Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 53–57

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Simultaneous determination of magnolin and epimagnolin A in rat plasma by liquid chromatography with tandem mass spectrometry: Application to pharmacokinetic study of a purified extract of the dried flower buds of *Magnolia fargesii*, NDC-052 in rats

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 28 March 2009
Accepted 30 March 2009
Available online 7 April 2009

Keywords:
Magnolin
Epimagnolin A
Rat plasma
LC/MS/MS
Purified extract of the dried flower buds of *Magnolia fargesii* (NDC-052)

ABSTRACT

A purified extract isolated from the dried flower buds of *Magnolia fargesii* (NDC-052) is currently being evaluated for phase III clinical trials as a new anti-asthma drug. A rapid, sensitive and selective liquid chromatography–tandem mass spectrometric (LC/MS/MS) method for the simultaneous determination of magnolin and epimagnolin A, the major bioactive components of NDC-052, in rat plasma was developed. After liquid–liquid extraction with tolterodine as an internal standard, magnolin and epimagnolin A were separated on a Luna phenyl-hexyl column with the mobile phase of 70% methanol in 10 mM ammonium formate. The analytes were detected by an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curves were linear over the concentration range of 50–2500 ng/mL for magnolin and epimagnolin A in rat plasma. The intra- and inter-day coefficients of variation and relative errors for magnolin and epimagnolin A at four QC concentrations were 1.5–11.4% and 5.9–12.5%, respectively. The lower limits of quantification for magnolin and epimagnolin A were 50.0 ng/mL using 50 μL of plasma. This method was successfully applied to the pharmacokinetic study of magnolin and epimagnolin A after an oral administration of NDC-052 in male Sprague–Dawley rats.

1. Introduction

The Chinese crude drug shin-i, the dried flower buds of *Magnolia fargesii* Cheng (Magnoliaceae), has been used for the treatment of empyema, nasal congestion, sinusitis and allergic rhinitis [1]. Essential oils, lignans, neolignans and sesquiterpenes have been found in the flower buds of *Magnolia fargesii*. Some of these lignans, i.e., magnolin, epimagnolin A, fargesin, dimethylpinoresinol, dimethylliroresinol, aschantin, magnone A and B isolated from this plant showed the anti-inflammatory, antihistamine, anticomplementary, Ca^{2+}-antagonist, platelet activating factor (PAF)-antagonistic and inhibitory activity of the expression of the cell adhesion molecules ICAM-1 and VCAM-1 [2–7].

A purified extract isolated from the dried flower buds of *Magnolia fargesii* (NDC-052) was developed to enhance and control the contents of the bioactive lignans such as magnolin and epimagnolin A [8]. NDC-052 inhibited various inflammation mediators such as PAF, cytosolic phospholipase A2, hexosaminidase, lypoxygenase, complement system and leukotriene C4 production [4,6,8]. Oral administration of NDC-052 at dose of 50 mg/kg for 8 weeks reduced the airway resistance in the chronic asthma model using guinea pigs (our unpublished data). Currently, NDC-052 is being evaluated for phase III clinical trials in Korea as a new anti-asthma drug.

The evaluation of the pharmacokinetics and bioavailability of herbal medicinal drugs can link data from pharmacological assays to clinical effects and also help the design of rational dosage regimens [9]. The FDA general requirements for *in vivo* bioavailability data in a purified chemical drug are applicable to botanical drug products [10]. However, the type of bioavailability and pharmacokinetic study for a specific botanical drug product depends on the information on the active constituent, if known, the complexity of the drug substance, and the availability of analytical methods.

Magnolin was metabolized to 4′-*O*-demethylmagnolin, 4″-*O*-demethylmagnolin and their glucuronides in the rats [11]. There was no report on bioavailability data of magnolin and epimagnolin A in the experimental animals as well as humans. To the best of our knowledge, the bioanalytical method has not been reported for the simultaneous determination of magnolin and epimagnolin A from plasma. LC–mass spectrometry (LC/MS) is recognized as a powerful tool for the simultaneous determination of the active compounds of

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doi:10.1016/j.jpba.2009.03.036

herbal drug in biological samples due to the selectivity, sensitivity, robustness and sample throughput [9,12,13].

