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Sensitive and selective liquid chromatography–tandem mass spectrometry method for the quantification of aniracetam in human plasma

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Abstract

A rapid, sensitive and selective LC–MS/MS method was developed and validated for the quantification of aniracetam in human plasma using estazolam as internal standard (IS). Following liquid–liquid extraction, the analytes were separated using a mobile phase of methanol–water (60:40, v/v) on a reverse phase C_{18} column and analyzed by a triple-quadrupole mass spectrometer in the selected reaction monitoring (SRM) mode using the respective $[M+H]^+$ ions, *m/z* 220 → 135 for aniracetam and *m/z* 295 → 205 for the IS. The assay exhibited a linear dynamic range of 0.2–100 ng/mL for aniracetam in human plasma. The lower limit of quantification (LLOQ) was 0.2 ng/mL with a relative standard deviation of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. The validated LC–MS/MS method has been successfully applied to study the pharmacokinetics of aniracetam in healthy male Chinese volunteers.

Keywords: Aniracetam; Liquid chromatography–tandem mass spectrometry; Human plasma; Pharmacokinetic

1. Introduction

Aniracetam, Fig. 1A, a 2-pyrrolidinone derivative as a cognitive performance enhancer, has therapeutic efficacies towards emotional disturbances, sleep disorders and behavior abnormalities (delirium and nocturnal wandering) occurring as sequelae of cerebral infarction [1,2]. Interestingly, the compound is particularly effective for poststroke depression accompanied with both sleep disorders and anorexia [2]. Aniracetam has been shown to possess mechanisms for positively modulating cholinergic and glutaminergic nervous systems, as well as increasing synaptic efficacy and energy metabolism [2].

Following oral administration to humans, anitracetam was rapidly absorbed from the gastrointestinal tract and eliminated primarily by the liver. Aniracetam is extensively metabolized in the body according to two-species-specific pathways. In humans, it is mainly (70%) biotransformed into *N*-anisoyl-GABA, and the pathway leading to *p*-anisic acid and 2-pyrrolidinone accounts for the remaining 30% of the dose [3–5]. Owing to the rapid metabolic degradation of aniracetam, its peak plasma concentrations are very low. Therefore, a sensitive analytical method is needed for the determination of this drug in human plasma. Guenzi and Zanetti developed a HPLC-UV method with LLOQ of 5 ng/mL to study the bioavailability in healthy volunteers. Unfortunately, this method required a column-switching system which was very complex and unsuitable for routine application. It also needed relatively long running time (about 26 min per sample), which was not suitable for high-throughput analysis when a large number of samples need to be quantitated [6]. Song determined the plasma concentration of aniracetam by HPLC-UV and the LLOQ of the method was 2 ng/mL [7]. These assays were not sensitive enough to enable pharmacokinetic study when followed the dosage of 400 mg/day aniracetam to humans.

A rapid and sensitive LC–MS/MS method for the quantification of aniracetam in human plasma was developed in this paper. The procedure exhibited excellent performance in terms of high selectivity and rapid analytical speed (5.5 min per sam-

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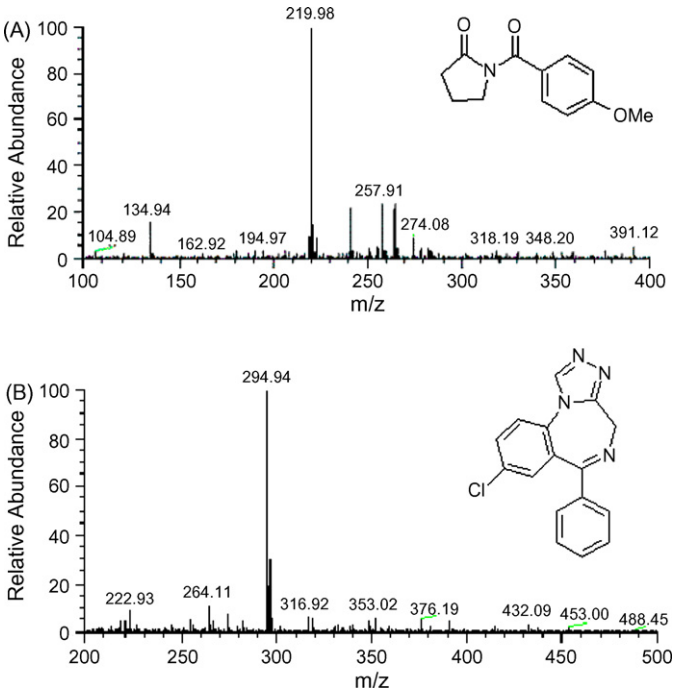

Fig. 1. Full scan ESI (+) precursor ion mass spectra of aniracetam (A) and the IS (B) and their chemical structures.

ple). Although the simultaneous determination of main active metabolites was missing, the method developed is useful when evaluating the bioavailablity/bioequivalence of different dosage forms in which the parent drug is mainly considered to illustrate the pharmacokinetic profiling.

