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Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of ε-Acetamidocaproic Acid in Rat Plasma

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A simple and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of ε-acetamidocaproic acid (AACA), the primary metabolite of zinc acexamate (ZAC), in rat plasma by using normetanephrine as an internal standard. Sample preparation involved protein precipitation using methanol. Separation was achieved on a Gemini-NX C_{18} column (150 mm × 2.0 mm, i.d., 3 μm particle size) using a mixture of 0.1% formic acid-water : acetonitrile (80 : 20, v/v) as the mobile phase at a flow rate of 200 μl/min. Quantification was performed on a triple quadrupole mass spectrometer employing electrospray ionization and operating in multiple reaction monitoring (MRM) and positive ion mode. The total chromatographic run time was 4.0 min, and the calibration curves of AACA were linear over the concentration range of 20~5000 ng/ml in rat plasma. The coefficient of variation and relative error at four QC levels were ranged from 1.0% to 5.8% and from −8.4% to 6.6%, respectively. The present method was successfully applied for estimating the pharmacokinetic parameters of AACA following intravenous or oral administration of ZAC to rats.

***Key words*:** Zinc acexamate (ZAC), ε-Acetamidocaproic acid (AACA), Liquid chromatography tandem mass spectrometry (LC-MS/MS), Rat, Pharmacokinetics

INTRODUCTION

Zinc acexamate (ZAC) is the first zinc compound clinically used for peptic ulcer (1,2). ZAC showed ulcer protective effects in several experimental animal ulcer models by reducing the gastric acid output, enhancing the mucosal protective factors, restoring the continuity of the damaged mucosa (2). ZAC also showed an anti-ulcer activity with anti-secretory and gastroprotective properties with minimal side effects in less than 2% of the treated patients (3,4). ZAC protects from the gastric mucus in different models of experimental ulcer induced by non-steroidal anti-inflammatory drugs (NSAID) (5,6) and promotes the healing of chronic gastric lesions (3), as results of stabilization of biological cell membrane integrity (7), augmentation of mucus production (8), increase in sulfated macromolecules secreted by gastric glands (3), and inhibition of gastric mucosal prostaglandin E_2 production (9). In addition, oral administration of ZAC has the restorative effect on bone loss because zinc is an essential nutritional factor in the growth related to differentiation into osteoblastic cells (10).

ZAC is a salt between a zinc atom and two molecules of ε-acetamidocaproic acid (AACA; pKa of 4.75). AACA is classified as NSAID in PubChem Compound (http://pubchem.ncbi.nlm.nih.gov), and was used to avoid pulmonary fibrosis in patients treated with bleomycin (11). Most of reports for AACA were related to ZAC, and the role of AACA was understood as a salt which facilitating the absorption or distribution of zinc ion. For example, in streptozotocin-induced diabetic rats, ZAC administration restored the bone components significantly but zinc sulfate did not (12). The authors explained the difference by easily enter-

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ing of ligand-binding zinc into bone cells compared with inorganic zinc salt (12). However, the same group reported that zinc sulfate enhanced Runx2, OPG and regucalcin mRNA expression in osteoblastic MC3T3-E1 cells while ZAC did not (13). These controversial results imply that the role of AACA in ZAC is not limited to the simple salt change, and the possible pharmacological effects of AACA may be considered. It has been also reported that AACA has a wound healing effect on intestinal or skin wounds (14,15), and is effective for the treatment of exit-site granuloma with gentamicin (16). A metabolite of AACA, ε-aminocaproic acid, is effective for the prevention of plasmin activity, which is related to the development of gastric and duodenal ulcers (17).

For the understanding of the activities of ZAC, the pharmacokinetic evaluation of AACA has been carried out by our research group (18,19), and for this purpose, a new analytical method to monitor AACA in biological samples was also developed by ourselves because of the rarity of this type of methods (20). However, unfortunately, we could not have any chance to report information regarding performance of our own analytical method, yet. Therefore, here, we provide analytical performance information of our method to determine AACA in biological samples using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Based on our validation results, this method is proven very rapid, sensitive, quantitative and reproducible, and consequently, the credibility of our previous pharmacokinetic/toxicokinetic reports on AACA using this method is also enhanced.

