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Simultaneous determination of ginkgolides A, B, C and bilobalide in plasma by LC–MS/MS and its application to the pharmacokinetic study of *Ginkgo biloba* extract in rats

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Abstract

A new liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of ginkgolides (includes ginkgolide C for the first time) and bilobalide in plasma is presented. Ketoprofen was used as an internal standard, and sample pre-treatment consisted of a liquid–liquid extraction. Chromatographic separation was achieved on a 5 μm Shiseido C8 column (150 mm × 2.0 mm i.d., particle size 5 μm) with a mobile phase consisting of methanol/6 mM ammonium acetate (60/40, v/v) at a flow rate of 0.3 ml/min. A tandem mass spectrometric detection was conducted using multiple reaction monitoring (MRM) under negative ionization mode with an atmospheric pressure chemical ionization (APCI) interface. The method was validated in terms of intra- and inter-day precision (<12.7%), accuracy (within ±7.0%), linearity, specificity and stability. In addition, matrix effects of ginkgolides and bilobalide in plasma were evaluated in different reconstitution solvents. Smaller matrix effects were observed for reconstitution solvents containing less organic solvent. The method has been successfully applied to a pharmacokinetic study of *Ginkgo biloba* extract in rats after intravenous administration. This is the first report of pharmacokinetic data for ginkgolide C.

Keywords: Ginkgolides; Bilobalide; LC–MS/MS; Simultaneous analysis; Matrix effect; Pharmacokinetic

1. Introduction

Ginkgo is considered to be the oldest surviving tree species on Earth and has been used medicinally for many centuries in China. In recent years, standardized extracts of ginkgo leaf are amongst the top-selling phytomedicines in the world [1]. In China, *Ginkgo* leaf and its standardized extracts are listed in the national pharmacopoeia. Various preparations made of *Ginkgo biloba* extract, including tablets, capsules, oral solutions and injectable solutions are available in hospital pharmacies and non-hospital drug stores.

The pharmacological activities of *Ginkgo biloba* extract that have been reported include antioxidant, anti-ischemic and neuro-protective effects and cardiovascular, cerebrovascular and peripheral circulatory benefits, and are used to prevent and treat diseases such cerebral and vascular insufficiency, cognitive deficits and other age-associated impairments [2,3].

The ginkgo terpenoids are the main pharmacological components in *Ginkgo biloba* extract. Ginkgo terpenoids include a sesquiterpenoid (bilobalide) and diterpenoids (ginkgolides). Ginkgolides, including A, B, and C, are potent and specific antagonists of platelet activating factor (PAF) [4,5] and bilobalide is believed to function as a neuro-protectant [6]. Structurally, the ginkgolides differ only in the number and position of hydroxyl groups. The structures of these compounds are shown in Fig. 1.

Though several methods have been described in the literature for the determination of ginkgo terpenoids, few data are available on the pharmacokinetics of *Ginkgo biloba* components. Biber and Koch [7] succeeded in this task using gas chromatography/mass spectrometry (GC/MS) after derivatization of ginkgolides, and Yang et al. [8] developed a similar method to study the pharmacokinetics of ginkgolide A and B in human volunteers. Li and Wong [5] estimated ginkgolide concentra-

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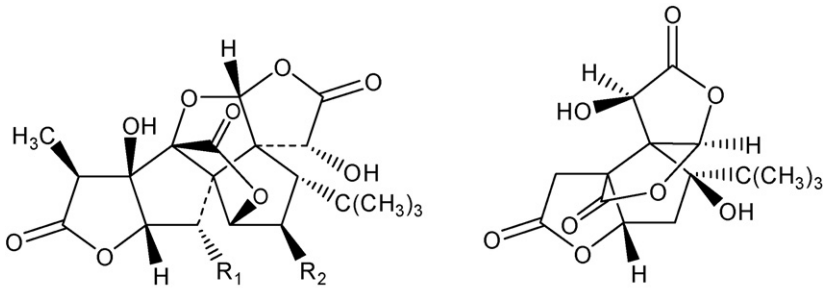

Ginkgolide	R1	R2
A	H	H
B	OH	H
C	OH	OH

Bilobalide

Fig. 1. Structure of ginkgolides A, B, C and bilobalide.

