *m*/*z* 423 and *m*/*z* 492 for ginkoglide B and glibenclamide were chosen for quantification, respectively (Fig. 2).

Mobile phase composition was found to be critical factor for achieving good chromatographic peak shape and sensitivity. In the present study, MS responses at different concentrations of ammonia water (1, 0.1, 0.01 and 0.001%) in the aqueous phase were compared and optimized. Better response was shown when the aqueous phase containing 0.01% ammonia water. Glibenclamide was selected as an internal standard (IS) because of its similar ionization condition, appropriate *m*/*z* value, and good recovery efficiency (95%) as compared to ginkoglide B.

3.2. Method validation

3.2.1. Selectivity

Potential interference from endogenous substances was investigated by the analysis of six blank plasma samples from different dogs. Representative chromatograms of blank plasma, blank plasma spiked with ginkgolide B and IS, and plasma sample from dog after treated with ginkgolide B were shown in Fig. 2. No interferences from endogenous substances with analyte or IS were detected. The LC-MS method has high specificity because only the objective ions derived from the analytes of interest are monitored. Therefore, compounds with different ions could not be detected under the present MS conditions. Each sample required less than 9 min of chromatographic run time (Fig. 3).

3.2.2. Linearity and lowest quantitation limit

Visual inspection of the plotted five calibration curves and correlation coefficients (>0.999) confirmed that the calibration curves were linear over the concentration ranges 0.1–20 ng/ml. The following equations were obtained:

$$y = 8.54(\pm 0.37)x + 1.02(\pm 0.26)$$

The lowest quantitation limit, defined as the lowest concentration analyzed with accuracy within ±20% and a precision <20%, was 0.1 ng/ml for determination of ginkoglide B in plasma.