MATERIALS AND METHODS

Materials and chemicals. AACA and normetanephrine (internal standard, IS) were purchased Sigma-Aldrich Corporation (St. Louis, MO, USA). Water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and used without further purification.

Preparation of stock Solution, calibration standards, and quality control samples. A stock solution of AACA (5 mg/ml) was prepared in 50% methanol and dilutions of stock solution were made with 50% methanol. Standard solutions of AACA in rat plasma were prepared by spiking with an appropriate volume (less than 0.1% of total plasma volume) of the diluted stock solution, giving final concentrations of 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. All the solutions were stored at 4°C and were brought to room temperature before use.

Quality control (QC) samples of AACA were prepared at concentrations of 20, 60, 500, 4000 and 5000 ng/ml by same method used for standard solutions. The IS solution was also prepared in 50% methanol and diluted with 50% methanol to give a final concentration of 100 μg/ml. Aliquots of spiked plasma samples were taken in eppendorf tubes and stored at −70°C.

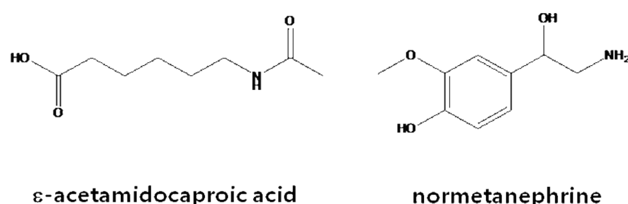

Fig. 1. Chemical structures of ε-acetamidocaproic acid (AACA) and normetanephrine (IS).

Sample preparation. A 5 μl aliquot of IS (100 μg/ml) and 200 μl of methanol were added to a 50 μl aliquot of plasma. After vortex mixing for 30 sec, the mixture was centrifuged at 8,000 rpm for 10 min. The supernatant (100 μl) was transferred to clean eppendorf tube and 200 μl of 50% methanol was added. After vortex mixing for 10 sec, the solution was centrifuged at 8,000 rpm for 10 sec. A 10 μl aliquot of the solution was injected onto the LC-MS/MS system.

Apparatus and chromatographic conditions. The liquid chromatography was carried out by using an Accela (Thermo Fisher Scientific Inc., Waltham, MA, USA) system composed of a solvent delivery module and an autosampler, and was connected to a Discovery Max (Thermo Fisher Scientific Inc.) quadrupole tandem mass spectrometer via an electrospray ionization source. System control and data analysis were carried out with Xcalibur (Thermo Fisher Scientific Inc.). Chromatographic separation was achieved using a reversed-phase HPLC column (Gemini-NX C_{18}, 150 mm × 2.0 mm i.d., 3 μm particle size, Phenomenex, Torrance, CA, USA) protected by a guard column (Phenomenex C_{18}, 4 mm × 2 mm, Phenomenex). A degasser (Degassit, Varian inc., Palo Alto, CA, USA) and a column oven (Metatherm, MetaChem Technologies, Torrance, CA, USA) were used online. The mobile phase of 0.1% formic acid-water/acetonitrile (80 : 20, *v/v*) was run at a flow rate of 200 μl/min.

The mass spectrometer was operated in positive ion mode. Multiple reaction monitoring (MRM) of the precursor-product ion transitions from *m/z* 174.0 to *m/z* 114.0 for AACA and from *m/z* 184.2 to *m/z* 134.1 for normetanephrine was used for quantitation. Collision energy was 14.0 and 19.0 volts for AACA and normetanephrine, respectively. The optimized conditions were: ESI needle spray voltage (5000 V), sheath gas pressure (30 unit), auxiliary gas pressure (10 unit), capillary temperature (205°C), collision gas (Ar) pressure (1.0 mTorr), skimmer offset (10 V), chrom filter peak width (10 s). The scan was performed in centroid mode with SIM width, 0.700 FWHM, scan time,

0.200 sec. and scan width 0.5 Da.