tions in animal plasma using a bioassay based on the capacity of ginkgolide to inhibit the binding of PAF to its receptor. Mauri et al. [9,10] used liquid chromatography/atmospheric pressure chemical ionization-ion trap mass spectrometry (LC–APCI-ITMS) to study the pharmacokinetics of ginkgolide A, B and bilobalide in both animals and human volunteers. Hua et al. [11] developed a LC-ESI/MS for analysis of ginkgolide B in dog plasma. However, no method for the determination of ginkgolide C has previously been reported in the literature and pharmacokinetic data are thus not available. Since ginkgolide C is abundantly present in *Ginkgo biloba* extract, it was deemed necessary to develop a method for the determination of this ginkgolide in plasma.

Ginkgolide C was found to be the most polar and to have the weakest chromatographic retention amongst the four compounds. It was vulnerable to matrix effects and difficult to measure in plasma. It has previously been mentioned [9,10,12,13] that ginkgolide C could not be detected in plasma samples and only ginkgolides A, B or bilobalide were measured in those studies. However, the present study shows that ginkgolide C is detectable after the intravenous administration of *Ginkgo biloba* extract to rats.

The matrix effects of ginkgolides A, B, C and bilobalide in plasma were also investigated in this project since no report on this aspect has previously been reported in any published methods. In fact, all four compounds suffered from significant matrix effects in plasma and in order to reduce these matrix effects, different reconstitution solvents were evaluated in terms of 'absolute' and 'relative' matrix effect.

In this article, a new method for the simultaneous determination of ginkgolide A, B, C and bilobalide in plasma and its application to a pharmacokinetic study of *Ginkgo biloba* extract in rats are described.

2. Experimental

2.1. Chemicals and reagents

Ginkgolides A, B, C, bilobalide and ketoprofen were all obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol and methyl *tert*-butyl ether (MTBE) were products of Sigma–Aldrich (Steinheim, Germany). Formic acid was obtained from TEDIA Co. (Fairfield, IA, USA). Ammonium acetate and other chemical reagents of analytical grade or better were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). Purified water used throughout the study was commercially available (Wahaha®, Hangzhou Wahaha Co., Ltd, China). The *Ginkgo biloba* leaf extract product standardized to contain 9.24% of ginkgo terpenoids and 24.25% of flavonoids was provided by Sine Promod Pharmaceutical Co., Ltd. (Shanghai, China).

2.2. Chromatographic conditions

An HPLC system, consisting of a Shimadzu LC-10ADvp pump, a Shimadzu SIL-HTc auto sampler, a Shimadzu CTO-10Avp column oven and a Shiseido C8 column (150 mm × 2.0 mm, 5 μm) equipped with a guard column (C18, 4 mm × 3.0 mm, Phenomenex Co,. Ltd., Torrance, CA, USA) was used for the chromatographic separation of ginkgolides A, B, C, bilobalide and the internal standard. The mobile phase was composed of methanol and 6 mM ammonium acetate at a ratio of 60/40 (v/v), and its flow rate was set at 0.3 ml/min. The temperatures of the analytical column and auto sampler were maintained at 40 °C and 5 °C, respectively. Under these conditions, the retention times for ginkgolides A, B, C, bilobalide and the internal standard were 1.8 ± 0.1 min, 1.9 ± 0.1 min, 1.6 ± 0.1 min, 1.7 ± 0.1 min and 2.4 ± 0.2 min, respectively. The total run time for a LC–MS/MS analysis was 3.5 min.

2.3. Mass spectrometric conditions

An API 3000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Ontario, Canada) with an atmospheric pressure chemical ionization (APCI) interface operated in negative ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The operation conditions were optimized by infusing diluted stock solutions of each analyte as

Table 1
Optimized mass parameters for ginkgolides A, B, C, bilobalide and the internal standard

Analyte	MRM (*m/z*)	Dwell time (ms)	DP (v)	CE (V)	FP (v)	EP (v)	CXP (v)
Bilobalide	325.0 → 162.9	75	−15	−28	−375	−10	−5.0
Ginkgolide B	423.2 → 367.0	75	−20	−22	−375	−10	−5.0
Ginkgolide C	439.2 → 383.0	75	−20	−25	−375	−10	−5.0
Ginkgolide A	407.2 → 351.0	75	−40	−22	−375	−10	−5.0
Ketoprofen (IS)	253.2 → 208.9	75	−20	−10	−375	−10	−5.0

follows: nebulizing gas flow 10 L/min, curtain gas flow 8 L/min, collision gas flow 6 L/min, source temperature 375 °C. The specific parameters for each analyte are shown in Table 1.