The purpose of this paper was to develop the selective, sensitive, rapid and reliable LC/MS/MS assay using liquid–liquid extraction for the simultaneous determination of magnolin and epimagnolin A in rat plasma. This method was successfully applied to the pharmacokinetic study of magnolin and epimagnolin A after oral administration of NDC-052 in male Sprague–Dawley rats.

2. Experimental

2.1. Materials

Magnolin and epimagnolin A were isolated from the dried and pulverized flower buds of *Magnolia fargesii* by using our previous method [8] and their purities were more than 99.0%. The dried flower buds of *Magnolia fargesii* were purchased from Il-Shin Pharm. Co. (Taejon, Korea) and the voucher specimen is deposited in Natural Product Biosynthesis Research Unit, Korea Research Institute of Biology & Biotechnology. Tolterodine tartrate (internal standard) was a gift from Dong-A Pharm. Co. (Yongin, Korea). Methanol and methyl *tert*-butyl ether (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free rat plasma containing sodium heparin as the anticoagulant was obtained from rats.

NDC-052 was a purified extract isolated from the dried flower buds of *Magnolia fargesii* [8] and the contents of magnolin and epimagnolin A in NDC-052 were 18.0% and 9.0% by LC/MS/MS method, respectively.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of magnolin, epimagnolin A and tolterodine (1 mg/mL) were prepared in dimethylsulfoxide. Working standard solutions of magnolin and epimagnolin A were prepared by diluting each primary solution with acetonitrile. The internal standard working solution (7.5 μg/mL) was prepared by diluting an aliquot of stock solution with acetonitrile. All standard solutions were stored at 4 °C in polypropylene bottles in the dark when not in use.

Rat plasma calibration standards of magnolin and epimagnolin A (50, 100, 200, 500, 1000, 2000, and 2500 ng/mL) were prepared by spiking appropriate amount of the working standard solutions into a pooled rat plasma. Quality control (QC) samples at 50, 150, 800 and 1800 ng/mL were prepared in bulk by adding 50 μL of the appropriate working standard solutions (2, 6, 32 and 72 μg/mL) to drug-free rat plasma (1950 μL). The QC samples were aliquoted (50 μL) into polypropylene tubes and stored −20 °C until analysis.

2.3. Sample preparation

50 μL of blank plasma, calibration standards and QC samples were mixed with 10 μL of internal standard working solution and 300 μL of 50 mM potassium phosphate buffer (pH 7.4) in 1.5 mL-polypropylene tubes. The samples were extracted with 1000 μL of methyl *tert*-butyl ether by vortex-mixing for 3 min at high speed and centrifuged at 13,000 × *g* for 5 min. The organic layer was pipette transferred and evaporated to the dryness at 35 °C for 10 min using a vacuum concentrator (EZ-plus, Genevac, UK). The residues were dissolved in 30 μL of 70% methanol by sonicating for 3 min, transferred to injection vials, and 5 μL were injected onto LC/MS/MS system.

