Journal of Chromatography B, 897 (2012) 50–54

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Determination of aniracetam's main metabolite, N-anisoyl-GABA, in human plasma by LC–MS/MS and its application to a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 29 November 2011
Accepted 4 April 2012
Available online 12 April 2012

Keywords:
N-anisoyl-GABA
LC–MS/MS

ABSTRACT

A simple and rapid high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method has been developed and validated for the determination of 4-p-anisamidobutyric acid (ABA; or N-anysoyl-γ-aminobutiryc acid, N-anisoyl-GABA), a major active metabolite of aniracetam, in human plasma. After protein precipitation of plasma sample with methanol, ABA and the internal standard lisinopril were separated on a Venusil ASB C_{18} column at 25 °C. The mobile phase consisted of methanol–ammonium acetate (10 mmol/L) (30:70, v/v). The detection was performed on a triple quadrupole tandem mass spectrometer with an ESI source in negative ion mode. Multiple reaction monitoring (MRM) using the precursor→product ion combinations of m/z 235.8→m/z 106.6, and m/z 403.8→m/z 113.6 was used to quantify ABA and lisinopril, respectively. This is the first LC–MS/MS method for ABA with advantages of short analysis time (4.5 min per sample run) and high selectivity attributable to the MRM detection and optimized HPLC conditions. The response was linear in a concentration range of 0.0485–19.4 μg/mL in plasma. The extraction recovery of ABA was between 89.1% and 100.7%. The precision (RSD) and accuracy (RE) of the method were evaluated to be within 7.3% and from 2.5% to 6.9%. The validated method has been applied to the pharmacokinetic study after a single oral administration of aniracetam dispersible tablets to human beings.

1. Introduction

Aniracetam (1-p-anisoyl-2-pyrrolidinone) has been used for the treatment of emotional disturbances, sleep disorders, cognition impairments and behavioral abnormalities in patients with cerebrovascular diseases, progressive supranuclear palsy, Parkinson's and Alzheimer's diseases. It is very rapidly absorbed from the intestinal tract and widely metabolized after oral administration with extremely low bioavailability [1–5]. About 70% of the parent drug is biotransformed to its active metabolite, 4-p-anisamidobutyric acid (ABA; or N-anysoyl-γ-aminobutiryc acid, N-anisoyl-GABA) (Fig. 1A), while other two metabolites, p-anisic acid (AA) and 2-pyrrolidinone (PD), account for the remaining 30% of the dose [6,7]. The pharmacokinetic data and its behavioral efficacy indicate that ABA may primarily contribute to the therapeutic effects in humans, mainly by acting on the reticulothalamic cholinergic pathway [8]. Therefore, it is necessary to monitor ABA plasma concentration in clinic. Although the pharmacokinetics of ABA or aniracetam after oral or intervenous administration was published [9,10], there was no report about the pharmacokinetic profile of ABA in humans after administration of aniracetam dispersible tablets. The present study was aimed to investigate the pharmacokinetics of ABA for aniracetam dispersible tablets, so as to provide reference information for the clinical application of this new formulation. Guenzi and Zenetti [11] developed an HPLC method to determine the concentration of ABA in human plasma with a whole run time of 12 min. Lv et al. [12] reported an HPLC–MS method to determine ABA plasma concentration with a whole run time of 8 min and applied it to a bioequivalence study. In order to satisfy the requirements of high-throughput analysis, the present study developed a more rapid and simple method to determine ABA in human plasma using liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the first time. The assay was validated and applied to a clinical pharmacokinetic study in 12 healthy volunteers after oral administration aniracetam in dispersible tablets.

2. Materials and methods

2.1. Chemicals and reagents

ABA (purity, 98.95%) was purchased from China National Pharmaceutical Industry Corporation Ltd. (Beijing, PR China) and the internal standard, lisinopril (purity, 91.2%), was provided by the National Institutes for Food and Drug Control (Beijing, PR China).