Method validation. For the determination of linear range, 8 non-zero calibration samples were used. The linear regression of the ratio of peak area of AACA to that of IS versus the concentration of AACA were done with a weighting of $1/X^2$ (least-squares linear regression analysis, where X is the analyte concentration). The lower limit of quantitation (LLOQ) was defined as the lowest concentration of AACA with acceptable precision and accuracy ($< \pm 20\%$). For other concentrations, precision and accuracy should be within ± 15% (20).

The precision and accuracy of this analysis method were determined by the concentration of the QC samples which were calculated from the simple linear equation at each day using regression analysis of spiked plasma calibration standard with reciprocate of the drug concentration as a weighting factor ($1/\text{concentration}^2$, i.e. $1/X^2$). Five replicates of QC samples were measured during a single run and this was done on different five days. The accuracy and precision were evaluated in terms of relative error (RE) and % of the coefficient of variation (% CV), respectively.

The matrix effect of AACA was determined by comparing the mean peak areas of the analyte spiked at three concentrations (60, 500 and 4000 ng/ml) into the extracts originating from six different lots of rat blank plasma sample (set 1) to the mean peak areas for the neat solutions of the analyte in 50% methanol (set 2). The recovery of AACA was determined by comparing the mean peak areas of analytes spiked before extraction into the same six different sources as set 1 (set 3) with those of the analytes spiked post-extraction into different blank plasma extracts at three concentrations, 60, 500 and 4000 ng/ml (set 1). The matrix effect and recovery of IS were determined in one concentration (10 μg/ml) using same way for AACA.

Stock solution stability was performed by comparing area response of stability sample of analyte and internal standard with the area response of sample prepared from fresh stock solutions. The stocks were found to be stable for a minimum 2 weeks. The stability in extracted sample (post-preparative stability), the stability in rat plasma after three freeze/thaw cycles, the long-term stability in rat plasma at -70°C for 2-week and 1-week, and the short-term stability in rat plasma at room temperature for 24 hr (bench top stability) were measured using five replicates of QC samples at three concentrations (60, 500 and 4000 ng/ml), respectively.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the upper limit of quantification (ULOQ). Dilution integrity experiment was carried out at 2.5 times the ULOQ concentration for all the analytes. Five replicates each of 1/3 and 1/5 concentration were prepared and their concentrations were calculated by applying the dilution factor of 3 and 5 against the freshly prepared calibration curve.

Application to a pharmacokinetic study in rats. All animal procedures were based on the guidelines issued by Dankook University Institutional Animal Care and Use Committee, which adheres to the guidelines issued by the Institution of Laboratory of Animal Resources. The carotid artery (for blood sampling) and the jugular vein (for iv administration only) of male Sprague-Dawley rats (body weight 350~400 g, Orient Bio Inc., Seoul, Republic of Korea) were cannulated with polyethylene tubing (Clay Adams, Parsippany, NJ, USA) under anesthesia using Zoletil 50 (10 mg/kg i.m.). Each cannula was exteriorized to the dorsal side of the neck. Each rat was housed individually in a rat metabolic cage (Daejong Scientific Company, Seoul, Republic of Korea) and allowed for 4~5 hr to recover from anesthesia before the study began. Zinc acexamate at dose of 20 mg/kg was administered by intravenous infusion over 1 min via the jugular vein or by oral administration (total injection volume was approximately 0.6 ml, n = 5, respectively). Blood samples were collected from the carotid artery at 0 (pre-dose), 5, 15, 30, 60, 75, 120, 180, 240, 300 and 360 min after dosing. Approximately 120 μl of blood was withdrawn at each time point, and a similar amount of heparinized saline was back flushed. The blood was immediately centrifuged at 12000 rpm to obtain 50 μl of plasma. The plasma was stored at -70°C until analysis.