2.4. LC/MS/MS analysis

The LC/MS/MS system consisted of a Nanospace SI-2 pump, SI-2 column oven, an SI-2 autosampler (Shiseido, Tokyo, Japan) and a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd., UK). The separation was performed on a phenyl-hexyl column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using 70% methanol in 10 mM ammonium formate at a flow rate of 0.2 mL/min. The column and autosampler tray were maintained at 50 and 4 °C, respectively. The analytical run time was 5.5 min. The electrospray ionization source settings for the analysis of magnolin, epimagnolin A and tolterodine were as following: capillary voltage 3.5 kV; desolvation temperature 350 °C; ion source temperature 120 °C; cone voltages 25 V for magnolin, 25 V for epimagnolin A and 40 V for tolterodine. Fragmentation of molecular ions ($[M+H]^+$) for magnolin, epimagnolin A and tolterodine was performed at collision energy of 20, 20 and 30 eV, respectively, by collision-activated dissociation with argon as the collision gas. Quantification of the ions was performed by multiple reaction monitoring (MRM) mode: m/z 417.2–399.3 for magnolin and epimagnolin A, and m/z 326.4–147.6 for tolterodine. Data processing was performed by MassLynx Version 4.0 (Micromass UK Ltd., UK).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on 3 different days to complete the method validation. In each batch, QC samples at 50, 150, 800 and 1800 ng/mL were assayed in sets of five replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from the true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision.

To evaluate the three freeze/thaw cycle stability and room temperature storage stability, five replicates of QC samples at each of the low and high concentrations (150 and 1800 ng/mL, respectively) were subjected to three freeze/thaw cycles or the storage for 4 h at room temperature. Post-preparative stability was evaluated by reinjection of QC samples after 24 h storage under autosampler conditions.

The absolute matrix effects for magnolin, epimagnolin A and tolterodine were assessed by comparing mean peak areas of the analytes spiked at four concentrations, i.e., 50, 150, 800 and 1800 ng/mL into plasma extracts originating from five different rats to mean peak areas for the neat solutions of the analytes in 70% methanol [14]. The relative matrix effect was assessed by the variability in the peak areas of magnolin, epimagnolin A and tolterodine spiked post-extraction into five different plasma extracts, expressed as CVs (%). Recoveries of magnolin, epimagnolin A and tolterodine were determined by comparing the mean peak areas of the extract of analyte-spiked plasma with those of the analyte-spiked post-extraction into five different blank plasma lots at four concentrations.

2.6. Application

The present LC/MS/MS method was used to evaluate the pharmacokinetics of magnolin and epimagnolin A after an oral administration of NDC-052 to male Sprague–Dawley rats (body weight 240–300 g, Orient ENG Inc., Sungnam, Korea). Animals were kept in plastic cages with free access to standard rat diet (Samyang Co., Seoul, Korea) and water. The animals were maintained at a temperature of 22–25 °C with a 12 h light/dark cycle and relative humidity of 50 ± 10%. The rats were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and cannulated with polyethylene tubing (0.58 mm i.d. and 0.96 mm o.d., Clay Adams Co., Parsip-