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http://dx.doi.org/10.1016/j.jchromb.2012.04.007

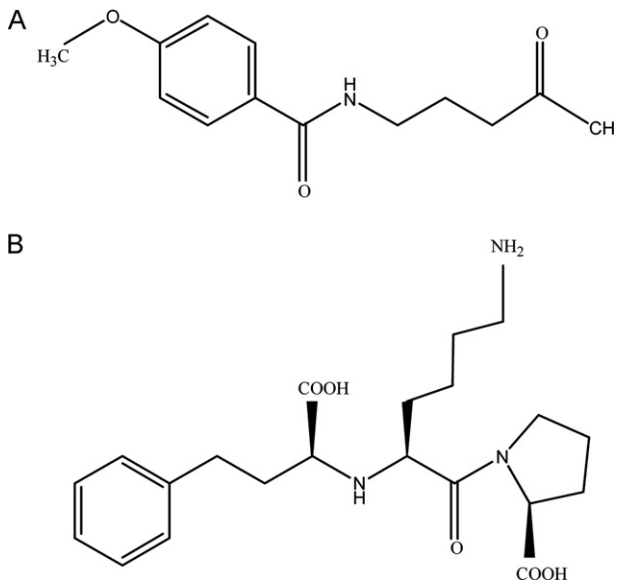

Fig. 1. Chemical structures of ABA (A) and internal standard lisinopril (B).

Ammonium acetate of chromatographic grade was purchased from Dikma Corporation (Richmond Hill, USA), while methanol of chromatographic grade was purchased from Tedia Company Inc. (Fairfield, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Instrumentation and operation conditions

A Waters ACQUITY™ Ultra-Performance Liquid Chromatography system (Waters Corp., Milford, MA, USA) was used to perform the separation of ABA and lisinopril. The separation was achieved on a Venusil ASB C_{18} column (100 mm × 2.1 mm, 5 μm; Agela, USA) maintained at 25 °C. The mobile phase consisted of methanol–ammonium acetate (10 mmol/L) (30:70, v/v) at a flow rate of 0.2 mL/min. The injection volume was 10 μL.

A triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) was equipped with an electrospray ionization interface (ESI) for analytical detection. The ESI source was set in negative ionization mode. Multiple reaction monitoring (MRM) was used to monitor precursor to product ion transition of m/z 235.8→106.6 for ABA, and m/z 403.8→113.6 for lisinopril with scan time of 0.20 s per transition. The optimal MS parameters were as follows: capillary voltage 1.5 kV, cone voltage 35 V for ABA and 13 V for IS, source temperature 110 °C and desolvation temperature 450 °C. Nitrogen was used as the desolvation gas with a flow rate of 150 L/h. The optimized collision energy for ABA and IS was 20 and 25 eV, respectively.

The data acquisition and sample quantification were operated using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of calibration standards and quality control samples

Stock solutions of ABA (388 μg/mL) and the IS (98 μg/mL) were separately prepared in methanol. The stock solution of ABA was diluted with 50% methanol to obtain the serial working solutions for preparation of the calibration standards. The IS working solution (9.80 μg/mL) was prepared by diluting the stock solution with 50% methanol. All solutions were kept at 4 °C and were brought to room temperature before use.

The calibration standard samples of ABA (19.4, 9.70, 2.91, 0.970, 0.485, 0.194, 0.0970, 0.0485 μg/mL) were prepared as follows: 50 μL serial working solutions at required concentrations were added into Eppendorf micro tubes, and evaporated to dryness at 45 °C under a gentle stream of nitrogen, then to the residue was added 0.1 mL blank plasma and mixed. The QC samples were prepared with blank plasma at low, medium and high concentration levels of 0.0972, 1.94 and 15.5 μg/mL which were used in the developing of analytical method and during the pharmacokinetic study. The calibration standard samples and QC samples were then processed with the same procedure as for plasma samples described in Section 2.4.

2.4. Sample preparation

An aliquot of the plasma sample (100 μL) was transferred to an Eppendorf micro tube for processing. The IS (50 μL) and 300 μL methanol were added and vortex-mixed for 1 min. After centrifugation for 10 min at 13,000 rpm, a 10 μL aliquot of the supernatant was injected onto the LC–MS/MS system for analysis.

2.5. Method validation

This method was validated according to the USFDA document with respect to selectivity, linearity, precision and accuracy, recovery, matrix effect and stability [13].