RESULTS AND DISCUSSIONS

Sample preparation and liquid chromatography. For simple sample preparation, protein precipitation was attempted using acetonitrile and methanol and the same solvents were also tested for the decision of the mobile phase in LC study. The best LC peak symmetry was achieved when less than 20% of any organic solvent was used as the mobile phase and the retention time of AACA under these conditions showed only an increase of about 0.5 min compared to cases of using 40% of the same solvent for the mobile phase. When using acetonitrile for protein precipitation, the LC peak of AACA was split into two peaks under methanol-based mobile phase conditions. All other combinations with acetonitrile and methanol for the preparation of LC mobile phase and the precipitation agent did not show the peak split of AACA. However, the LC peak shapes of AACA and IS were the best when using methanol for precipitation and acetonitrile for the preparation of the mobile phase.

Mass spectrometry. Our mass spectrometry conditions could sensitively detect AACA and IS in positive ion mode. The most abundant protonated ion ($[M+H]^+$) peak in the full mass spectra of AACA and IS were *m/z* 174.0 and *m/z*

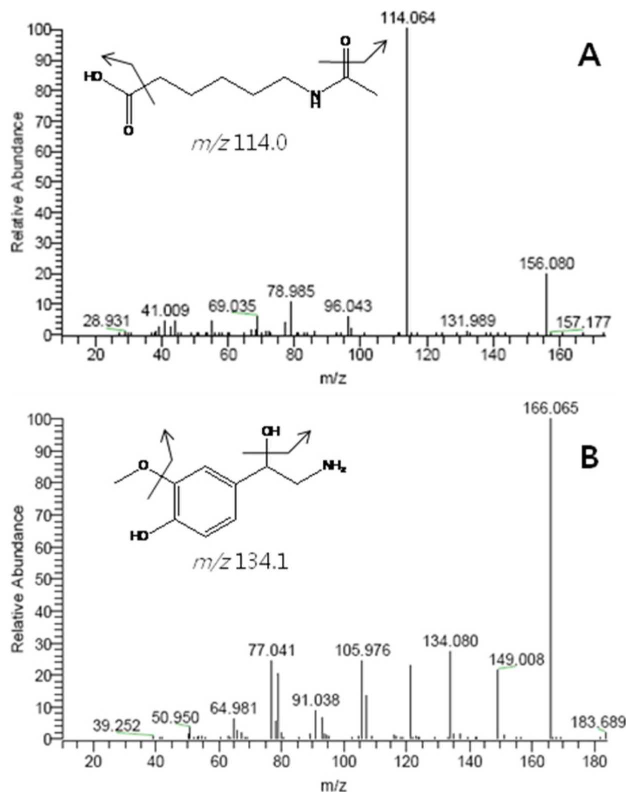

Fig. 2. Product ion mass spectra used in multiple reaction monitoring for (A) AACA (precursor ion m/z 174.0) and (B) normetanephrine (precursor ion m/z 184.2).

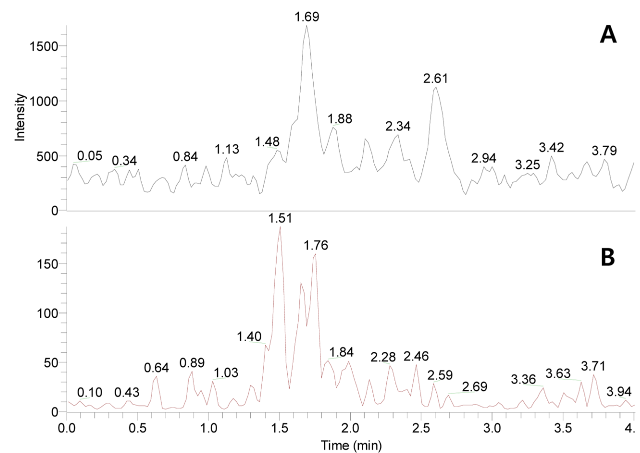

Fig. 3. Representative chromatograms of drug-free rat plasma (A: AACA, B: IS).