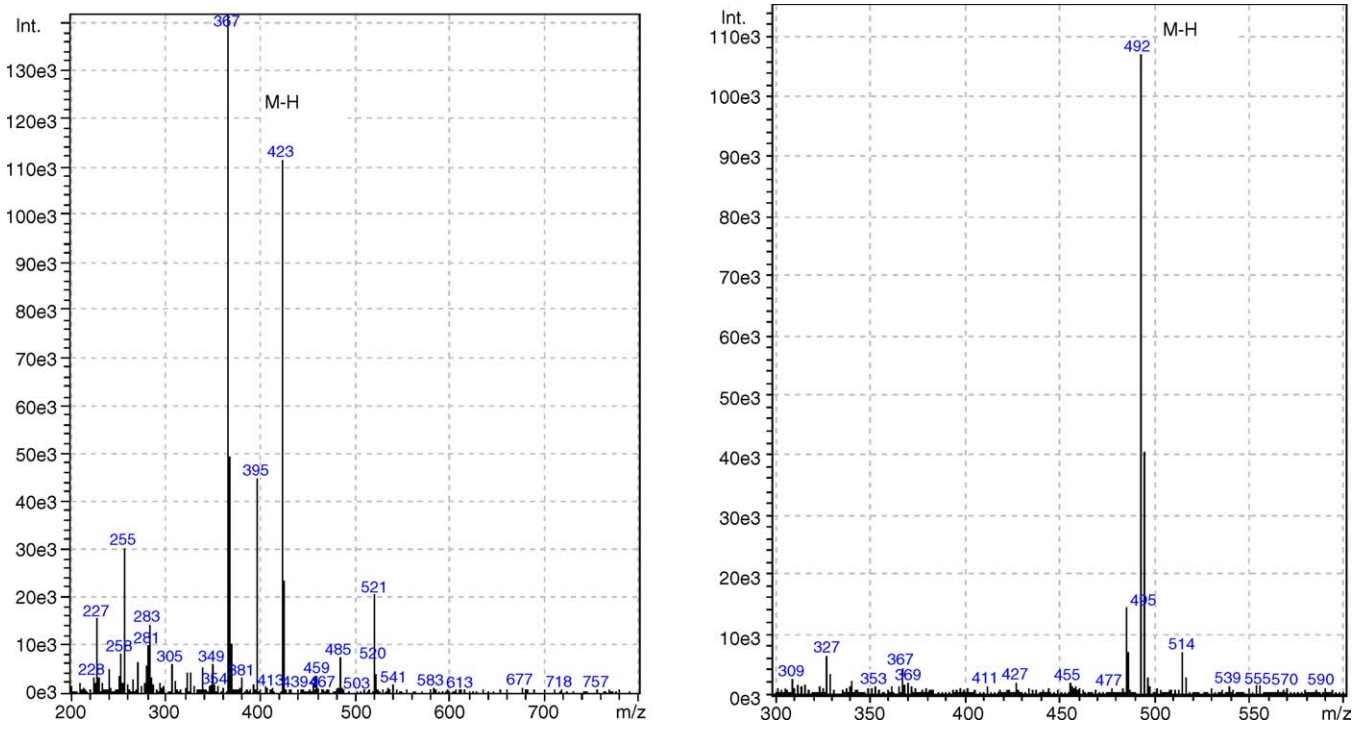

Fig. 2. Mass spectra of: (a) ginkolide B and (b) glibenclamide (IS).

3.2.3. Precision and accuracy

The intra-day accuracy and precision of the assay was measured by analyzing five replicates of 0.2, 2.0, and 20.0 ng/ml spiked quality controls samples of ginkolide B.

Intra-day accuracy of the method for ginkolide B ranged from 100.5 to 101.5%, while the intra-day precision ranged from 2.22 to 9.45% at concentration of 0.2, 2.0 and 20.0 ng/ml (Table 2a). The inter-day precision of the assay was measured by analyzing 15 replicates of 0.2, 2.0 and 20.0 ng/ml quality controls of ginkolide B obtained from days 1–3. Inter-day accuracy of the method for ginkolide B ranged from 99.5 to 102.5%, while the inter-day precision ranged from 3.04 to 11.2%, at concentration 0.2, 2.0 and 20.0 ng/ml (Table 2b).

Table 2
(a) Intra-day and (b) inter-day precision and accuracy of measurement of ginkgolide B when used negative ion detection with selective ion monitoring

Ginkgolide B		Accuracy (%)	Precision (%R.S.D.)
Nominal concentration (ng/ml)	Calculated concentration, mean ± S.D. (ng/ml)		
Part (a)			
0.2	0.203 ± 0.011	101.5	5.42
2.0	2.01 ± 0.19	100.5	9.45
20.0	20.2 ± 0.45	101.0	2.22
Part (b)			
0.2	0.205 ± 0.023	102.5	11.2
2.0	1.99 ± 0.13	99.5	6.53
20.0	20.1 ± 0.61	100.5	3.04

Five replicates at each concentration level ($n = 5$)

3.2.4. Recovery and stability

The absolute recoveries of ginkgolide B at concentrations of 0.2, 2 and 20 ng/ml ($n = 5$) from dog plasma were determined to be 98.6 ± 9.1, 93.1 ± 11.3 and 98.8 ± 12.4%, respectively. QC samples of ginkgolide B obtained by extraction showed no significant degradation after at least 24 h at room temperature (8.4% deviation of the spiked values). Standard stock solution of ginkgolide B was shown to remain stable for at least 2 months at 4 °C.

3.2.5. Freeze and thaw stability

The freeze and thaw stability was determined by analyzing ginkgolide B samples at three concentrations after three freeze–thaw cycles. The coefficients of variation were all within 10% for both ananlytes and the deviation from the expected concentration ranged from −2.4 to −5.7 for ginkgolide B. These results shown that ginkgolide B are stable in the frozen dog plasma and during the freeze–thaw cycles.

3.3. Pharmacokinetic study

Ginkgolide B was given to eight dogs by intragastric administration at the dosage of 0.1 mg/kg. Blood samples (0.5 ml) were collected by venepuncture prior to dosing and 0.17, 0.33, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h post-intragastric administration and were immediately centrifuged (2000 × g for 10 min) to separate the plasma fractions. The five highest concentrations of the samples were diluted using blank dog plasma.