2.4. Preparations of standards and quality control (QC) samples

Four separate primary stock solutions for ginkgolides A, B, C and bilobalide were prepared in methanol at 80 μg/ml. The stock solutions were then mixed together and serially diluted with water to produce a series of standard or QC working solutions at the desired concentrations. The calibration standards were freshly prepared by adding 50 μl of the standard working solutions into 200 μl of pooled plasma to provide final concentrations at 2 ng/ml, 5 ng/ml, 10 ng/ml, 40 ng/ml, 100 ng/ml, 200 ng/ml and 400 ng/ml, respectively. Low, medium and high level QC samples were prepared at 5 ng/ml, 40 ng/ml and 200 ng/ml, respectively.

Ketoprofen (IS) was also prepared as a stock solution of 50 μg/ml in methanol and diluted with water to yield a working standard of 2.5 μg/ml.

All solutions described above were stored at 4 °C.

2.5. Samples preparation

Thawed plasma samples were briefly vortex-mixed and 50 μl of water was added to aliquots of 200 μl of each sample except the calibration standards. Twenty microlitres of internal standard working solution were then added to all samples except the blank, and the final concentration of ketoprofen was 500 ng/ml. To all samples, 5 ml of methyl *tert*-butyl ether were added, and the tubes were capped and vortexed at high speed for 3 min, then centrifuged at 3000 rpm for 10 min. The upper organic layer was transferred to another glass tube and evaporated to dryness under a gentle stream of nitrogen. The dried extract was reconstituted with 100 μl of water and then transferred into a plastic HPLC vial. A 20 μl aliquot was injected into the LC–MS/MS system for analysis.

2.6. Method validation

The current LC–MS/MS assay was validated for specificity, linearity, intra-day and inter-day precision, accuracy, extraction efficiency and stability. Selectivity of the method was confirmed by analyzing different lots of blank plasma without the presence of internal standards. Three validation batches were assayed to assess the precision and accuracy of the method. Each batch contained one set of calibration standards and five replicates of QC samples at low, medium and high concentration levels, and was processed on a separate day. The linearity of each curve was confirmed by plotting the peak area ratio (y) of the analyte to IS versus analyte concentration (x). The accuracy of the assay was expressed by [(mean observed concentration)/(spiked concentration)] × 100%.

Stability was evaluated under conditions mimicking situations likely to be encountered during sample storage, preparation and the analytical process, including −20 °C storage in plasma for 44 days, three cycles of freeze and thaw, and extracted samples storage in an autosampler (5 °C) for 24 h.

2.7. Matrix effect

The 'absolute' matrix effect was evaluated by comparing the peak areas of analytes added post-extraction with those of the standards in the reconstitution solvent. The coefficients of variation [CV%] of the mean peak areas of analytes added post-extraction indicate the presence of 'relative' matrix effects, which derived from the variance in five different lots of blank plasma matrix. In method development, three different reconstitution solvents, mobile phase, 10% methanol and water were closely examined for their matrix effect.

2.8. Pharmacokinetic study

The suitability of the developed assay was demonstrated in a pharmacokinetic study of *Ginkgo biloba* extract after intravenous administration to rats. Six Sprague-Dawley rats (three males, three females), weighing 300 ± 50 g, were supplied by the Experimental Animal Center of Shanghai Institute of Pharmaceutical Industry. The rats were weighed and an injectable solution of *Ginkgo biloba* extract was administered intravenously via a tail vein at a dose of 8 mg/kg. The solution was prepared in normal saline containing 0.4% Poloxamer (F-188) at a concentration of 2.5 mg/ml. The content of ginkgolides A, B, C and bilobalide per dose were 303.2 μg, 168.8 μg, 100.3 μg and 98.0 μg, respectively.

Serial blood samples (∼0.5 ml each at 0 min, 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min and 180 min after administration) were collected in heparinized tubes by puncture of the orbital sinus of the rats under ether anesthesia. Plasma was obtained by centrifugation at 12000 rpm for 8 min, and stored in polypropylene tubes at −20 °C until assay.