2.5.1. Selectivity

A selectivity study is to investigate whether endogenous constituents and other substances existing in samples will interfere with the detection of analyte and IS. The selectivity of this method was assessed by analyzing the blank plasma from six different sources and those containing ABA and IS, particularly to exam any possible interference at the retention times of ABA and the IS.

2.5.2. Linearity and LLOQ

The linearity was determined by the observed peak area ratios of analyte to IS versus the spiked concentrations of ABA based on three independent seven-point calibration curves, the deviation criteria of these back-calculated concentrations from the spiked concentrations were set within ±15%, except for the lower limit of quantification (LLOQ) which was defined as the lowest concentration in the calibration curve with acceptable precision (RSD) of no more than 20% and accuracy within ±20% of RE.

2.5.3. Precision and accuracy

Precision and accuracy assays were carried out in six replicates at three QC levels on the same day and three batches on three consecutive validation days. The precision was expressed as relative standard deviation (RSD) and the accuracy as relative error (RE).

2.5.4. Extraction recovery and matrix effect

The extraction recovery of ABA was determined by comparing the responses from blank plasma samples spiked with ABA before extraction with those from blank plasma samples spiked after extraction, the later was achieved by transferring the supernatant of protein precipitated blank plasma into another tube and adding 50 μL. ABA working solution. This experiment was performed at three QC levels with six replicates.

The matrix effect was evaluated by comparing the peak areas of the post-extracted blank plasma spiked with ABA working solutions with those of corresponding standard solutions. The experiments were performed at the three QC levels, in triplicate. And the same procedure was performed for IS.

2.5.5. Stability

Stability tests of the analyte were assessed using triplicate spiked samples at low and high QC levels under various conditions: 4 h at room temperature, three freeze–thaw cycles, stored at −20 °C for a month and at 4 °C 12 h after pretreatment. The stability of stock solutions of ABA and internal standard was tested after 1 month of storage under refrigeration conditions. The analytes were considered stable when 85–115% of the initial concentrations were found.

2.6. Pharmacokinetic study

The established analytical method was used to investigate the pharmacokinetic profile of ABA in healthy young men after oral administration of 400 mg aniracetam in dispersible tablets. Blood samples (about 3 mL) were collected into heparinized polythene tubes at 0, 0.083, 0.167, 0.25, 0.33, 0.42, 0.58, 0.75, 1.00, 1.33, 1.67, 2.00, 2.50, 3.00 h after administration, respectively. The plasma was immediately separated by centrifugation at 3000 rpm for 10 min and stored at −20 °C until analysis. The pharmacokinetic parameters of ABA were calculated by DAS 2.1 software (Mathematical Pharmacology Professional Committee of China). Non-compartmental analysis was used to determine standard pharmacokinetic parameters of ABA. The AUC was calculated using trapezoidal rule and extrapolated to infinity. The data are presented as mean ± SD.

3. Results and discussion

3.1. Method development

3.1.1. Selection of internal standard

Several compounds were tested as the internal standard including phenacetin, trimetazidine, benazepril, enalapril and lisinopril under the present experiment conditions. However, phenacetin and trimetazidine were not eluted out the column by the described mobile phase, and benazepril had low response, while a bad shape was observed for enalapril, which made them impossible to be the appropriate IS. Finally, lisinopril was selected as the IS because of its chromatographic behavior, MS response, polarity and extraction efficiency which were similar to those of ABA. The chemical structure of lisinopril is presented in Fig. 1B, similar to ABA in having acid, benzene ring and amide groups.

3.1.2. Selection of MS conditions

In this study, ESI was chosen as the ionization source. To optimize the ESI conditions for detection of ABA, both positive and negative ion detection modes were tried. In the precursor ion spectra, $[M+H]^+$ and $[M+Na]^+$ ions were observed in positive ion mode and $[M-H]^-$ in negative ion mode for ABA. It was also found that both the analyte and IS had better response in negative ion detection mode with low background noise level, thus the negative ion mode was employed for detection in the followed study. Furthermore, no appropriate product ions could be easily found for $[M+H]^+$ and $[M+Na]^+$, while $[M-H]^-$ provided product ion with high response under selected conditions. Therefore, $[M-H]^-$ was finally chosen to be the acceptable precursor ion. After optimization of the collision energy, the collision induced dissociation was carried out using 20 eV and 25 eV collision energy for the ABA and IS, respectively, to obtain the maximum intensity of product ions. The MRM transition with maximum sensitivity was m/z 235.8→106.6 for ABA, m/z 403.8→113.6 for the IS. Full-scan product ion spectra of $[M-H]^-$ of these compounds are shown in Fig. 2.