2. Experimental

2.1. Chemicals and reagents

Aniracetam reference standard (purity 99.0%) was supplied by Jiangsu Fangqiang Pharmaceutical Factory (Jiangsu, PR China). Estazolam reference standard (internal standard, IS) was obtained from Hubei Pharmaceutical Factory (Hubei, PR China). HPLC grade methanol was purchased from VWR International Company (Darmstadt, Germany). Ethyl acetate was purchased from Nanjing Chemical Reagent No. 1 Factory. Other chemicals were all of analytical grade. Water was distillated twice before use.

2.2. Preparation of stock and working solutions

Standard stock solutions of aniracetam and estazolam (IS) were prepared in 10 mL methanol at concentrations of 1.0 mg/mL and were stored at 4 °C, respectively.

Working solutions of aniracetam were prepared daily in methanol–water (60:40, v/v) by appropriate dilution at 1, 2.5, 5, 25, 50, 250 and 500 ng/mL.

The stock solution of estazolam was further diluted with methanol–water (60:40, v/v) to prepare the working internal standard solution containing 1 μg/mL of estazolam.

2.3. LC–MS/MS instrument and conditions

Liquid chromatographic separation and mass spectrometric detection were performed using the Finnigan™ TSQ Quantum Discovery MAX™ LC–MS/MS system consisted of a Finnigan™ Surveyor LC pump, a Finnigan™ Surveyor autosampler and combined with a triple-quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corporation). The chromatographic separation was on a Shimadzu Shim-pack VP-ODS C_{18} (150 mm × 2.0 mm, 5 μm) analytical column at 40 °C. The isocratic mobile phase composition was methanol–water (60:40, v/v), which was pumped at a flow rate of 0.2 mL/min.

The tandem MS system is equipped with an ESI source, and run with the Xcalibur 2.0 software (Thermo Electron Corporation). The mass spectrometer was operated in positive ion and SRM mode with precursor to product qualifier transition *m/z* 220 → 135 for aniracetam and *m/z* 295 → 205 for estazolam. Spray voltage was optimized at 5000 V, transfer capillary temperature at 300 °C, sheath gas and auxiliary gas (nitrogen) pressure at 30 and 8 arbitrary unites (set by the LCQ software, Thermo Electron Corporation), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr and collision energy was 18 and 35 V for aniracetam and estazolam, respectively. The scan width for SRM was 0.01 *m/z* and scan time was 0.3 s. The peak width settings (FWHM) for both Q1 and Q3 were 0.7 *m/z*.

2.4. Sample preparation

The plasma samples were prepared by liquid–liquid extraction. The working internal standard solution (10 μL × 1 μg/mL) and 5 mL ethyl acetate were added into a 1 mL aliquot of the collected plasma sample from a human volunteer and then was vortexed for 2 min. After centrifuged at 3000 rpm for 10 min, the upper organic phase was transferred to another 10 mL centrifuge tube and evaporated to dryness under stream of nitrogen gas in water bath at 40 °C. The dried extract was redissolved in 200 μL mobile phase. An aliquot of 10 μL was injected into the LC–MS/MS system.

2.5. Standard curves

Calibration curves were prepared on 5 different days by spiking blank plasma with proper volume of one of the working solutions to produce the standard curve points equivalent to 0.2, 0.5, 1, 5, 10, 50 and 100 ng/mL of aniracetam. The following assay procedures were the same as described above. In each run, a blank plasma sample (processed without the IS) was analyzed to confirm absence of interference but not used to construct the calibration function.

2.6. Preparation of quality control samples

Quality control (QC) samples were prepared daily by spiking blank plasma with proper volume of one of the working solution mentioned above to produce a final concentration equivalent to 0.5 ng/mL (low level), 10 ng/mL (middle level) and 100 ng/mL

(high level) of aniracetam. The following procedures were the same as described above.