3. Results and discussion

3.1. Specificity and selectivity

Abundant deprotonated molecules of the ginkgo terpenoids that formed the base peak of each mass spectrum were observed from Q1 scans during the infusion of the neat solution in negative mode. Four $[M-H]^-$ precursor ions, *m/z* 325.0 for bilobalide, *m/z* 407.2 for ginkgolide A, *m/z* 423.2 for ginkgolide B and *m/z* 439.2 for ginkgolide C, were subjected to collision induced dissociation (CID). The product ion tandem mass spectra of the deprotonated molecules of the ginkgo terpenoids are shown in Fig. 2. The major ion of *m/z* 163 for the deprotonated bilobalide may be due to loss of both a *tert*-butyl group and a hydroxyl group, and the additional elimination of two carbon dioxide groups from the other rings. However, this hypothesis needs further confirmation with detailed studies. For the deprotonated ginkgolides, the loss of two carbon monoxide molecules, $[M-H-2CO]^-$, was the most abundant fragment pathway. Therefore, the mass transition patterns, *m/z* 325.0 → 162.9, *m/z* 407.2 → 351.0, *m/z* 423.2 → 367.0 and *m/z* 439.2 → 383.0 were selected to monitor bilobalide, ginkgolides A, B and C, respectively. A MS/MS channel of *m/z* 253.2 → 208.9 was chosen to monitor the internal standard, ketoprofen.

Under the current LC–MS/MS conditions, the four analytes were well separated from interferences in the matrix blank. Chromatograms of different lots of blank plasma were found to contain no endogenous peak co-eluted with any of the analytes or with the internal standard. Representative chromatograms of blank samples with or without the presence of analytes and internal standard are shown in Fig. 3. In addition, the "cross-talk" between channels used for monitoring the analytes and IS was evaluated by analysis of their individual solution at high concentration. The responses in the MRM mass transition channels used for quantification were monitored. No "cross-talk" or interference between the analytes and IS was observed.

3.2. Matrix effect

The *matrix* effect in three different reconstitution solvents, mobile phase, 10% methanol and water was investigated during method development. As seen in Table 2, a significant matrix effect was observed when using the mobile phase as the reconstitution solvent. By using 10% methanol or water as reconstitution solvent, both the 'absolute' and the 'relative' matrix effect [CV%] were considerably reduced. The results indicated that the less organic solvent in the reconstitution solution, the smaller the observed matrix effect. This phenomenon may due to less matrix interference when water is used compared with mobile phase. In addition, the spread of the peaks was reduced and the sensitivity was also improved using water as the reconstitution solvent. The extraction efficiency was also evaluated by comparing the absolute peak areas of analytes added pre-extraction with those of post-extraction. The mean extraction efficiencies for bilobalide, ginkgolides A, B and C were greater than 77%, 90%, 95% and 87%, respectively.

3.3. Linearity and sensitivity

Seven-point calibration curves ranging from 2 ng/ml to 400 ng/ml were prepared on three separate days. The curves were obtained by plotting the peak area ratio of the analytes to IS against the corresponding concentration of the analytes in the freshly prepared plasma calibrators. The regression parameters of slope, intercept and correlation coefficient were calculated by $1/x$-weighted linear regression in Analyst 1.1 software used in Sciex API 3000. Excellent linearity was achieved with correlation coefficients greater than 0.998 for all validation batches (Table 3). The concentrations of calibration standards were back calculated. Concentrations for QC samples were calculated from

Table 2
Matrix effects for ginkgolides A, B, C, bilobalide and internal standard in different reconstitution solvents

