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Determination of 1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA free acid) in rat plasma, urine and feces by liquid chromatography with UV and tandem mass spectrometric detection

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ABSTRACT

BAPTA free acid was identified as the main metabolic product of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(actoxymethyl ester) (BAPTA-AM), a neuroprotective agent in cerebral ischemia, in rats. In this paper, liquid chromatography-ultraviolet (LC-UV) and mass spectrometry/mass spectrometry (LC–MS/MS) methods were employed for the determination of BAPTA free acid in rat urine and feces and rat plasma, respectively. By liquid–liquid extraction and LC-UV analysis, a limit of quantitation of 1000 ng/ml using 0.2 ml rat urine for extraction and 250 ng/ml using 1 ml rat fecal homogenate supernatant for extraction could be reached. The assay was linear in the range of 1000–50,000 ng/ml for rat urine and 250–10,000 ng/ml for rat fecal homogenate supernatant. Because the sensitivity of the LC-UV method was apparently insufficient for evaluating the pharmacokinetic profile of BAPTA in rat plasma, a LC–MS/MS method was subsequently developed for the analysis of BAPTA free acid. By protein precipitation and LC–MS/MS analysis, the limit of quantitation was 5 ng/ml using 0.1 ml rat plasma and the linear range was 5.0–500 ng/ml. Both methods were validated and can be used to support a thorough preclinical pharmacokinetic evaluation of BAPTA-AM liposome injection.

1. Introduction

BAPTA-AM, the first cell-permeable Ca^{2+} chelator designed by Tsien [1], has been demonstrated to be neuroprotective in models of cerebral ischemia, both in vitro and in vivo, by a number of researchers [2–4]. To solve the problems arising from its poor solubility in acceptable pharmaceutical solvents, a liposome-entrapped BAPTA-AM formulation has been developed as a neuroprotectant for cerebral ischemia stroke [5]. It was shown in the preclinical studies that, after intravenous administration of liposome injection (3.0 mg/kg) to rats, the concentration of BAPTA-AM rapidly decreased below 1 ng/ml in the systemic circulation after 1.5 h [6] and trace level of BAPTA-AM existed in excreta [7]. The final hydrolysis product of BAPTA-AM, BAPTA free acid (Fig. 1), was detected as the only measurable metabolite in rat excreta using a full scan LC–MS analysis (shown in this paper). To support a thorough preclinical pharmacokinetic evaluation, it is essential to have a sensitive bioanalytical method for the determination of BAPTA.

Reversed-phase liquid chromatography methods have been previously reported for the separation of the long-chain aliphatic esters of BAPTA and their impurities [8,9]. In our previous paper, a LC–MS/MS method was developed for the determination of BAPTA-AM in rat plasma [6]. An enzyme-linked immunoassay (ELISA) used to be reported for determination of the relative loading of BAPTA into cells [10]. To our knowledge, there is still lack of a validated sample pretreatment and analytical method for the determination of BAPTA concentration in biological fluids. Compared with BAPTA lipophillic derivatives, it is more challenging to develop rapid and sensitive analytical techniques for the detection of BAPTA free acid due to special properties of tetraacetic acid group such as strong acid and extremely hydrophilicity. This report describes an integrated method development strategy for the determination of BAPTA free acid in rat plasma, urine and feces by liquid chromatography with UV and tandem mass spectrometric detection.

2. Experimental

2.1. Chemical and reagents

BAPTA and the internal standard (I.S.) for LC-UV, 3,5-dinitrobenzoic acid, were purchased from the Sigma (St. Louis, MO, USA). BAPTA-AM liposome and the internal standard (I.S.) for

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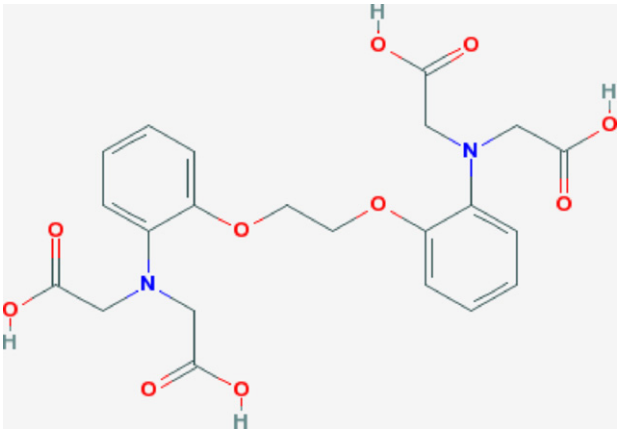

Fig. 1. Chemical structure of BAPTA.

LC–MS/MS, BAPTA acetoxymethyl diester, were provided by Hefei Healstar Medicine Research Institute (Hefei, China). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA) until achieving a resistivity of 18.2 MΩ cm. Other chemicals were all of analytical grade and were used as received.