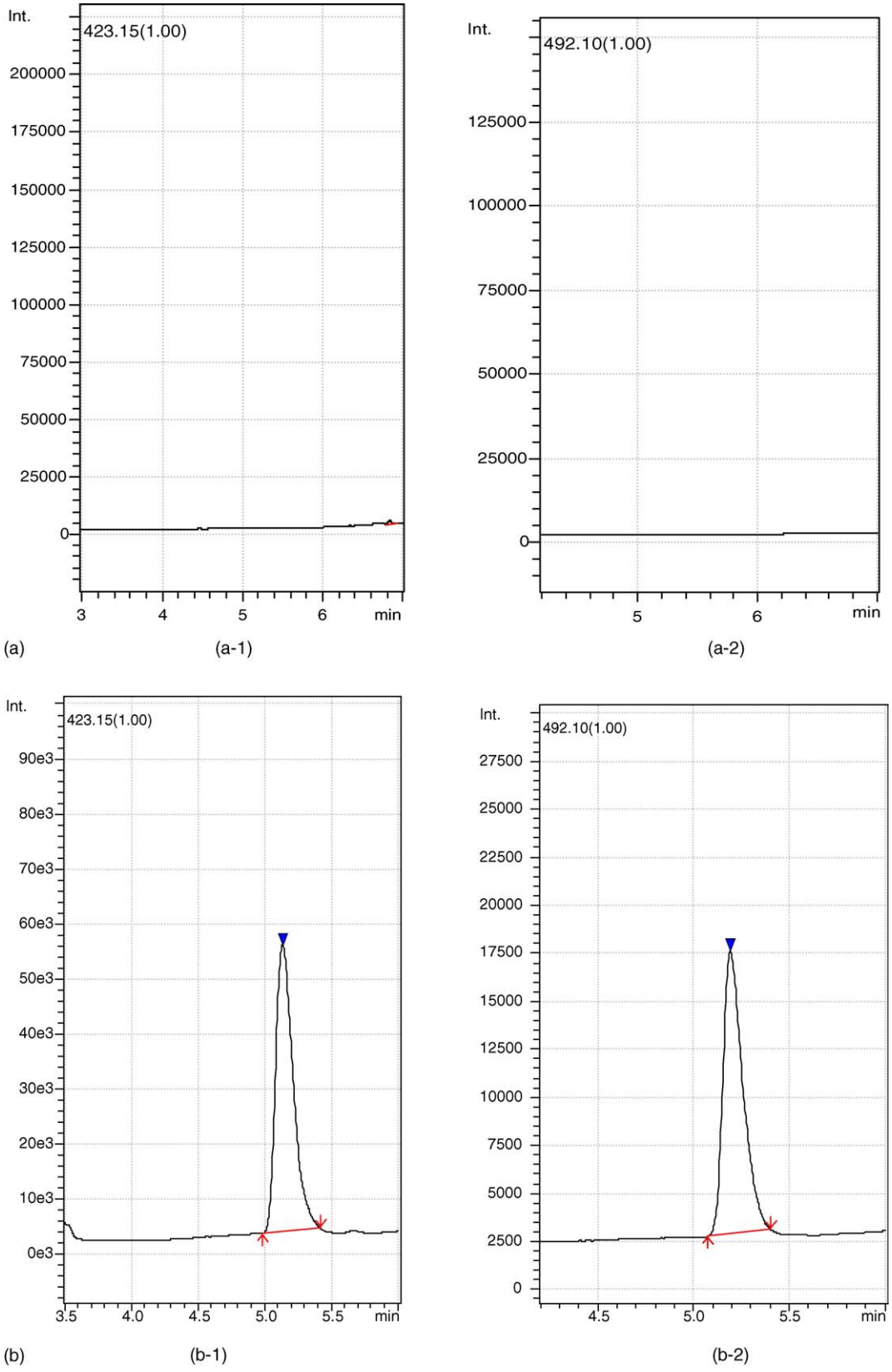

Fig. 3. Representative chromatograms of: (a) blank plasma, (a-1) monitored *m*/*z* ratio was 423.15 (ginkgolide B) and (a-2) monitored *m*/*z* ratio was 492.10 (IS); (b) blank plasma spiked with ginkgolide B (b-1) and IS (b-2); (c) ginkgolide B and IS in plasma sample from dog treated with ginkgolide B.