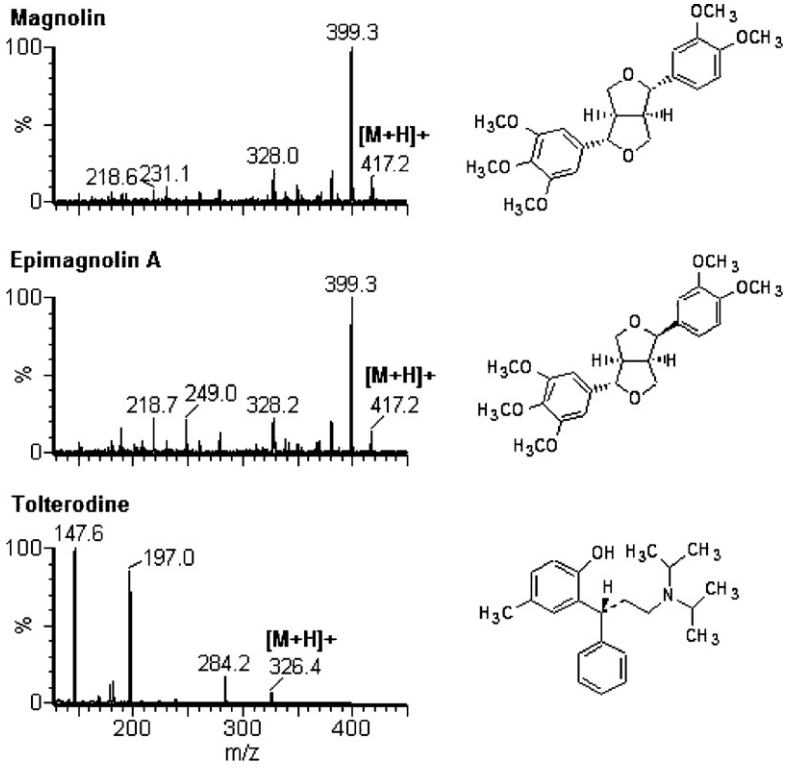

Fig. 1. Product ion mass spectra of magnolin, epimagnolin A and tolterodine (internal standard).

pany, NJ, USA) in the carotid artery for blood sampling. Each rat was housed individually in a rat metabolic cage and allowed to recover from anesthesia for 1 day before the study began. The rats were not restrained at any time during the study. Each cannula was flushed with heparinized 0.9% NaCl-injectable solution (20 U/ml) to prevent blood clotting. NDC-052 was dissolved in a mixture of dimethylsulfoxide: propylene glycol: water (1:2:1, v/v/v) and orally administered at a dose of 22.2 mg/kg in the rats ($n = 6$). Arterial blood samples were collected at 0 (control), 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after drug administration. Plasma samples were harvested by centrifugation at 3000 × g for 10 min and stored at −20 °C until analysis.

The following pharmacokinetic parameters for magnolin and epimagnolin A were calculated using a non-compartment analysis (WinNonlin, Pharsight, Mountain View, CA, USA): the area under the plasma concentration–time curve (AUC) and terminal elimination half-life ($t_{1/2}$). The maximum plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were determined by visual inspection from each plasma concentration–time plot for magnolin and epimagnolin A. Pharmacokinetic parameter values were expressed as mean ± S.D.

3. Results and discussion

3.1. LC/MS/MS

The electrospray ionization of magnolin, epimagnolin A and tolterodine produced the abundant protonated molecular ions ($[M+H]^+$) at m/z 417.2, 417.2 and 326.4, respectively without any evidence of fragmentation and adduct formation. MH^+ ions from magnolin, epimagnolin A and tolterodine were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 1). The product ion at m/z 399.3 was prominently formed from MH^+ ions of magnolin and epimagnolin A due to a loss of H_2O. The prominent product ion for tolterodine was m/z 147.6 (the loss of 3-diisopropylamino and 1-phenyl groups from $[M+H]^+$). The quantification of the analytes was performed using the MRM data acquisitions due to the high selectivity and sensitivity, where the precursor and product ions are monitored. Two pairs of MRM transitions were selected: m/z 417.2 → 399.3 for magnolin and epimagnolin A and m/z 326.4 → 147.6 for tolterodine (internal standard).

The typical MRM chromatograms for the separation of magnolin and epimagnolin A on a Luna phenyl-hexyl column with the mobile phase of 70% methanol in 10 mM ammonium formate are shown in Fig. 2. There was no interference peak at the retention times of magnolin (4.4 min), epimagnolin A (4.9 min) and tolterodine (4.2 min) in the analysis of blank plasma samples obtained from twenty different rats, indicating the selectivity of the present method (Fig. 2(a)). Sample carryover effect was not observed.

3.2. Method validation

Calibration curves were obtained over the concentration range of 50–2500 ng/mL of magnolin and epimagnolin A in rat plasma. Linear regression analysis with a weighting of 1/concentration^2 gave the optimum precision and accuracy of the corresponding calculated concentrations at each level (Table 1). The low CV value for the slope of magnolin (8.5%) and epimagnolin A (8.0%) indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-day precision and accuracy data for QC samples containing magnolin and epimagnolin A. Intra- and inter-day CV values ranged from 1.5% to 9.9% for magnolin and from 3.4% to 8.2% for epimagnolin A at four QC levels. The intra- and inter-day RE values were −5.0% to 5.6% for magnolin and −3.4% to 6.4% for epimagnolin A at four QC levels. These results indicated that the present method has the acceptable accuracy and precision. The lower limit of quantification (LLOQ) for magnolin and epimagnolin A was set at 50.0 ng/mL using 50 μL of rat plasma.