2.2. Standard solutions

Standard stock solutions of BAPTA were prepared by dissolving the standard in 10 mM sodium tetraborate buffer (pH 9.2 at 25 °C) at the final concentration of 1 mg/ml and were stored at 4 °C. The BAPTA stock solution was diluted with water to prepare a series of working standard solutions before use. The I.S. solution of 3,5-dinitrobenzoic acid was prepared by dissolving the compound with water and then was diluted to obtain 50 μg/ml I.S. working solution. The I.S. solution of BAPTA acetoxymethyl diester was prepared by dissolving the compound with acetonitrile and then was diluted to obtain 20 ng/ml I.S. working solution (in acetonitrile). Both I.S. stock solutions were stored at 4 °C.

2.3. Preparation of calibration standards and quality control samples

Fecal samples were dried, weighed, homogenized with 10 mM sodium tetraborate buffer (pH 9.2 at 25 °C) in the ratio 1:20, and centrifuged to obtain fecal homogenate supernatant. Calibration standards were prepared by adding appropriate working standard solution into 0.1 ml blank rat plasma, 0.2 ml blank rat urine, or 1 ml blank rat fecal homogenate supernatant in a glass conical tube. This provided a calibration standard series for each method and matrix (urine: 1000, 2500, 5000, 10,000, 25,000, 50,000 ng/ml; fecal homogenate supernatant: 250, 500, 1000, 2500, 5000, 10,000 ng/ml; plasma: 5.0, 12.5, 25, 50, 125, 250, 500 ng/ml). Quality control (QC) samples were prepared by aliquoting the standards (from separate weighing) to blank matrix to produce concentration pools as listed in Table 1 for each method and matrix. QC samples were stored frozen at −20 °C and each QC sample was analyzed in singlet.

2.4. Sample preparation

2.4.1. Rat urine and fecal homogenate supernatant samples

The calibration standards, QC samples and test samples (0.2 ml for urine and 1 ml for fecal homogenate supernatant) were mixed with 20 μl I.S. working solution (3,5-dinitrobenzoic acid, 50 μg/ml in water). Before sample preparation, 1.8 ml of 600 mM phosphate buffer (pH 3.0 at 25 °C) was added to urine samples and 50 μl of 2 M hydrochloric acid was added to fecal homogenate supernatant samples. After vortex mixing for 30 s, the urine mixture or fecal homogenate supernatant was extracted with 3 ml ethyl acetate by vortexing for 60 s. After centrifugation at 3000 rpm for 5 min, 2 ml of supernatant was transferred to another glass conical tube, and evaporated to dryness at 45 °C under a gentle stream of nitrogen gas. The residue was reconstituted in 0.1 ml 10 mM sodium tetraborate buffer (pH 9.2 at 25 °C), vortexed for 60 s and centrifuged for 5 min at 12,000 rpm. The supernatant was transferred to an auto-sampler vial, and 20 μl was injected for LC-UV or full scan LC–MS analysis.

2.4.2. Rat plasma samples

The calibration standards, QC samples and test samples (0.1 ml plasma) were mixed with 0.2 ml I.S. working solution (BAPTA acetoxymethyl diester, 20 ng/ml in acetonitrile). After vortex mixing for 30 s, the sample was centrifuged at 16,000 rpm for 5 min at 4 °C. The supernatant was transferred to an auto-sampler vial, and 20 μl was injected for LC–MS/MS analysis.

2.5. Chromatographic conditions

2.5.1. LC-UV

The LC system employed for the LC-UV method consisted of an Agilent 1100 Series (Agilent Technologies, Palo Alto, USA) liquid chromatograph equipped with an autosampler (model G1313A) and a VWD detector (model G1314A). Chromatographic separation was performed on a Gemini C_{18} column (150 mm × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) at 25 °C. The mobile phase consisted of 0.1% (V/V) phosphoric acid–acetonitrile (75:25, V/V) at a flow-rate of 1 ml/min. Detection was set at a wavelength of 210 nm.

2.5.2. Full scan LC–MS and LC–MS/MS

The LC–MS/MS system consists of a Surveyor LC pump, a Surveyor auto-sampler, a TSQ Quantum Ultra AM triple-quadrupole tandem mass spectrometer and Xcalibur 1.2 software for data acquisition and analysis (Thermo Finnigan, San Jose, USA). The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 4.5 kV. The nitrogen sheath gas and

Table 1
Summary of precision and accuracy of BAPTA in rat plasma, urine, and feces (n = 3 assays, 5 replicates per assay).