Analyte	Analyte concentration (ng/ml)	Mobile phase	10% Methanol	Water
		Matrix effect (%)[a] [CV%[b], $n=5$]	Matrix effect (%)[a] [CV%[b], $n=5$]	Matrix effect (%)[a] [CV%[b], $n=5$]
Bilobalide	5.13	−25.8 [24.5]	−20.0 [10.1]	−8.8 [4.7]
	41.0	−50.9 [44.7]	17.2 [5.6]	0.1 [5.6]
	205	−35.6 [17.4]	−4.8 [4.1]	−1.0 [12.0]
Ginkgolide B	5.10	−14.3 [53.4]	2.3 [7.1]	−7.7 [8.0]
	40.8	−77.9 [104.2]	8.1 [7.5]	−2.1 [4.5]
	204	−61.2 [54.6]	−1.8 [4.8]	6.4 [4.1]
Ginkgolide C	5.00	−31.1 [33.2]	−9.2 [7.7]	7.6 [6.7]
	40.0	−73.5 [111.9]	12.5 [18.2]	2.8 [10.8]
	200	−62.4 [33.0]	−11.5 [8.7]	−0.3 [10.7]
Ginkgolide A	5.11	−10.8 [31.2]	7.5 [6.3]	−7.6 [11.3]
	40.9	−36.8 [41.3]	23.1 [0.9]	0.7 [4.5]
	205	−13.6 [22.7]	11.1 [2.3]	7.3 [2.3]
IS	500	−30.4 [14.8]	6.6 [3.0]	6.2 [12.3]

[a] Matrix effect (%): (mean peak areas of analytes added post-extraction in five different lots of plasma)/(peak areas of standard) × 100 − 100.
[b] CV%: coefficient of variation of the mean peak areas of analytes added post-extraction in five different lots of plasma.