2.7. Method validation

2.7.1. Assay specificity

Blank plasma samples of the healthy human used for testing specificity of the method were obtained from six different sources. Each blank sample was tested for the visible interference. The potential matrix effect on the ionization of the analytes was evaluated by comparing the peak area of analytes resolved in blank sample (the final solution of blank plasma after extraction and reconstitution) to that of standard solutions at the same concentration. Three different concentration levels of aniracetam (0.5, 10 and 100 ng/mL) were evaluated by analyzing five samples at each set. The matrix effect of IS (10 ng/mL in plasma) was evaluated by the same method.

2.7.2. Linearity

Calibration curves were generated by using the ratios of the analyte peak area to the IS peak area versus concentration and were fitted to the equation $y = bx + a$ by weighted least-squares linearity regression.

The lower limit of detection (LOD) and the LLOQ were determined as the concentrations with a signal-to-noise ratio of 3 and 10, respectively. Each back-calculated concentration should meet the following acceptable criteria: no more than 20% deviation at LLOQ and no more than 15% deviation above LLOQ.

2.7.3. Precision and accuracy

The intra-batch precision and accuracy was determined by analyzing five sets of spiked plasma samples of aniracetam at each QC level (0.5, 10 and 100 ng/mL) in a batch. The inter-batch precision and accuracy was determined by analyzing five sets of spiked plasma samples of aniracetam at each QC level (0.5, 10 and 100 ng/mL) in three consecutive batches. The concentration of each sample was calculated using standard curve prepared and analyzed on the same day.

2.7.4. Extraction recovery

The absolute recovery of aniracetam through the extraction procedures was determined at three concentrations (0.5, 10 and 100 ng/mL). A known amount of aniracetam was added to blank human plasma prior to extraction as described in the section of "sample preparation", and then the IS (estazolam) was added after extraction to eliminate bias introduced by sample processing. As standards, firstly blank samples were extracted and evaporated to dryness, the same concentration levels of aniracetam and IS were added to the residues and evaporated to dryness, then the residue was dissolved in 200 μL of mobile phase prior to analysis. The extraction recovery was calculated by comparing the peak area ratio of aniracetam/estazolam of extracted samples to the peak area ratio of aniracetam/estazolam of standards.

2.7.5. Stability

The short-term stability of aniracetam was assessed by determining QC plasma samples kept at room temperature for 24 h, which exceeded the routine preparation time of samples. The long-term stability was evaluated by determining QC plasma samples kept at low temperature (−20 °C) for 10 days. The post-preparative stability was measured by determining QC samples kept under the auto-sampler conditions (15 °C) for 24 h. The freeze and thaw stability was tested by analyzing QC plasma samples undergoing three freeze (−20 °C)-thaw (room temperature) cycles on consecutive days. The stock solution stability of aniracetam and the IS were evaluated by analyzing their working solutions kept at room temperature for 24 h, respectively.

3. Results and discussion

3.1. Selection of IS

It is important to select a suitable IS for getting good accuracy. According to the literature published, aniracetam and the compound with structural similarity to aniracetam were less stable in plasma at room temperature for 24 h owing to the effect of enzyme [6]. During the experiment, it was not found that aniractam was less stable when the plasma samples were prepared immediately after thawed. To insure the reliability of the results, the compound with structural similarity to the aniracetam was not selected. Estazolam was adopted in the end because of its similarity of retention time, ionization and extraction efficiency to aniracetam. Additionally, there was less endogenous interference at the retention time of estazolam in blank plasma. The structures of aniracetam and estazolam are shown in Fig. 1.

3.2. Sample preparation

Liquid–liquid extraction was necessary and important because this technique could not only purify but also concentrate the sample. Diethyl ether, ethyl acetate and *n*-hexane-isopropanol (95:5, v/v) were all attempted and ethyl acetate was finally adopted because it showed high extraction efficiency, and the extracted endogenous compounds didn't interfere with the determination of aniracetam and IS.