Biological matrix	Added concentration (ng/ml)	Measured concentration (ng/ml)	Bias (%)	Within-batch R.S.D. (%)	Between-batch R.S.D. (%)
Plasma	12.5	12.08 ± 0.79	−3.4	6.57	5.48
	50	48.36 ± 2.44	−3.3	5.05	4.29
	250	253.1 ± 5.24	1.2	2.07	3.02
Feces	1000	934.8 ± 66.1	−6.5	7.07	10.7
	5000	4999 ± 162	0	3.24	8.52
	10,000	10,614 ± 218	6.2	2.06	3.23
Urine	2500	2324 ± 128	−7.1	5.46	11.2
	10,000	10,133 ± 593	1.3	5.85	6.47
	25,000	25,062 ± 685	0.3	2.73	4.15

Table 2
Gradient profile for LC–MS/MS analysis.

Time (min)	0.5% formic acid (%, V/V)	Methanol with 0.5% formic acid (%, V/V)
0	80	20
1.00	20	80
4.00	20	80
4.01	80	20
6.00	80	20

the auxiliary gas were set at 35 psi and 5 psi, respectively. The heated capillary temperature was 350 °C. The collision energy in the in-source collision induced dissociation (CID) mode was set at 10 eV. For CID, argon was used as the collision gas at a pressure of 1.2 mTorr. Chromatographic separation was performed on a Gemini C_{18} column (150 mm × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) at 25 °C. The liquid flow rate was set at 1 ml/min, and 30% of the eluent was split into the inlet of the mass spectrometer.

For full scan LC–MS analysis, the mobile phase consisted of 0.05% (V/V) trifluoroacetic acid–acetonitrile (68:32, V/V). Full scan mass spectrum was obtained over a range of *m/z* 400–800. For LC–MS/MS analysis, the gradient from 20% to 80% methanol with 0.5% formic acid was used as an eluent (Table 2) and a divert valve directed the LC flow in the first 3.0 min of the chromatographic run to the waste container and afterwards to the ion source. The quantitation was accomplished using the selected reaction monitoring (SRM) for the transitions: BAPTA $[M+Na]^+$ *m/z* 499.0 → *m/z* 441.0 (30 eV) and the I.S. $[M+Na]^+$ *m/z* 643.0 → *m/z* 571.0 (35 eV), with a dwell time 0.3 s per transition. Both Q1 and Q3 peak widths were set at 0.7 Th.

2.6. Pharmacokinetic study

Sprague–Dawley rats (220–250 g) used in preclinical pharmacokinetic evaluation of BAPTA-AM liposome were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Rat chow and water were given ad libitum. BAPTA-AM liposome dissolved in physiological saline was administered to six rats by tail vein at a dose of 3 mg/kg. Blood samples (about 0.3 ml) were collected in heparinized polythene tubes before administration and post-dose at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 6.0, 9.0, 12.0 h. The plasma was separated out by centrifugation at 5000 rpm for 3 min and was stored at −20 °C until analysis. Excretion was studied in another four SD rats, raised in cages separately. Rats were housed with free access to food and water, except for the final 12 h before an intravenous dose of 3 mg/kg (access to water was ad libitum during the experiment). Feces and urine were collected after administration in different periods (0–4, 4–8, 8–12, 12–24, 24–36, 36–48 h). The amount of feces and urine collected over each period was recorded, respectively, and then feces and urine was stored at −20 °C until analysis.

3. Results

3.1. Metabolite identification

Full scan LC–MS chromatograms (Fig. 2) indicated the presence of a single metabolite eluting at 4.6 min, in rat excreta, after administration of 3 mg/kg BAPTA-AM liposome. By comparison of retention time and mass spectrum with the authentic standard, this metabolite was unambiguously identified as the final hydrolysis product of the parent compound, BAPTA free acid.

3.2. Method validation

The specificity of the method was demonstrated by comparing chromatograms of biological samples from blank rats (no BAPTA-AM dose), each as a blank sample and a spiked sample. As shown in Figs. 3 and 4, no significant interference was found either in LC-UV chromatograms or in LC–MS/MS chromatograms using corresponding method, showing the excellent selectivity of these methods. For LC-UV, the retention times of BAPTA and I.S. were about 8.0 min and 11.5 min, respectively. For LC–MS/MS, they were 4.4 min and 4.8 min due to the different chromatographic conditions.

Quantitation for the LC-UV method was accomplished by the BAPTA/I.S. peak-area ratio (*f*) *versus* the added BAPTA concentrations (*C*, ng/ml). The calibration curve was computed by unweighted least-squares linear regression analysis. Data generated from the LC–MS/MS were analyzed by generating calibration curves of the peak-area ratio *versus* the added concentrations using weighted ($1/C^2$) least-squares linear regression analysis. The mean calibration curves obtained were described by the following equations ($n = 3$): $f = 1.40 \times 10^{-4}(\pm 8.30 \times 10^{-6})$ $C + 9.78 \times 10^{-2}(\pm 3.94 \times 10^{-2})$ ($r = 0.999$) for urine, $f = 7.80 \times 10^{-4}$ $(\pm 2.85 \times 10^{-5})$ $C + 5.37 \times 10^{-2}(\pm 3.77 \times 10^{-2})$ ($r = 0.999$) for fecal homogenate supernatant, $f = 1.58 \times 10^{-3}(\pm 8.48 \times 10^{-5})$ $C - 4.33 \times 10^{-6}(\pm 1.55 \times 10^{-5})$ ($r = 0.996$) for plasma.