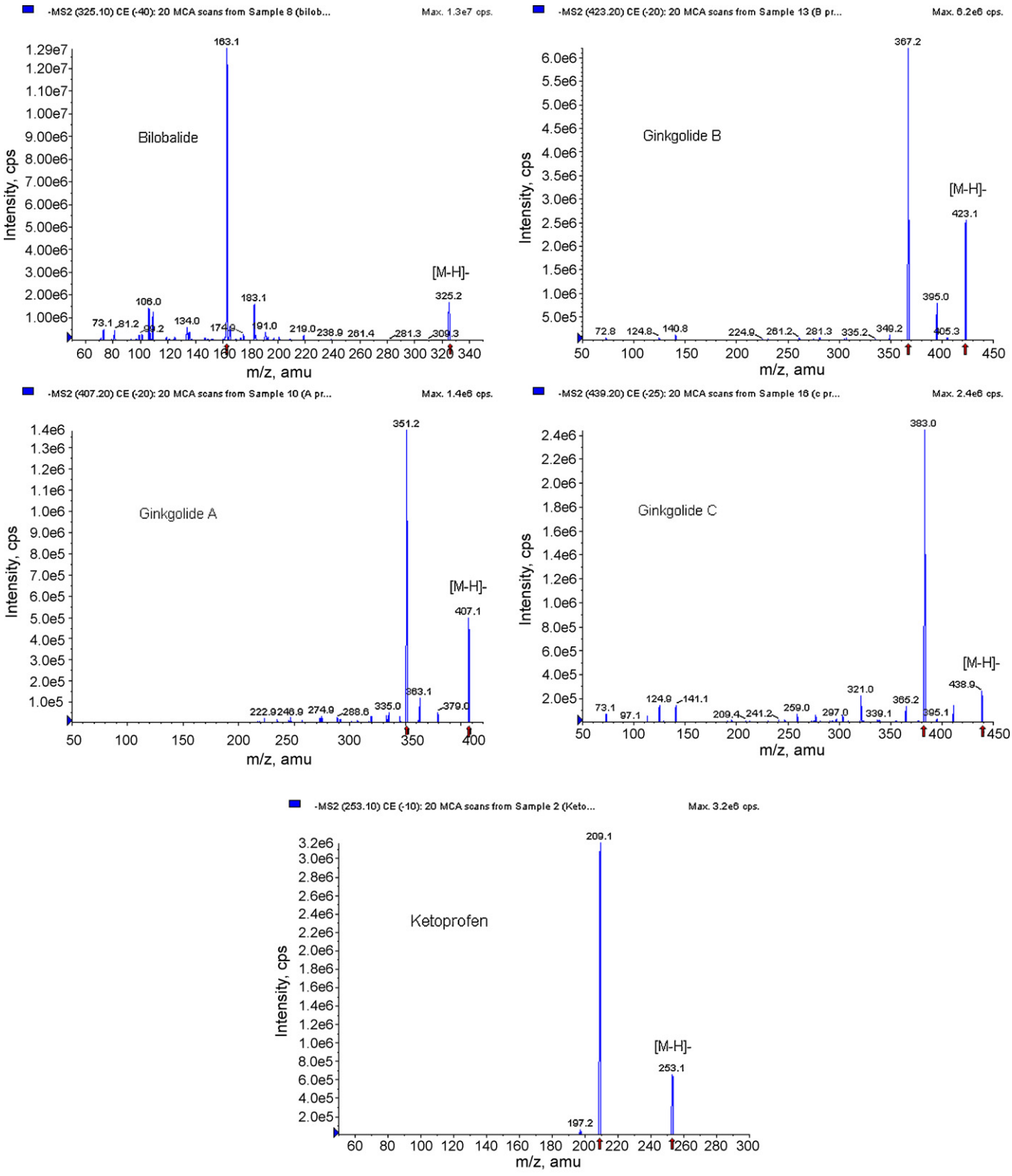

Fig. 2. MS/MS product ion mass spectra of the deprotonated molecules $[M-H]^-$ of bilobalide, ginkgolides A, B, C and ketoprofen (IS).

the resulting peak area ratios and the regression equation of the calibration curves.

Though the validated LLOQ (2 ng/ml) of the method is sensitive enough for our present pharmacokinetic study, the good signal to noise (shown in Fig. 3) obtained at this concentration still allows lowering the LLOQ of the method or decreasing the volume of plasma used.