3.3. LC–MS/MS conditions

The LC–MS/MS method for the determination of aniracetam in human plasma was investigated. Tandem mass spectrum analysis was carried out by electrospray ionization. Both positive and negative ion full scan were done to check the optimum response of aniracetam and estazolam, there was no signal response for aniracetam in negative ions and the response of positive ions was stronger than negative ions for estazolam, so positive ions mode was chosen. In the precursor ion full scan spectra, the most abundant ions were protonated quasimolecular ion $[M+H]^+$ with *m/z* 220 for aniracetam and *m/z* 295 for IS. And the product

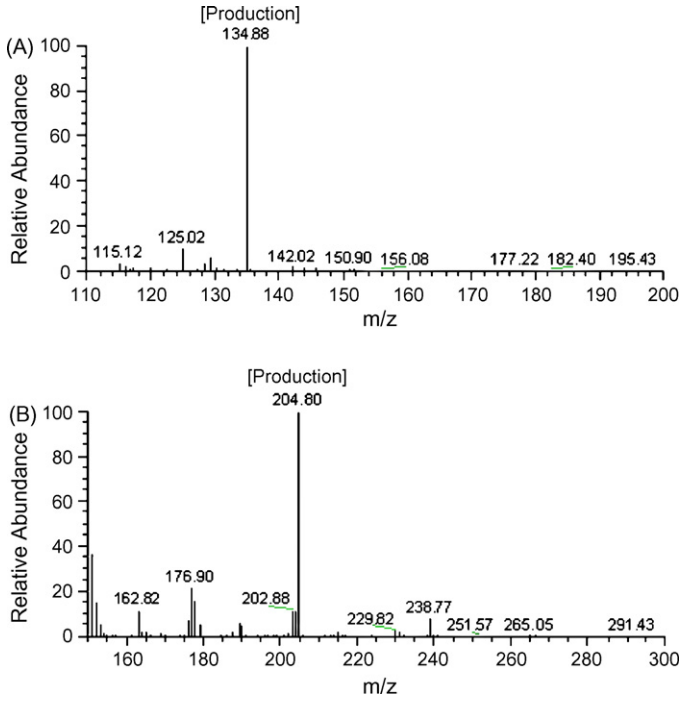

Fig. 2. Full scan ESI (+) product ion mass spectra of aniracetam (A) and the IS (B).

ions scan spectra was also investigated for using SRM mode, the most prominent product ions were *m/z* 135 for aniracetam and *m/z* 205 for the IS, respectively. Other main mass spectrometry parameters, such as spray voltage, capillary temperature, sheath gas and auxiliary gas pressure, source CID, collision gas pressure and collision energy, were also optimized by continuous infusion of a standard solution of aniracetam (1 μg/mL) and the IS (1 μg/mL) with a TSQ Quantum electronically controlled integrated syringe and the TSQ Quantum Tune program. Finally, the transition ions of *m/z* 220 → 135 for aniracetam, *m/z* 295 → 205 for the IS were set as detecting ions for obtaining maximum sensitivity. The positive ion ESI mass spectrum and the MS/MS product ion spectrum of these compounds are shown in Figs. 1 and 2.

3.4. Method validation

3.4.1. Assay specificity

Blank plasma samples showed signal-to-noise ratios <3 at the retention time of aniracetam (2.6 min) and the IS (4.2 min) (shown in Fig. 3A) which means no interference of the analytes was observed. All the ratios of the peak area of analytes resolved in blank sample (the final solution of blank plasma after extraction and reconstitution) to that of standard solutions at the same concentration were between 85 and 115%, which means that no significant matrix effect for aniracetam and the IS was implied in the method.

3.4.2. Linearity and LLOQ

The method exhibited excellent linear response over the selected concentration range of 0.2–100 ng/mL by weighted