The limit of quantitation (LOQ) was estimated from the lowest concentration standard in the calibration curve with acceptable accuracy and precision (bias or RSD ≤ 20%). By LC-UV analysis, a LOQ of 1000 ng/ml using 0.2 ml rat urine for extraction and 250 ng/ml using 1 ml rat fecal homogenate supernatant for extraction could be reached depending on the biological sample size available. A significant improvement in detection limit was observed for the LC-ESI–MS/MS method with a LOQ of 5 ng/ml when using 0.1 ml plasma.

To assess within-batch and between-batch precision and accuracy, five QC samples at three concentration levels were prepared for each method and matrix and the procedure was repeated on three consecutive days. The accuracy was evaluated by comparing the known concentrations with those measured against the calibration curve (bias) and the precision by relative standard deviation (R.S.D.). Both precision and accuracy were less than 15% in all cases as can be concluded from Table 1, for the LC-UV and LC-ESI–MS/MS methods, respectively.

The extraction recovery of BAPTA was determined by spiked samples at three concentration levels with five replicates. For rat plasma samples, it was calculated by comparing the peak areas from spiked samples (as described in Section 2.4.2) to the same amounts of BAPTA standard solutions (12.5, 50, 250 ng/ml). For rat urine and fecal samples, spiked samples (urine: 2500, 10,000, 25,000 ng/ml, feces homogenate supernatant: 1000, 5000, 10,000 ng/ml) were prepared as described in Section 2.4.1. The extraction recoveries of BAPTA in rat urine and fecal samples were calculated by the following equation: (peak area of spiked sample/peak area of BAPTA standard solutions) × 3/2 × 100%. As a result, the average extraction recoveries were 90.62–96.45% for rat plasma samples by protein precipitation and were 77.56–86.01% and 86.76–92.46% for rat urine and fecal homogenate supernatant samples by liquid–liquid extraction, respectively. Matrix effects in LC-ESI–MS/MS analysis at three concentration levels (12.5, 50, 250 ng/ml) were determined by the following equation: (peak area of post-extraction plasma blanks spiked with BAPTA standard solutions/peak area of BAPTA standard solutions) × 100%. No significant matrix effect (96.87 ± 9.41%, 102.0 ± 3.27%, 91.79 ± 5.30% at three concentration levels using three replicates) was observed for BAPTA using LC-ESI–MS/MS method.

QC samples at three concentration levels were used to investigate the stability of BAPTA in rat plasma, urine and fecal homogenate supernatant under the experimental conditions. Results were expressed for each concentration level as the percentage of the added concentration, which was referred to as 100%.