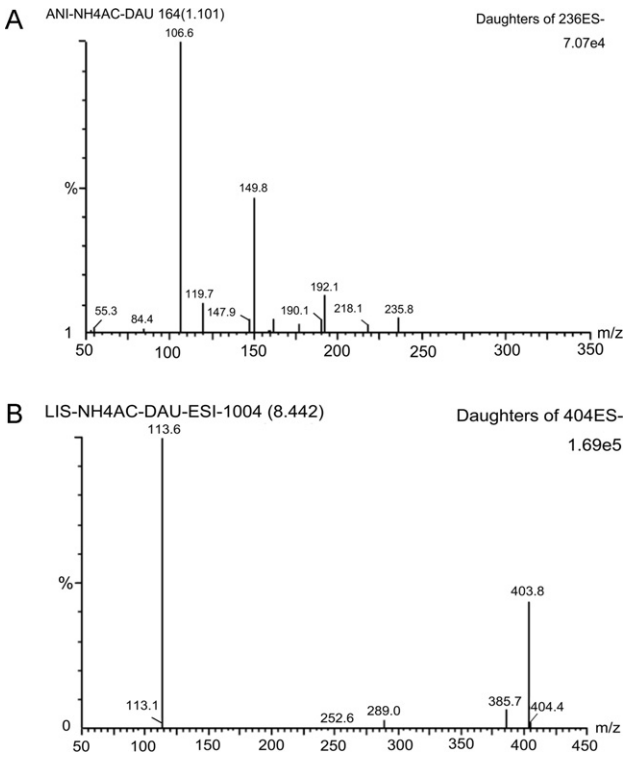

Fig. 2. Full-scan product ion spectra of $[M-H]^-$ of ABA (A), lisinopril (B).

3.1.3. Selection of mobile phase

The separation and ionization of ABA and IS were affected by the composition of mobile phase. Therefore, the selection of mobile phase is important for improving peak shape, detection sensitivity and shortening run time. Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. Much lower detection response was presented when acetonitrile was adopted. Therefore methanol was chosen as the organic phase. The proportion of methanol in mobile phase affected the retention time and peak shape, with 30% methanol generating the best result. When ammonium hydroxide was added in the mobile phase, the response of ABA was not significantly improved. Both ABA and the IS were found to have highest response and better peak shapes in the mobile phase containing 10 mmol/L ammonium acetate which was beneficial to the ionization of the analytes. The finally employed mobile phase was methanol–ammonium acetate (10 mmol/L) (30:70, v/v), which provided retention time of 2.95 min for ABA and 2.80 min for the IS, and a total run time of 4.5 min for each sample much shorter than those reported in literature [11,12]. This method meets the requirement for high sample throughput in bioanalysis.

In order to avoid source pollution, provide perfect peaks and accurate the integration of peak areas, a switchover technology was employed. The eluate between 0 and 2.4 min was switched away from the MS to remove any possible endogenous constituents from the plasma samples.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was determined by analyzing six batches of blank human plasma with and without analytes. The retention times for ABA and IS were 2.95 and 2.80 min, respectively. The chromatograms of samples showed no significant interference at the retention time of ABA and internal standard, which

Table 1
Summary of inter- and intra-day precision and accuracy data for assay of ABA.

	Added C (μg/mL)	Found C (mean ± SD, μg/mL)	Precision (%)	Accuracy (%)
Intra-day (n = 6)	0.0972	0.0972	7.3	6.5
	1.940	2.030	3.1	2.5
	15.5	15.4	5.1	4.6
Inter-day (n = 18)	0.0972	0.0989	6.2	3.7
	1.940	2.081	3.1	6.1
	15.5	15.6	4.9	6.9

demonstrated that protein precipitation in this assay produced clean sample solutions for the quantification of ABA and IS (Fig. 3).