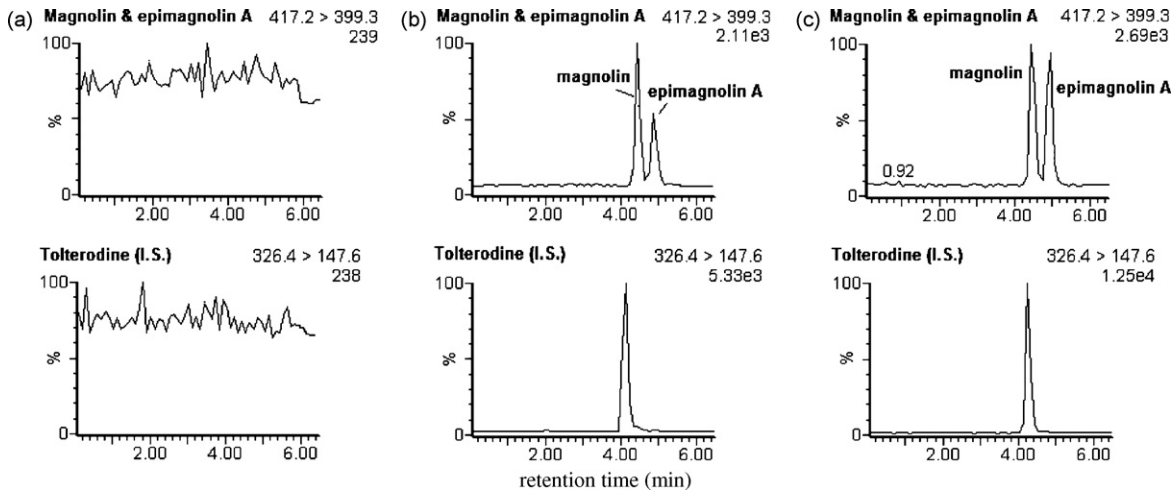

Fig. 2. MRM chromatograms of (a) a blank rat plasma, (b) rat plasma sample spiked with 50 ng/mL of magnolin and epimagnolin A, and (c) a plasma sample obtained at 2 h after oral administration of NDC-052, a purified extract of dried flower buds of *Magnolia fargesii* at a dose of 22.22 mg/kg in rat.

Table 1
Calculated concentrations of magnolin and epimagnolin A in calibration standards prepared in rat plasma ($n = 3$).

Statistical variable	Theoretical concentration (ng/mL)								
	50.0	100	200	500	1000	2000	2500	Slope	r^2
Magnolin									
Mean (ng/mL)	45.1	93.9	193	521	1044	1891	2568	0.0047	0.9976
CV (%)	9.5	7.4	5.6	8.4	7.7	2.9	4.5	8.5	
RE (%)	−9.8	−6.1	−3.5	4.2	4.4	−5.5	2.7		
Epimagnolin A									
Mean (ng/mL)	47.3	96.6	196	508	1014	1967	2519	0.0033	0.9990
CV (%)	12.0	7.9	8.4	7.7	8.7	5.1	4.5	8.0	
RE (%)	−5.4	−3.4	−2.0	1.6	1.4	−1.7	0.8		

Representative chromatograms at LLOQ are shown in Fig. 2(b) and the signal-to-noise ratios for magnolin and epimagnolin A are higher than 10.