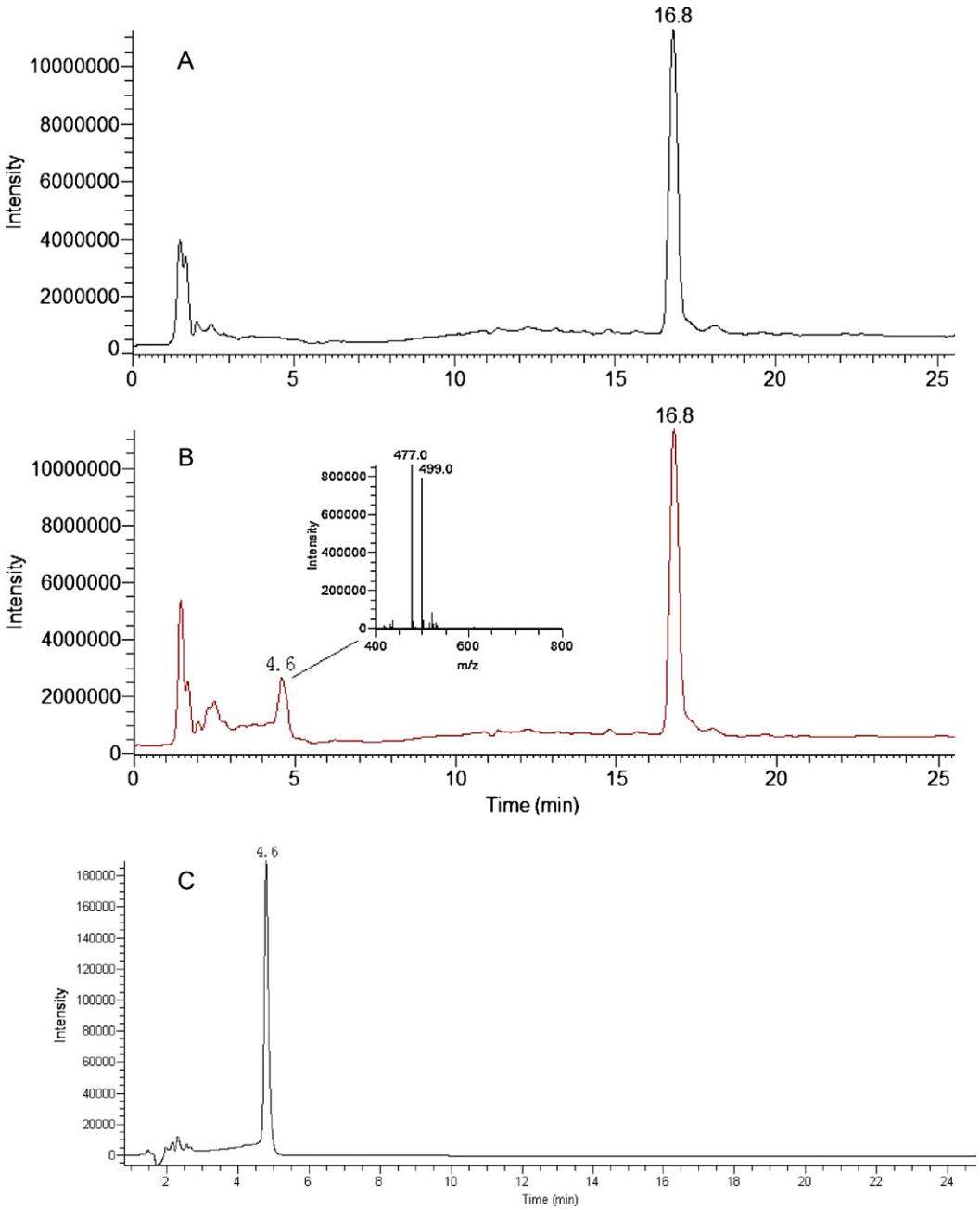

Fig. 2. Full scan LC–MS chromatograms for BAPTA identification. (A) Blank rat urine; (B) rat urine after administration of 3 mg/kg BAPTA-AM liposome; (C) BAPTA standard solution. Mobile phase: 0.05% (V/V) trifluoroacetic acid–acetonitrile (68:32, V/V); scan range: *m*/*z* 400–800.

There was no evidence of instability of BAPTA during each sample preparation and analytical procedure. The stability of BAPTA standard stock solution was assessed by a LC-UV method. The standard stock solution of BAPTA sodium tetraborate buffer solution was stable for a minimum of 1 month when stored at room temperature in the dark (data not shown).

3.3. Application of the validated methods

Each sample for pharmacokinetic and excretion studies of BAPTA in rat was analyzed a single time using the respective method. Fig. 5 shows mean plasma concentration (±S.D.)–time curves of BAPTA after administration of 3 mg/kg BAPTA-AM liposome injection. The fecal and urinary excretion amounts of BAPTA were calculated from the BAPTA concentration and the amount of feces and urine collected over each period. The percentage of BAPTA excretion was calculated from the administrated dose of BAPTA-AM. It was found that the cumulative fecal and urinary excretion of BAPTA in 48 h was 32.96% and 7.86% of dose, respectively.

4. Discussion

4.1. Chromatography

The primary issue for analytical method development was the chromatographic behavior of BAPTA free acid. According to the results in our study, BAPTA was difficult to be eluted from the column and peak shape was severely distorted and broadened under conventional reversed-phase chromatographic conditions. The problem could be caused by secondary interactions during