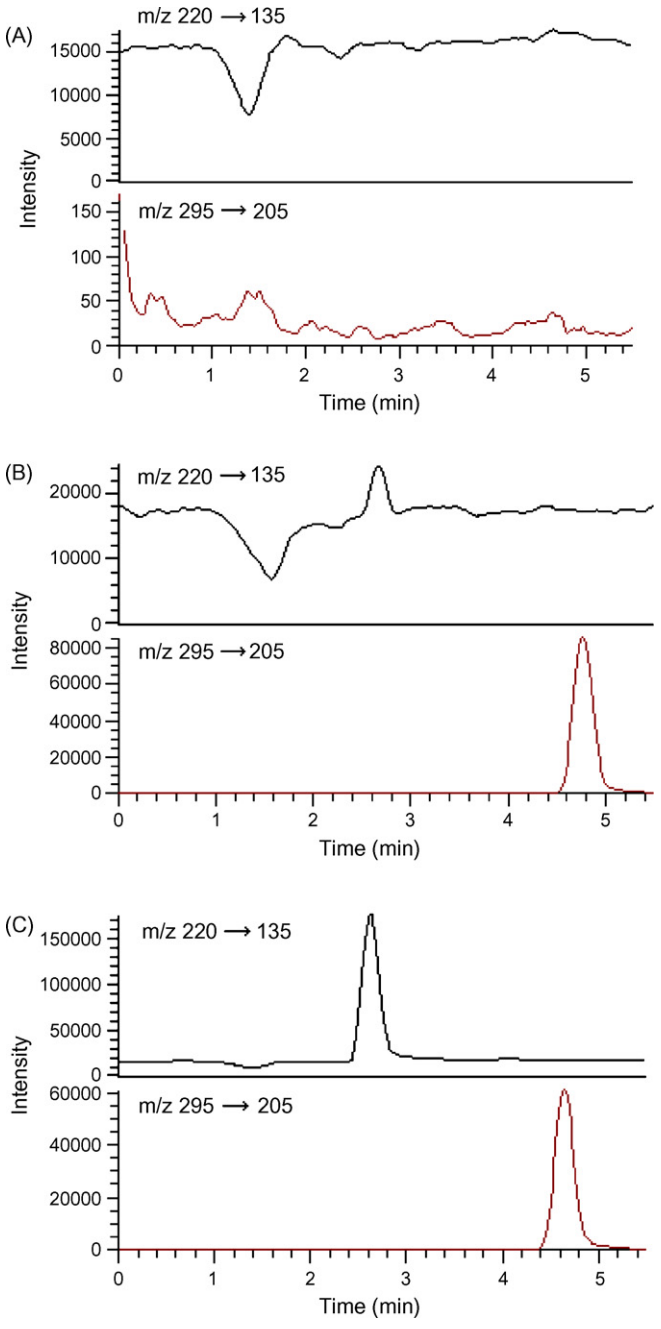

Fig. 3. Representative SRM chromatograms for aniracetam and the IS resulting from analysis of (A) blank plasma (drug and IS free); (B) 0.2 ng/mL (LLOQ) of aniracetam in human plasma spiked with the IS; (C) a plasma sample obtained at 0.33 h from a subject after a single oral dose (400 mg) of aniracetam and the sample concentration was determined to be 9.74 ng/mL for aniracetam.

(1/*x*) least-squares linear regression analysis. The mean standard curve was typically described by the equation: $y = 0.241x + 0.0162$, $r = 0.9976$, where y corresponds to the peak area ratio of aniracetam to the IS and x refers to the concentration of aniracetam added to plasma. Results of five representative standard curves for LC–MS/MS determination of aniracetam are given in Table 1.

The lower limit of quantification for aniracetam proved to be 0.2 ng/mL, and the lower limit of detection was 0.1 ng/mL.

Table 1
The results of five calibration curves for determining aniracetam in human plasma

Concentration added (ng/mL)	Assay	0.2	0.5	1	5	10	50	100
	1	0.16	0.45	0.96	4.84	10.75	42.87	98.10
	2	0.23	0.48	1.05	5.24	10.63	43.00	91.62
Concentration found (ng/mL)	3	0.19	0.50	1.00	5.07	10.97	45.67	90.57
	4	0.20	0.50	1.05	5.11	11.47	48.14	95.80
	5	0.21	0.55	1.13	5.55	10.11	45.73	93.93
Mean (ng/mL)		0.20	0.50	1.04	5.16	10.79	45.08	94.01
Precision (%)		11.8	7.9	6.2	5.0	4.6	4.9	3.3
Accuracy (%)		99.3	99.3	103.9	103.3	107.9	90.2	94.0

Table 2
The precision and accuracy of the method for determining aniracetam in human plasma

Concentration added (ng/mL)	Intra-batch ($n=5$)			Inter-batch ($n=3$)		
	Concentration found (mean ± SD, ng/mL)	Accuracy (%)	Precision (%)	Concentration found (mean ± SD, ng/mL)	Accuracy (%)	Precision (%)
0.5	0.47 ± 0.03	93.6	6.7	0.50 ± 0.04	99.6	8.6
10	9.23 ± 0.47	92.3	5.1	9.60 ± 0.53	96.5	5.5
100	91.67 ± 3.00	91.7	3.3	93.76 ± 3.35	94.0	3.6

Fig. 3B shows the chromatogram of an extracted sample that contained 0.2 ng/mL (LLOQ) of aniracetam.