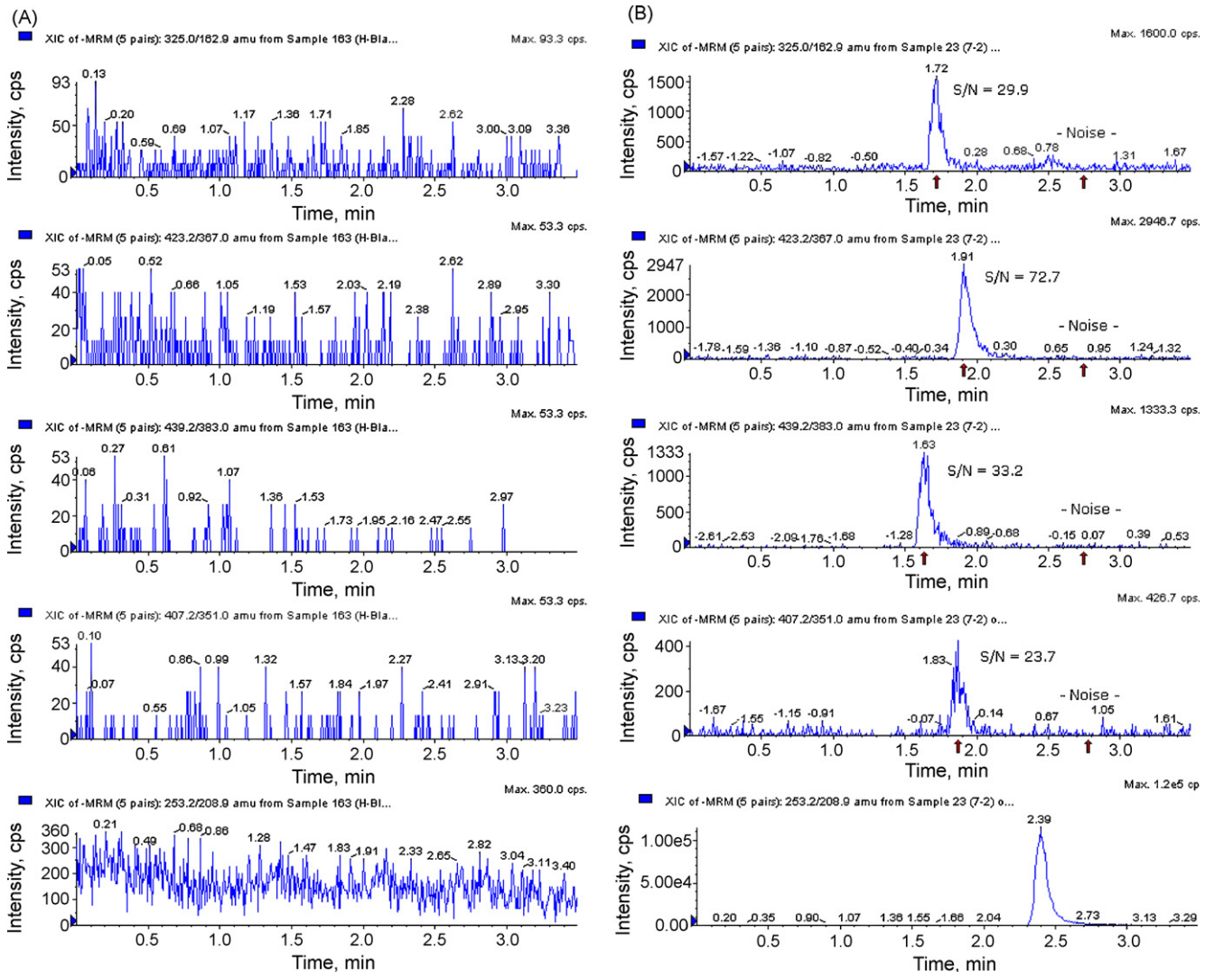

Fig. 3. Representative MRM chromatograms for (A) blank plasma; (B) LLOQ, 2 ng/ml for analytes with 500 ng/ml IS in plasma.

3.4. Precision and accuracy

The intra-day precision and accuracy was determined by the replicate analyses of QC samples ($n = 5$) at three level concentrations during the three separate days. One replicate of the QC samples at each concentration level from three separate validation batches was used to evaluate the inter-day precision. The assay precision and accuracy results are shown in Table 4. The

Table 3
Linearity for assay of ginkgo terpenoids in plasma

Analyte	Run	Linear range (ng/ml)	Intercept	Slope	Correlation coefficient
Bilobalide	1	2.19–438	−0.00231	0.00968	0.9989
	2		0.00683	0.00966	0.9999
	3		0.00672	0.00901	0.9997
Ginkgolide B	1	1.98–396	−0.0111	0.0284	0.9996
	2		−0.00856	0.0243	0.9999
	3		0.00359	0.0221	0.9997
Ginkgolide C	1	1.98–396	−0.00318	0.0147	0.9986
	2		−0.00363	0.0130	0.9996
	3		0.00711	0.0121	0.9995
Ginkgolide A	1	2.02–404	0.000787	0.00315	0.9995
	2		0.00139	0.00300	0.9997
	3		0.00328	0.00259	0.9999

Table 4
Intra-day and inter-day precision and accuracy for assay of ginkgo terpenoids in plasma

Analyte	Analyte concentration (ng/ml)	Intra-day ($n=5$)			Inter-day ($n=3$)		
		MC[a] (ng/ml)	RSD (%)	A[b] (%)	MC[a] (ng/ml)	RSD (%)	A[b] (%)
Bilobalide	5.48	5.592 ± 0.191	3.4	102.2	5.498 ± 0.431	7.8	100.4
	43.8	45.71 ± 1.38	3.0	104.4	42.86 ± 1.44	3.3	97.9
	219	233.1 ± 18.6	8.0	106.4	218.3 ± 10.1	4.6	99.7
Ginkgolide B	4.95	5.098 ± 0.270	5.3	103.0	4.889 ± 0.087	1.8	98.8
	39.6	40.04 ± 1.44	3.6	101.1	38.29 ± 0.83	2.2	96.7
	198	211.8 ± 7.9	3.7	107.0	199.6 ± 13.5	6.7	100.8
Ginkgolide C	4.95	5.143 ± 0.410	8.0	103.9	4.883 ± 0.616	12.6	98.7
	39.6	38.11 ± 1.42	3.7	96.2	37.93 ± 1.03	2.7	95.8
	198	194.3 ± 16.7	8.6	98.1	191.4 ± 4.8	2.5	96.7
Ginkgolide A	5.05	5.224 ± 0.455	8.7	103.4	4.898 ± 0.099	2.0	97.0
	40.4	39.74 ± 0.76	1.9	98.4	38.69 ± 0.58	1.5	95.8
	202	208.3 ± 4.8	2.3	103.1	197.5 ± 12.7	6.4	97.8

[a] MC: measured concentration.
[b] A: accuracy.