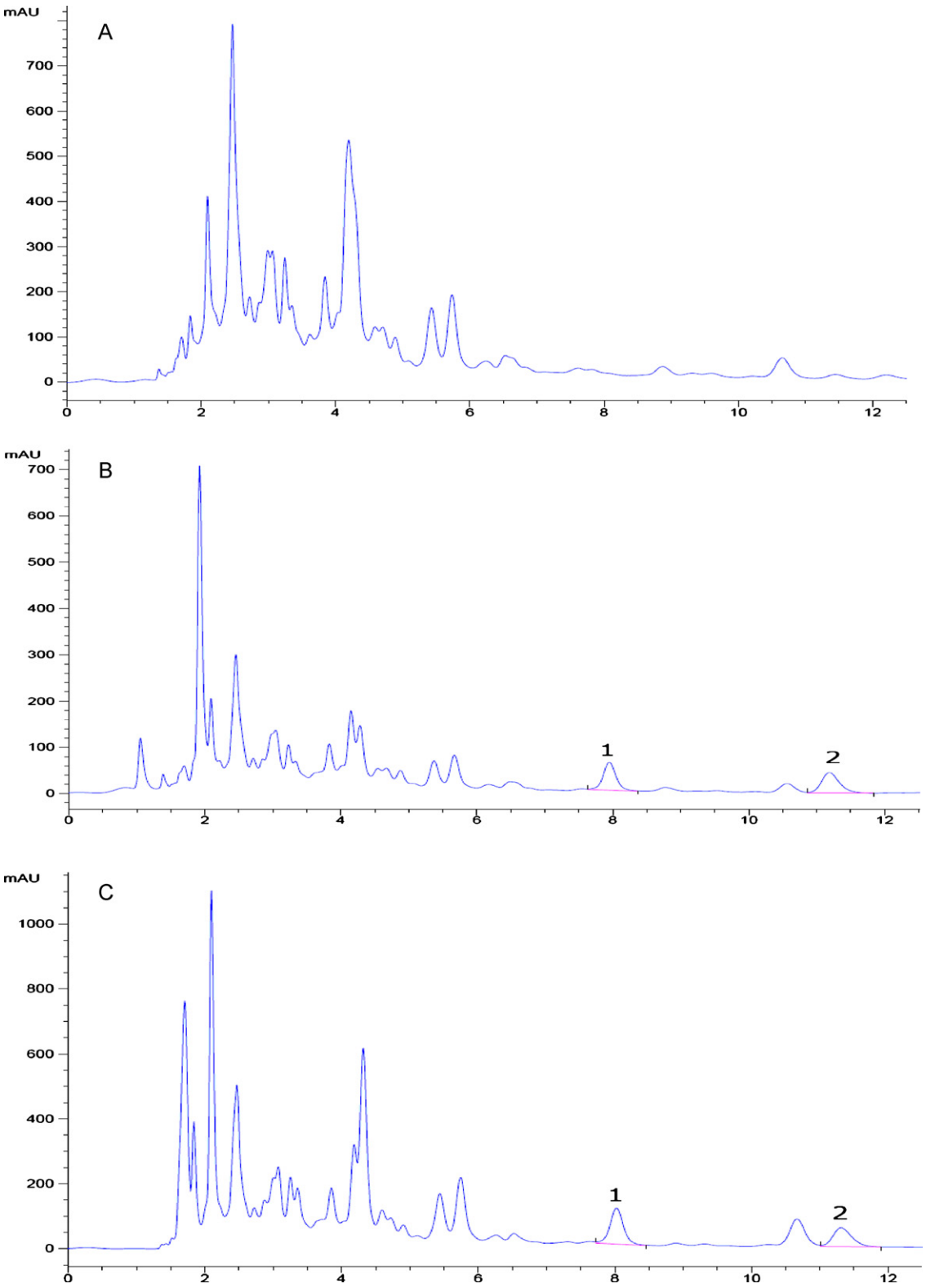

Fig. 3. LC-UV chromatograms of BAPTA and I.S. in rat fecal homogenate supernatant. (A) Blank rat fecal homogenate supernatant; (B) blank rat fecal homogenate supernatant spiked with BAPTA (1.0 μg/ml) and I.S. (50 μg/ml); (C) rat fecal homogenate supernatant at 12–24 h after administration (1.2 μg/ml) and spiked with I.S. (50 μg/ml); (1, BAPTA; 2, I.S.).

chromatographic process: hydrogen bonding between the residual silanol group and the carboxyl group, interaction between residual metal ions in the silica matrix and BAPTA molecule due to its high chelating potency [8], and intramolecular hydrogen bond between adjacent COOH groups and COO^- groups [11].

For LC-UV analysis, a good peak shape and faster chromatographic elution was obtained when using a strong acid containing mobile phase, 0.1% (V/V) phosphoric acid:acetonirile (75:25, V/V). Another advantage of this mobile phase system was that the UV-absorption of BAPTA at 210 nm increased with decreasing pH,