3.2.2. Linearity and LLOQ

Calibration curves were linear over the concentration range of 0.0485–19.4 μg/mL with the linear regression equation for the analyte of $y = 3.439 \times 10^{-1}x - 3.373 \times 10^{-3}$ (r = 0.9959) calculated by weighted ($1/x^2$) linear regression analysis, where y is the peak area ratio of ABA to IS and x is the concentration of ABA in plasma.

The LLOQ of the method was 0.0485 μg/mL for ABA in plasma, the precision (RSD%) was 9.8% (n = 6), and the accuracy (RE%) was 4.9% (n = 6), which was within the acceptable criteria.

3.2.3. Precision and accuracy

The precision and accuracy for ABA were evaluated by a one-way analysis of variance (ANOVA) at three levels of QC samples (Table 1). The intra- and inter-day precisions were measured to be within 7.3% and 6.2%, respectively, with relative error from 2.5% to 6.9%, indicating the acceptable accuracy and precision of the method developed.

3.2.4. Extraction recovery and matrix effect

The extraction recoveries of ABA and IS were 89.1 ± 4.5% (0.0972 μg/mL), 91.5 ± 5.5% (1.94 μg/mL), 100.7 ± 7.9% (15.5 μg/mL), and 93.9 ± 4.0% (IS), respectively.

The matrix effect evaluated for ABA was 100.1, 100.9, 104.6% (with the RSD% of 4.5, 3.2, 3.3%, respectively) at the three QC concentration levels. In addition, the matrix effect for IS was 103.0 ± 6.0%. These results indicated that the endogenous substances showed no significant effect on the ionization for both ABA and IS under the optimized LC–MS/MS conditions.

3.2.5. Stability

Stability results of ABA in QC samples are shown in Table 2, which indicate that ABA is stable in plasma samples under these tested conditions. The stability of ABA and the IS for 7 days at 4 °C was within −4.6% to 1.8% (RE%).

Table 2
Stability of ABA in human plasma under various storage conditions (n = 3).

Condition	Concentration (μg/mL)		RSD (%)	RE (%)
	Added	Found		
Ambient, 4 h	0.0972	0.0988	3.5	−3.1
	15.5	15.9	1.3	−3.4
Three freeze–thaw	0.0972	0.0996	4.3	1.5
	15.5	15.1	2.8	−1.8
−20 °C 40 days	0.0972	0.107	8.0	−4.3
	15.5	15.3	3.6	−0.2
After pretreatment, 4 °C, 12 h	0.0972	0.111	3.5	4.8
	15.5	16.5	3.9	7.4