3.4.3. Precision and accuracy

Data for intra-batch and inter-batch precision and accuracy of the method for aniracetam are presented in Table 2. The precision deviation values for intra-batch and inter-batch are all within 15% of the relative standard deviation (RSD) at each QC level. The accuracy deviation values for intra-batch and inter-batch are all within (100 ± 15)% of the actual values at each QC level. The results revealed good precision and accuracy.

3.4.4. Extraction recovery

The data of extraction efficiency measured for aniracetam and the IS in human plasma was consistent, precise and reproducible. The mean absolute extraction recovery of aniracetam at each QC level (0.5, 10 and 100 ng/mL) was (88.5 ± 7.8), (83.2 ± 5.2) and (85.7 ± 3.6)%, respectively.

3.4.5. Stability

Table 3 summarizes the results of the short-term stability, long-term stability, post-preparative stability and freeze and thaw stability of aniracetam. The data showed the reliable stability behavior of aniracetam under the condition tested.

3.5. Application

The validated method was successfully used to quantify aniracetam concentration in a bioequivalence study of a test and reference capsules of aniracetam which have the same drug ingredients and dosage forms but were made by different manufacturers. The clinical protocol of which was approved by Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College. Twenty Chinese healthy male volunteers participated in the preliminary pharmacokinetic study. After fasted overnight, twenty volunteers were administrated with aniracetam capsule (400 mg). Blood samples (3 mL) were sampled before intake and at 0.083, 0.17, 0.25, 0.33, 0.42, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5 h after oral administration. They were put into lithium heparin tubes and were immediately centrifuged at 1600 g for 10 min. The plasma obtained was frozen at −20 °C in coded polypropylene tubs until analysis. A representative chromatogram of a plasma sample obtained at 0.33 h from a subject who received a single oral dose (400 mg) of aniracetam is shown in Fig. 3C. The mean plasma concentration–time profiles of 20 volunteers are represented in Fig. 4. From the figure, it was found that the error bars (standard devitation) were mostly in excess of 100% which indicated the heterogeneity in the population for pharmacokinetics of aniracetam.

Table 3
The stability of aniracetam in human plasma under tested conditions

Concentration added (ng/mL)	Accuracy (mean ± SD%)		
	0.5 (ng/mL)	10 (ng/mL)	100 (ng/mL)
Short-term stability (24 h, room temperature)	111.16 ± 3.11	99.65 ± 6.18	95.39 ± 1.42
Long-term stability (10 days, −20 °C)	104.54 ± 7.25	95.38 ± 5.39	104.54 ± 3.53
Post-preparative stability (24 h, 15 °C)	109.79 ± 1.16	96.33 ± 1.49	92.62 ± 2.50
Freeze and thaw stability (3 cycles, −20 °C-room temperature)	105.79 ± 7.83	89.73 ± 4.88	88.49 ± 2.07

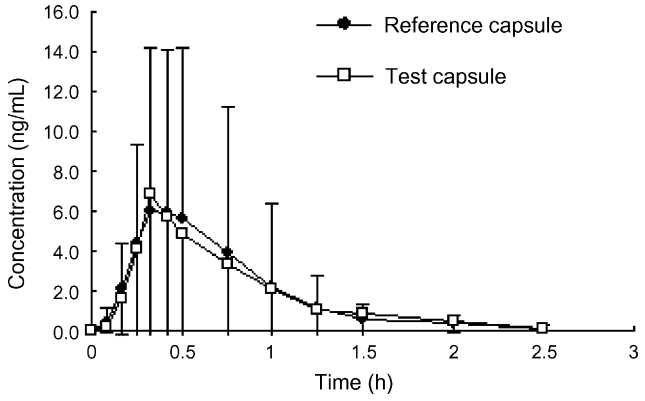

Fig. 4. Mean drug plasma concentration–time curve of aniracetam from 20 subjects after oral administration.

4. Conclusion

In summary, the method is described for the quantification of aniracetam in human plasma by LC–MS/MS in positive ionization mode using selective reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for the quantification of aniracetam at the range of 0.2–100 ng/mL in human plasma samples obtained from pharmacokinetic, bioavailability or bioequivalence studies. The method described is simple, rapid, sensitive, selective and fully validated according to commonly accepted criteria. All the superiorities of the method make it an attractive procedure in high-throughput bioanalysis of aniracetam.

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