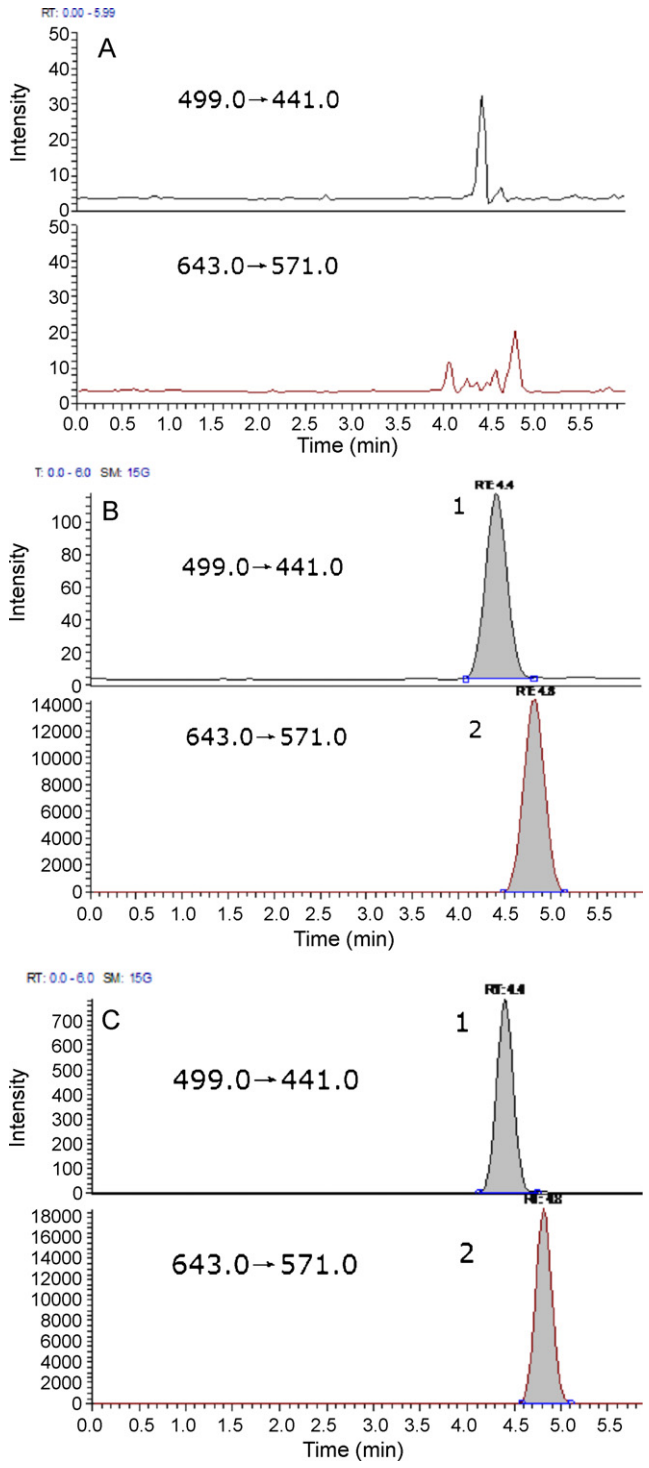

Fig. 4. LC–MS/MS chromatograms of BAPTA and I.S. in rat plasma. (A) Blank rat plasma; (B) blank rat plasma spiked with BAPTA (5.0 ng/ml) and I.S. (20 ng/ml); (C) rat plasma at 9.0 h after administration (25.8 ng/ml) and spiked with I.S. (20 ng/ml); (1, BAPTA; 2, I.S.).

probably due to more protonation of BAPTA free acid [12]. Although good peak shape can be achieved using various reversed-phase analytical columns with the optimized mobile phase, a Gemini C_{18} column was chosen due to its low pH tolerance.

For full scan LC–MS analysis, volatile trifluoroacetic acid, instead of non-volatile phosphoric acid, was selected as the mobile phase additive. However, trifluoroacetic acid caused severe ion suppression in LC–MS/MS analysis, leading to insufficient sensitivity for evaluating the pharmacokinetic profile of BAPTA in rat plasma. Since a strong acid containing mobile phase was unsuitable for mass spectrometric detection, gradient elution mode may be another effective way to improve the chromatographic performance of BAPTA free acid in LC–MS/MS analysis [8]. Through the optimization of chromatographic and mass spectrometric conditions, a steep gradient elution (20–80% methanol in 1 min) was developed for the LC–MS/MS method, which not only obtained sharp peak of BAPTA free acid but also improved assay sensitivity and speed.

4.2. Sample preparation

Sample preparation using protein precipitation was not appropriate for the LC-UV method due to the interference of endogenous components in rat urine and fecal homogenate supernatant and liquid–liquid extraction was chosen for sample preparation in this study. BAPTA was completely ionized under physiological pH conditions and cannot be extracted by any commonly used organic phase. When the pH value of biological samples was controlled under pH 3.5, high extraction recovery would be obtained using ethyl acetate as extraction solvent. However, the degradation of BAPTA was more severe when pH was below 2.0. Meanwhile, the lower pH, the more endogenous compounds would be extracted from biological matrices. In order to assure the specificity and sensitivity of the method, the pH of biological samples was accurately controlled at pH 3.0 using high buffer capacity solution before the extraction process. Furthermore, due to the poor solubility and stability of BAPTA in low pH solvent and common organic solvents, it was important to choose high pH buffer (sodium tetraborate buffer) to prepare the standard stock solutions of BAPTA and reconstitute the BAPTA in the residue after extraction.

Because of the sensitive nature of LC–MS/MS method, a simple protein precipitation method, instead of the time consuming liquid–liquid extraction procedure, was used for the preparation of plasma samples. Three types of precipitation reagents (methanol, acetonitrile and trichloroacetic acid) were investigated for the analysis of BAPTA in rat plasma. According to the experimental results, severe degradation of the analyte or internal standard in the supernatant was observed when methanol or trichloroacetic acid was chosen for sample preparation. On the contrary, samples after protein precipitation with acetonitrile were found to be stable for 12 h in the autosampler at 4 °C, along with high recovery of BAPTA by acetonitrile. Thus, protein precipitation with acetonitrile was most suitable for sample preparation of BAPTA in rat plasma.