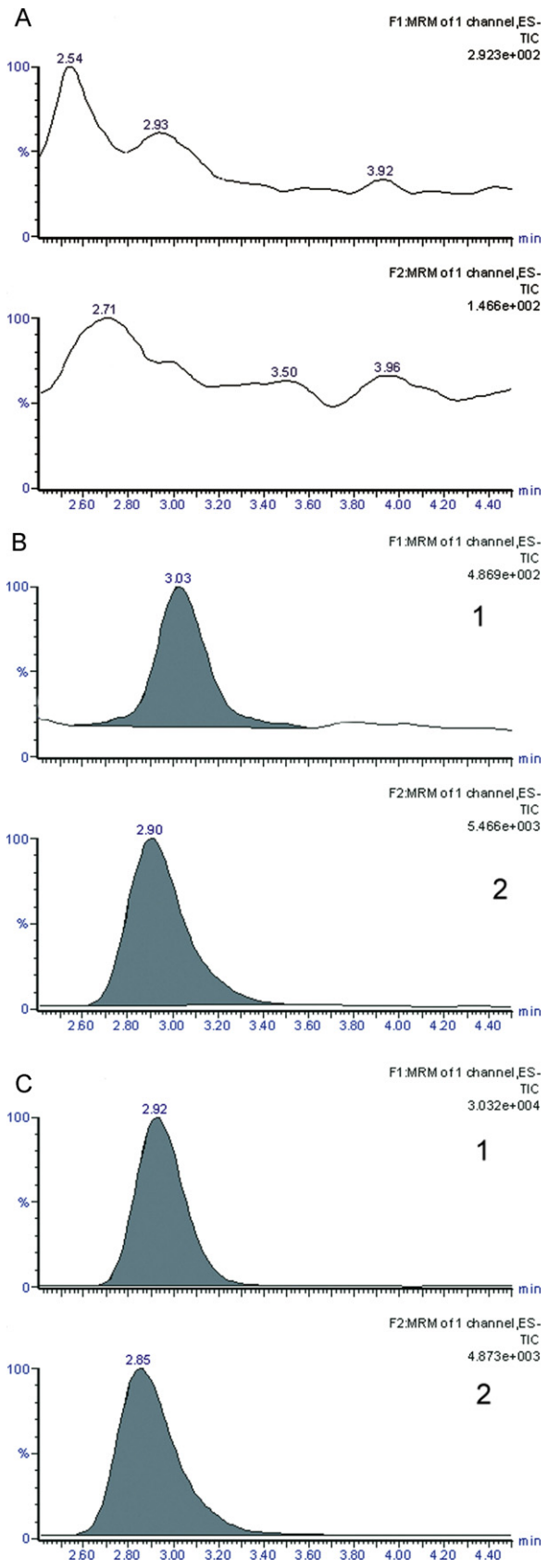

Fig. 3. UPLC–MS/MS chromatograms of matrix, ABA and IS. (A) Chromatogram of blank plasma. (B) Chromatogram of ABA at LLOQ and internal standard. (C) A representative chromatogram of human plasma sample (1. ABA; 2. lisinopril).

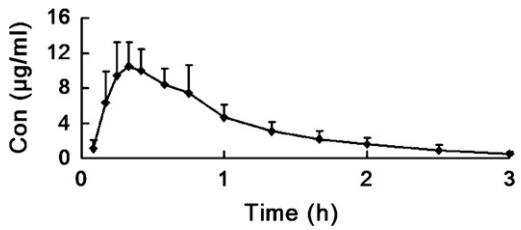

Fig. 4. Plasma concentration–time curves for ABA after administration of 400 mg aniracetam in dispersible tablets to human beings ($n = 12$).

Table 3
Estimated pharmacokinetic parameters of ABA after oral administration of aniracetam dispersible tablets (400 mg) to human beings ($n = 12$).

Parameter	Unit	Mean ± SD
C_{max}	μg/mL	13.11 ± 2.12
T_{max}	h	0.40 ± 0.17
$t_{1/2}$	h	0.68 ± 0.33
V_d	mL	31.07 ± 10.68
CL_p	mL/min	33.37 ± 5.86
$AUC_{0\to t}$	h μg/mL	11.50 ± 1.73

C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}; $t_{1/2}$, terminal elimination half life; V_d, apparent volume of distribution; CL_p, plasma clearance; $AUC_{0\to t}$, area under the plasma concentration–time curve from time 0 to t; SD, standard deviation.

3.3. Application to the pharmacokinetic study

There are several published literatures about the pharmacokinetic profile of ABA after oral administration of aniracetam in capsule form or tablet form, however, no one has investigated the pharmacokinetic characteristics of ABA after administration of aniracetam in dispersible tablets. This method has been applied to study the pharmacokinetics of oral administration aniracetam dispersible tablets to 12 healthy young men. The mean ABA concentration in plasma versus time profile is shown in Fig. 4. The corresponding pharmacokinetic parameters are shown in Table 3. After administration of a single dose of 400 mg aniracetam in dispersible tablets, the C_{max}, T_{max} and $t_{1/2}$ for ABA were 13.11 ± 2.12 μg/mL, 0.40 ± 0.17 h, and 0.68 ± 0.33 h, respectively, which were similar to those reported in the literatures [9,10], indicating the applicability of this method to the pharmacokinetic study of ABA after oral administration of aniracetam.

4. Conclusion

A simple, rapid and robust LC–MS/MS assay for the quantification of ABA in small volumes of human plasma (only 0.1 mL of plasma) was developed and validated. Compared with previously reported methods, the present one employed a simple and rapid extraction procedure for sample preparation, offered better selectivity and shorter run time attributable to the MRM detection and fast HPLC separation. The validated method meets the requirements of high-throughput in bioanalysis and has been successfully applied to a pharmacokinetic study of aniracetam dispersible tablets in humans.

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