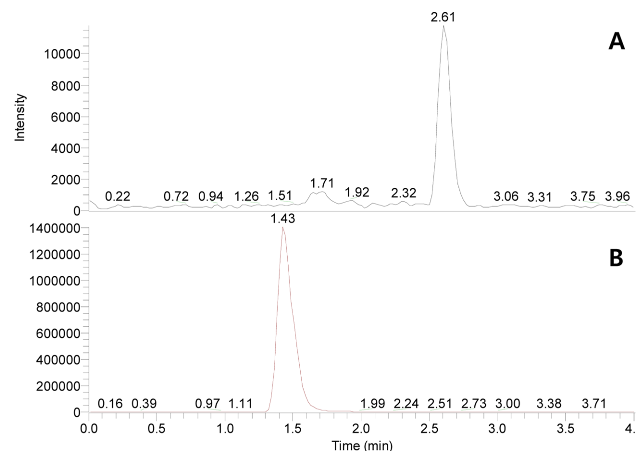

Fig. 4. Representative chromatograms of rat plasma spiked with AACA (20 ng/ml, LLOQ) and IS (A: AACA, B: IS).

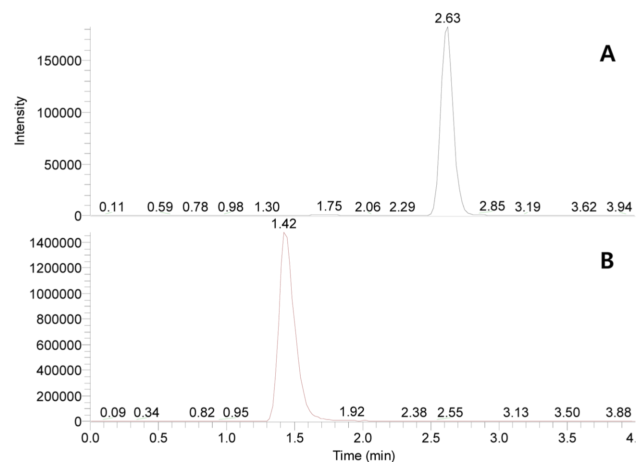

Fig. 5. Representative chromatograms of plasma sample 60 min after intravenous administration of 20 mg/kg of zinc axecamate (A: AACA, B: IS).

184.2, respectively, without any evidence of fragmentation and adduct formation. Fig. 2 shows MS/MS spectra and proposed fragmentation patterns after fragmentation of AACA (Fig. 2A) and IS (Fig. 2B) in the collision cell. AACA produced a strong fragment ion at *m/z* 114.0 ((*E*)-*N*-ethylidenepentan-1-amine) by loss of carboxyl group (-COOH) and hydroxyl group (-OH), and a fragment ion at *m/z* 134.1 (4-(2-iminoethylidene)cyclohexa-2,5-dienol) by loss of water (H_2O) and methoxy group ($-OCH_3$) from IS was also observed. Even though the most abundant fragment ion of IS was shown at *m/z* 166.1, it was not investigated further because of its severe interferences in blank sample during its MRM appications (data not shown). The mechanism of MRM transition of normethanphrine (IS) and specificity of *m/z* 134.1 ion was previously reported (21).

Sensitivity and specificity. Fig. 3 and 5 shows typical chromatograms of drug-free rat plasma, rat plasma validation sample at LLOQ with 20 ng/ml of AACA and IS, and plasma collected 60 min after intravenous administration of 20 mg/kg of zinc acexamate to rat. No significant interference from endogenous substances was observed in 5 different sources of rat plasma samples. Slight peaks were found at retention times near around that of IS (1.51 and 1.76 min) from blank tests (Fig. 3) but these were negligible com-

Table 1. Calculated concentrations of AACA in calibration standards prepared in rat plasma (n = 5 at each level)

	Theoretical concentration (ng/ml)								Slope	Intercept	r^2
	20	50	100	200	500	1000	2000	5000			
Mean	19.9	49.8	100.5	207.1	509.5	1034.8	1949.7	4694.3			
CV (%)	2.0	3.7	3.3	1.2	3.0	2.7	5.8	1.3	0.00136	0.004814	0.9987
RE (%)	–0.4	–0.3	0.5	3.5	1.9	3.5	–2.5	–6.1			

CV = Coefficient of Variation; RE = Relative Error.

pared with the peak intensity of IS in Fig. 4. The quantification limit for AACA in rat plasma was 20 ng/ml based on a signal-to-noise ratio of 10.0.