4.3. LC–MS/MS

Based on the chemical structure of BAPTA free acid, the starting point for method development was the attempt to choose the deprotonated molecule $[M-H]^-$ m/z 475.0 as the precursor ion for the quantitative assay in the negative ion ESI mode. However, the $[M-H]^-$ response was found to be significantly decreased as formic acid was added into the mobile phase. In the positive ion ESI mass spectrum of BAPTA, the protonated ion at m/z 477.0 and the sodium adduct ion at m/z 499.0 showed similar intensity under optimal mobile phase compositions. Evaluation of different precursor ions were performed by LC–MS/MS with the respective optimal ionization conditions and ion transitions in MS/MS. Higher background noise levels were observed for $[M+H]^+$ (m/z 477.0 → m/z 431.0) when compared with $[M+Na]^+$, leading to the highest sensitivity of $[M+Na]^+$ in LC–MS/MS. Therefore, the application of sodium adduct ion in LC–MS/MS was used for the determination of BAPTA in rat plasma. In order to ensure reproducibility of the results, a BAPTA derivative with similar MS/MS behavior as the analyte, was selected as internal standard for the LC–MS/MS method.

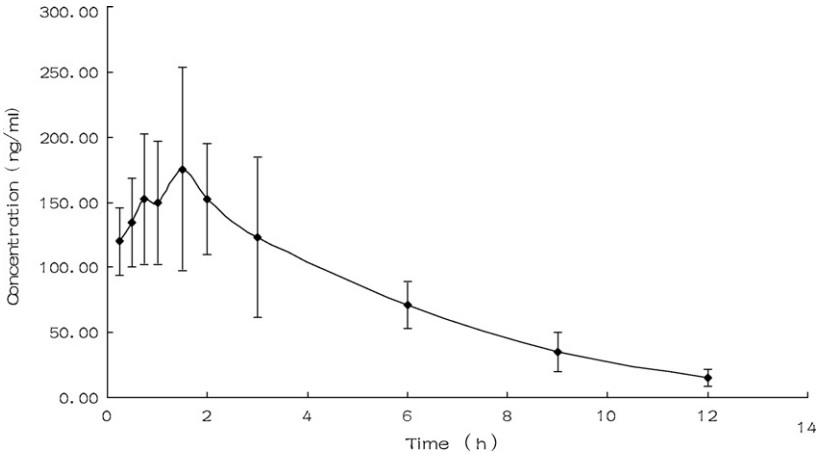

Fig. 5. Mean plasma concentration (±S.D.)–time profile of BAPTA after an intravenous dose of 3 mg/kg BAPTA-AM liposome to rats ($n = 6$).

4.4. Pharmacokinetics

It is generally presumed that BAPTA-AM is converted to BAPTA through enzymatic cleavage of the acetoxymethyl ester linkages by carboxylesterase based on the indirect evidence such as the change of pH value [1]. The full scan LC–MS analysis in this paper provided the direct evidence for BAPTA identification. It was found that, after administration of BAPTA-AM liposome in rat, BAPTA was the only measurable metabolite, mainly in feces, and also slightly in the urine. Our previous study showed that the biliary and urinary excretion of unchanged BAPTA-AM were negligible [7]. Therefore, the fecal excretion of BAPTA was the major elimination pathway of BAPTA-AM liposome injection in rat. Due to the rapid hydrolysis of BAPTA-AM by plasma carboxylesterase [6], BAPTA-AM plasma concentration–time profile could not support a thorough pharmacokinetic evaluation. On the contrary, BAPTA was stable and the concentration was relatively high in rat plasma. By considering the above factors, BAPTA was considered to be a good marker to study the pharmacokinetics of BAPTA-AM liposome injection in rats.

5. Conclusion

In this paper, LC-UV and LC–MS/MS methods were respectively employed for the analysis of BAPTA free acid in rat urine and feces and rat plasma. To improve the chromatographic performance of BAPTA free acid, a liquid chromatography using strong acid containing mobile phase was used for LC-UV method while a gradient elution was developed instead for the LC–MS/MS method. Sample extraction method was explored using liquid–liquid extraction and protein precipitation methods for LC-UV and LC–MS/MS, respectively. The LC-UV method developed for the excretion studies of BAPTA is simple and uses commonly available instrumentation. The described LC–MS/MS method combines the universality of liquid chromatographic separation with the sensitivity and selectivity of mass spectrometric detection and was demonstrated to be sensitive, selective, and reproducible for the determination of BAPTA free acid in rat plasma. Both methods were validated and can be used to support a thorough preclinical pharmacokinetic evaluation of BAPTA-AM liposome.

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