The absolute matrix effects, the ratio of mean peak areas of analytes spiked post-extraction to those of the same analyte standards multiplied by 100, were 100.1% for magnolin, 101.3% for epimagnolin A and 101.1% for tolterodine (Table 3). A value of 100% indicates that the response in the solvent and in the plasma extracts were the same and no absolute matrix effect was observed. A value of <100% indicates an ionization suppression and a value of >100% indicates an ionization enhancement. There was little absolute matrix effect for magnolin, epimagnolin A and tolterodine. The CVs of peak areas of magnolin, epimagnolin A and tolterodine spiked into each plasma extract originated from five rats were 5.0–8.5% for magnolin, 6.0–7.2% for epimagnolin A and 2.3–5.5% for tolterodine, suggesting that the relative matrix effects for magnolin, epimagnolin A and tolterodine were practically absent. The CVs of the peak area ratio of analytes/tolterodine (I.S.) for samples spiked post-extraction into plasma extracts from five different lots were 5.4–7.3% for magnolin and 2.6–5.3% for epimagnolin A at four concentration levels, indicating that the absolute and relative matrix effects might have practically no effect on the determination of magnolin and epimagnolin A spiked into five different lots of rat plasma using liquid–liquid extraction for sample preparation.

Table 2
Precision and accuracy of magnolin and epimagnolin A in quality control samples.

Statistical variable	Intra-day ($n = 5$)				Inter-day ($n = 3$)			
Magnolin								
QC (ng/mL)	50.0	150	800	1800	50.0	150	800	1800
Mean (ng/mL)	47.5	151	806	1812	52.8	155	806	1814
CV (%)	6.2	2.6	8.6	1.5	9.9	3.3	1.7	1.6
RE (%)	−5.0	0.7	0.8	0.7	5.6	3.3	0.8	0.8
Epimagnolin A								
QC (ng/mL)	50.0	150	800	1800	50.0	150	800	1800
Mean (ng/mL)	50.7	148	773	1847	50.8	159	820	1915
CV (%)	7.3	5.7	6.8	5.0	8.2	6.1	6.0	3.4
RE (%)	1.4	−1.3	−3.4	2.6	1.6	6.0	2.5	6.4

Methyl *tert*-butyl ether at neutral pH gave the best recovery of magnolin and epimagnolin A from rat plasma samples compared to the use of ethyl acetate and dichloromethane at neutral pH. As shown in Table 3, the overall extraction recoveries of magnolin and epimagnolin A were 84.2% and 87.5%, which were consistent over the concentration range of 50–1800 ng/mL. The recovery of tolterodine was 83.1%. Liquid–liquid extraction with methyl *tert*-butyl ether at neutral pH has been successfully applied to the extraction of magnolin and epimagnolin A from rat plasma.

Stability of magnolin and epimagnolin A during sample handling (three freeze/thaw cycles and short-term room temperature storage) and chromatography (re-injection) were evaluated and shown to be of insignificant effect (Table 4). Three freeze/thaw cycles and 4 h-room temperature storage of the QC samples before analysis had little effect on the quantification. The reanalysis of the reconstituted extracts stored for 24 h at 4 °C showed the acceptable accuracy and precision for QC samples.

3.3. Pharmacokinetics of magnolin and epimagnolin A in rats

The present method was successfully applied to the pharmacokinetic study of magnolin and epimagnolin A after oral

Table 3
Absolute matrix effect and recovery of magnolin, epimagnolin A and tolterodine (I.S.) in five different lots of rat plasma.

Nominal concentration (ng/mL)	Matrix effect[a] (%)			Recovery[b] (%)		
	Magnolin	Epimagnolin A	Tolterodine	Magnolin	Epimagnolin A	Tolterodine
50	99.8	100.1	100.2	83.4	86.4	83.6
150	101.5	100.9	100.9	82.2	85.3	82.1
800	99.4	102.2	100.5	84.3	88.9	81.8
1800	99.7	102.1	102.6	86.9	89.3	84.8
Mean	100.1	101.3	101.1	84.2	87.5	83.1

[a] Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction to the mean peak area of same analyte standards multiplied by 100.
[b] Recovery calculated as the ratio of the mean peak area of an analyte spiked plasma before liquid–liquid extraction to the mean peak area of an analyte spiked after liquid–liquid extraction of blank plasma multiplied by 100.