Table 5
Stability of bilobalide and ginkgolides in plasma

Analyte	Analyte concentration (ng/ml)	Three freeze and thaw cycles[a]	−20 °C storage for 44 days[a]	Extracted samples in autosampler (5 °C) for 24 h[a]
Bilobalide	5.48	104.0	94.2	113.4
	43.8	101.9	105.0	90.4
	219	100.2	95.3	107.3
Ginkgolide B	4.95	101.6	103.0	109.0
	39.6	100.0	99.6	96.4
	198	105.1	95.9	102.9
Ginkgolide C	4.95	105.5	115.0	101.6
	39.6	89.8	117.0	100.4
	198	100.0	106.0	102.0
Ginkgolide A	5.05	97.5	93.9	101.8
	40.4	107.3	94.2	97.2
	202	108.9	94.9	101.9

[a] Expressed as [(measured concentration)/(analyte concentration) × 100]%.

intra-day precision was within 8.7% and the inter-day precision was within 12.6%. The assay accuracy was 95.8–107.0% of the nominal values.

3.5. Stability

The stability of bilobalide and ginkgolides in plasma was investigated. The stability experiments were aimed at testing the effects of possible conditions that the samples might experience between collection and analysis. Stability results are summarized in Table 5. Three cycles of freeze and thaw for QC samples indicated that bilobalide and ginkgolides were stable in plasma. QC samples were stable when stored frozen at −20 °C for at least 44 days. Testing of autosampler stability of extracted samples indicated that bilobalide and ginkgolides were stable when kept in the autosampler (5 °C) for 24 h.

Table 6
Pharmacokinetic parameters of bilobalide, ginkgolides A, B and C in rats

Parameters	Units	Ginkgolide A	Ginkgolide B	Ginkgolide C	Bilobalide
$AUC_{(0-t)}$	μg h/L	228.2	140.7	110.3	33.25
$AUC_{(0-\infty)}$	μg h/L	258.6	157.6	115.3	39.06
$MRT_{(0-t)}$	h	0.96	0.87	0.72	1.05
$t_{1/2}$	h	0.97	1.02	0.67	1.13
Cl[a]	L/h kg	36.82	112.1	136.8	301.1
V[b]	L/kg	49.33	163.9	130.4	478.0

[a] Cl: clearance.
[b] V: apparent volume of distribution.

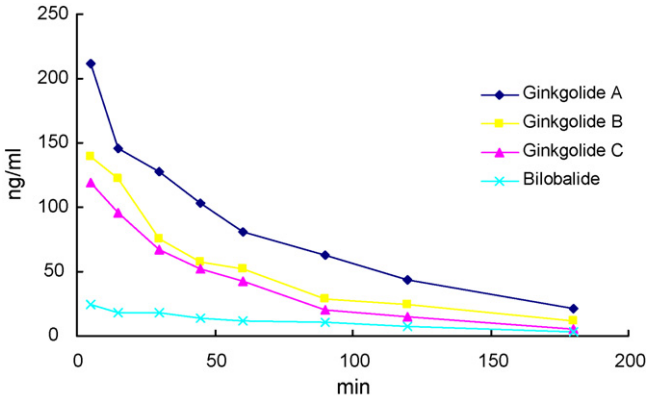

Fig. 4. Mean plasma concentration–time profiles of ginkgolides A, B, C and bilobalide ($n = 6$).

3.6. Pharmacokinetic study

The method was successfully applied to a pharmacokinetic study of *Ginkgo biloba* extract in rats. The mean plasma concentration–time profiles of ginkgolides A, B, C and bilobalide are shown in Fig. 4.

Pharmacokinetic parameters from a non-compartment model analysis (Drug and Statistics, DAS version 2.0) are summarized in Table 6.

4. Conclusion

In this paper, a LC–APCI-MS/MS method for simultaneous determination of ginkgolides A, B, C and bilobalide in plasma has been described. The method provides great improvement to previously published methods since ginkgolide C in plasma has been measured for the first time. In addition, matrix effects of ginkgolides and bilobalide were also carefully studied. As the results indicated, the matrix effects were significantly reduced via the novel use of water as a reconstitution solvent, and we suppose that it may also be useful for the determination of other compounds in bio-matrix. The current method also offers other advantages over existing methods, such as shorter analysis time (3.5 min), smaller sample volume (200 μl plasma) and simpler sample preparation process. This method has been successfully applied to a pharmacokinetic study of *Ginkgo biloba* extract after intravenous administration to rats. Lastly, pharmacokinetic data of ginkgolide C has been reported for the first time.

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