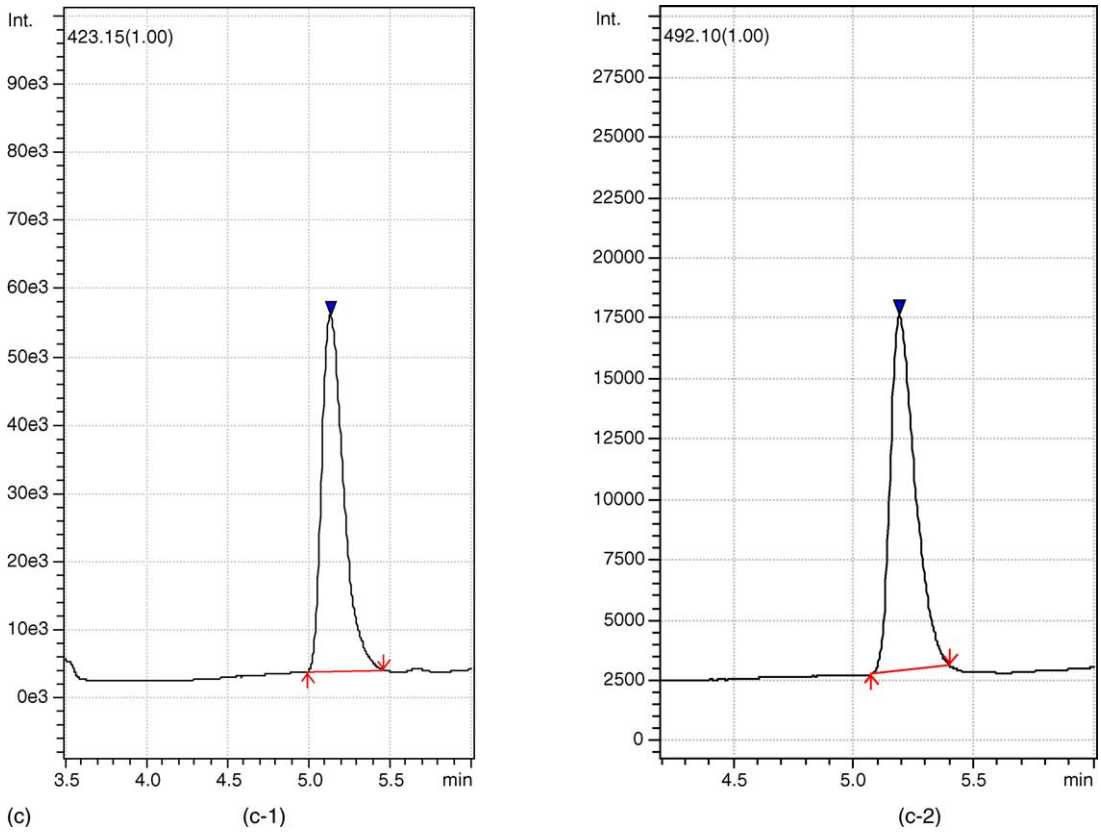

Fig. 3. (*Continued*).

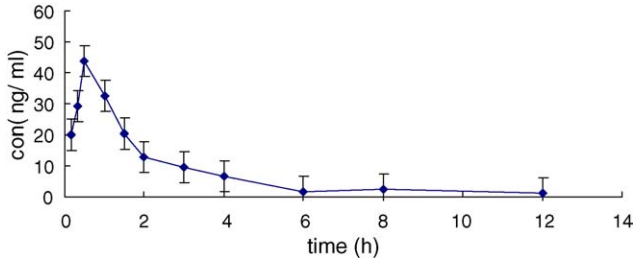

Fig. 4. Plasma concentration–time profile of ginkgolide B in dog after administration of a single dose of ginkgolide B.

The established method has been successfully applied to pharmacokinetic studies to determine the concentration of ginkgolide B in dog plasma. Fig. 4 shows the mean plasma concentration–time curve of ginkgolide B. The C_{max} and T_{max} of ginkgolide B were 43.8 ± 6.24 ng/ml and 0.5 h, and $t_{1/2}$ was 2.85 ± 0.54 h (Fig. 4).

4. Conclusions

In the present study, a simple and rapid LC–MS method to determine ginkgolide B levels in dog plasma was established and validated, which has been successfully applied to the pharmacokinetics studies. The developed assay showed acceptable precision, accuracy, linearity, stability and specificity.

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