Linearity. Eight-point standard curves were linear over the concentration range of 20~5000 ng/ml for AACA (Table 1). The correlation coefficients (r^2) of the standard curves generated during the validation ranged from 0.9981 to 0.9991. At all individual concentrations, RE values between nominal and measured concentrations ranged from −7.4 to 7.3% and CV values were less than 5.8%.

Precision and accuracy. The intra-batch and inter-batch precision and accuracy are shown in Table 2. The ranges of intra-batch and inter-batch precision (CV) of AACA in rat plasma were 1.7~5.8% and 1.0~4.5%, respectively, within the nominal concentration range of 20~5000 ng/ml (Table 2). The intra-batch and inter-batch accuracies (RE) of AACA were −8.4~6.4% and −6.5~6.6%, respecttively, within the concentration range of 20~5000 ng/ml (Table 2).

Recovery, matrix effect, stability and dilution integrity. In rat plasma, the matrix effects at three concentrations (60, 500, and 4000 ng/ml) of AACA were almost 100% (102.0, 102.0, and 99.4% from 60, 500, 4000 ng/ml of AACA, respectively), indicating that the ionization suppression or enhancement was negligible. The recovery values (101.1, 89.4, and 87.3% from 60, 500, 4000 ng/ml of AACA, respectively) were similar with those of matrix effects, but recovery values were smaller than matrix effect values by about 10% at high AACA concentration conditions.

Stock solution of AACA and IS were stable at room temperature for 24 hr and at 4°C for 30 days. After sample preparation, AACA was stable at 4°C up to 24 hr in autosampler. In rat plasma, AACA was stable after three freeze/thaw cycles. At room temperature, AACA was also stable for at least 24 hr. Long term stability was also good at −70°C for 1~2 weeks. The results for stability tests of AACA are summarized in Table 3.

The mean back calculated concentrations for 1/3 and 1/5 dilution samples were within 85~115% of their nominal concentrations. The CV values for 1/2 and 1/4 dilution samples were less than 5.0%.

Preclinical application. For additional validation of the method, a pharmacokinetic study in rats [intravenous or

Table 2. Intra-batch and inter-batch precision and accuracy of AACA in rat plasma

	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	CV (%)	RE (%)
Intra-batch[a]	20	18.3 ± 1.07	5.8	−8.4
	60	62.5 ± 1.11	1.8	4.1
	500	532.0 ± 12.91	2.4	6.4
	4000	3971.5 ± 67.23	1.7	−0.7
	5000	4872.1 ± 143.70	2.9	−2.6
Inter-batch[b]	20	19.5 ± 0.56	2.9	−2.3
	60	63.9 ± 1.13	1.8	6.6
	500	497.5 ± 22.41	4.5	−0.4
	4000	3822.0 ± 41.20	1.1	−4.5
	5000	4675.7 ± 46.02	1.0	−6.5

CV = Coefficient of Variation; RE = Relative Error.
[a]Mean of five replicates (n = 5) observations at each concentration.
[b]Mean of 25 replicates (n = 25) observations over five different analytical runs.

Table 3. Stability of AACA in rat plasma (n = 5 at each level)

Conditions	Theoretical concentration (ng/ml)	Final concentration (ng/ml)	RE (%)	CV (%)
Three freeze/thaw cycle (−70°C)	60	65.6	9.3	1.9
	500	539.2	7.8	2.4
	4000	3687.7	−7.8	2.4
Bench top stability (room temp., 24 hr)	60	65.2	8.7	2.1
	500	550.9	10.2	2.5
	4000	3657.2	−8.6	1.9
Post-preparative stability (4°C, 24 hr)	60	61.4	2.26	1.26
	500	501.2	0.24	3.55
	4000	3988.5	−0.29	2.27
Long term stability (−70°C, 1 week)	60	65.4	8.9	2.2
	500	523.8	4.8	4.8
	4000	3665.9	−8.4	3.3
Long term stability (−70°C, 2 weeks)	60	63.5	5.9	4.9
	500	510.3	2.1	5.8
	4000	3655.9	−8.6	1.1

CV = Coefficient of Variation, RE = Relative Error.

oral administration of ZAC (20 mg/kg)] was carried out. Its plasma concentration-time profile is shown in Fig. 6, and relevant pharmacokinetic parameters are listed in Table 4. According to comparisons of these parameters with those from our previous results (19), all parameters were similar in both studies except terminal half-life after oral administration (Table 4). Thus, this result can be considered as another evidence to validate the method itself as well as an important evidence to support our previous pharmacokinetic reports (18,19).