Table 4
Stability of magnolin and epimagnolin A in samples ($n = 5$).

Statistical variable	Theoretical concentration (ng/mL)			
	Magnolin		Epimagnolin A	
Three freeze/thaw cycles				
Mean (ng/mL)	160	1649	162	1911
CV (%)	6.1	8.4	9.4	6.1
RE (%)	6.3	−8.4	8.0	6.2
Short-term stability (4 h *at room temperature*)				
Mean (ng/mL)	157	1896	167	1995
CV (%)	9.3	5.2	2.8	2.9
RE (%)	4.7	5.3	11.3	10.8
Post-preparative stability				
Mean (ng/mL)	164	1853	162	1940
CV (%)	7.2	8.4	7.2	7.0
RE (%)	9.3	2.9	8.0	7.8

administration of NDC-052, a standardized extract of the dried flower buds of *Magnolia fargesii* at a dose of 22.22 mg/kg (equivalent to 4.0 mg/kg of magnolin and 2.0 mg/kg of epimagnolin A) to six male SD rats. Fig. 2(c) shows the representative MRM chromatograms of a plasma sample obtained at 2 h after oral administration of NDC-052 in the rat.

The mean plasma concentration–time profiles of magnolin and epimagnolin A in the rats are shown in Fig. 3 and the concentrations of magnolin and epimagnolin A in 24 h plasma samples were below LLOQ. C_{max}, T_{max}, AUC and $t_{1/2}$ of magnolin were 2493 ± 513 ng/mL, 0.54 ± 0.10 h, 7568 ± 1085 ng h/mL and 2.29 ± 0.64 h, respectively. C_{max}, T_{max}, AUC and $t_{1/2}$ of epimagnolin A were 1071 ± 224 ng/mL, 0.63 ± 0.14 h, 4340 ± 653 ng h/mL and 3.09 ± 0.70 h, respectively.

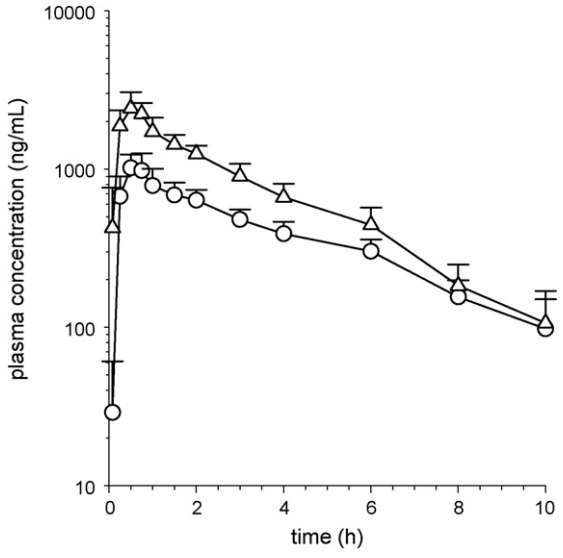

Fig. 3. Mean plasma concentration–time plots of magnolin (△) and epimagnolin A (○) after an oral administration of NDC-052, a purified extract of dried flower buds of *Magnolia fargesii* at a dose of 22.22 mg/kg in six male SD rats. Each bar represents the standard deviation.

4. Conclusion

A rapid, sensitive and selective LC/MS/MS method for the determination of magnolin and epimagnolin A in rat plasma has been successfully developed and validated using liquid–liquid extraction with methyl *tert*-butyl ether at neutral pH as sample clean-up procedure. The present method displayed acceptable sensitivity, precision, accuracy, selectivity, recovery and stability. The pharmacokinetics of magnolin and epimagnolin A were evaluated after oral administration of NDC-052, a purified extract of the dried flower buds of *Magnolia fargesii* to male SD rats, indicating that magnolin and epimagnolin A were well absorbed in the rats.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF) NRL Program grant funded by the Korea Government (MEST) (R0A-2008-000-20074-0).

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