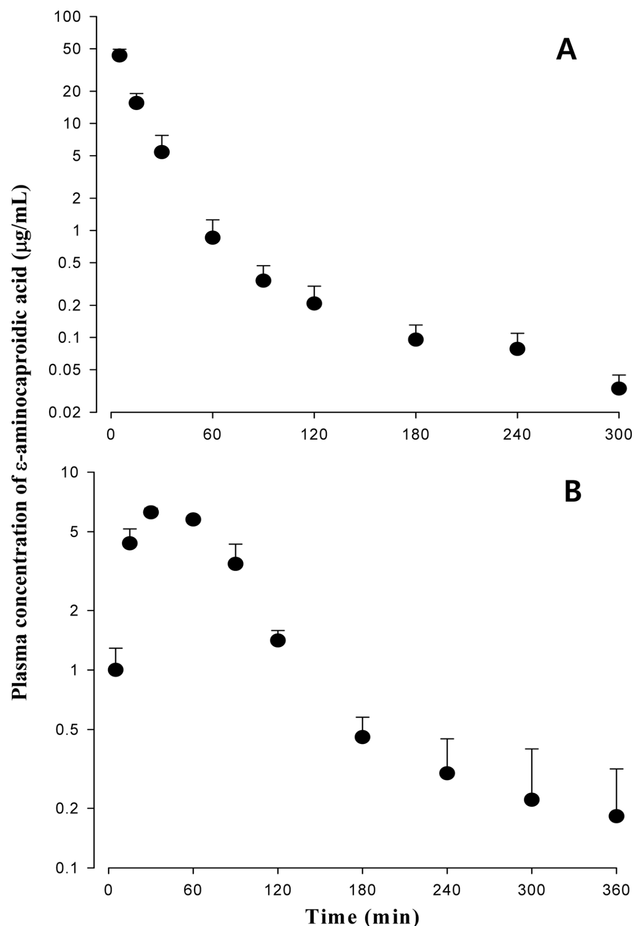

Fig. 6. Mean plasma concentration-time profile of AACA after intravenous injection (A) or oral administration (B) of zinc acexamate, 20 mg/kg, to rats (n = 5). Vertical bar represent SD.

Table 4. Pharmacokinetic parameters of AACA after intravenous or oral administration of zinc acexamate (20 mg/kg) to rats and their comparisons with those from a previous report

Parameters	Mean ± SD	
	New study	Previous study
Intravenous	n = 5	n = 8
AUC (μg min/ml)	1240 ± 169	1030 ± 129
Terminal half-life (min)	32.5 ± 7.89	28.7 ± 6.87
V_{ss} (ml/kg)	147 ± 42.4	217 ± 30.1
CL (ml/min/kg)	16.3 ± 2.12	19.9 ± 2.30
Oral	n = 5	n = 7
AUC (μg min/ml)	662 ± 50.1	548 ± 139
Terminal half-life (min)	172 ± 7.89	60.0 ± 27.5
C_{max} (ml/kg)	6.26 ± 0.319	4.20 ± 1.20
T_{max} (min)	30 (30~90)	60 (30~90)

SD = Standard Deviation.

In conclusion, we developed a simple and rapid method to quantitate AACA in biological samples. The protein precipitation with methanol for sample preparation and mass spectrometry used on the plasma samples gave a very clean chromatogram in which AACA and IS peaks were sufficiently resolved. The LLOQ of this method for AACA was 20 ng/ml, which was sufficient to detect terminal-phase levels after intravenous injection of a 20 mg/kg dose to rats. This method could be very useful for pharmacokinetic studies of AACA.

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