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Determination of piracetam in rat plasma by LC–MS/MS and its application to pharmacokinetics

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ABSTRACT: A sensitive and selective liquid chromatography–tandem mass spectrometry method for the determination of piracetam in rat plasma was developed and validated over the concentration range of 0.1–20 μg/mL. After addition of oxiracetam as internal standard, a simplified protein precipitation with trichloroacetic acid (5%) was employed for the sample preparation. Chromatographic separation was performed by a Zorbax SB-Aq column (150 × 2.1 mm, 3.5 μm). The mobile phase was acetonitrile–1% formic acid in water (10:90 v/v) delivered at a flow rate of 0.3 mL/min. The MS data acquisition was accomplished in multiple reaction monitoring mode with a positive electrospray ionization interface. The lower limit of quantification was 0.1 μg/mL. For inter-day and intra-day tests, the precision (RSD) for the entire validation was less than 9%, and the accuracy was within the 94.6–103.2% range. The developed method was successfully applied to pharmacokinetic studies of piracetam in rats following single oral administration dose of 50 mg/kg. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: LC–MS/MS; piracetam; rat plasma; pharmacokinetics

Introduction

Piracetam (2-oxopyrrolidine-1-acetamide, Fig. 1) is a nootropic drug used to treat memory impairment. Several methods for the determination of piracetam have been reported, such as micellar electrokinetic chromatography with ultraviolet detection (MEKC-UV) (Yeh *et al.*, 2006), capillary electrophoresis (CE) (Lamparczyk *et al.*, 1997), gas chromatography (GC) (Alebić-Kolbah *et al.*, 1990) and liquid chromatography with ultraviolet detection (LC-UV) (Curticapean and Imre, 2007; Doheny *et al.*, 1996; Louchahi *et al.*, 1995; Mascher and Kikuta, 1989; Rameis *et al.*, 1994; Saletu *et al.*, 1995). However, these methods performed with limited sensitivity and specificity, and required a relatively long analysis time to attain sufficient chromatographic separation.

In recent years, the high sensitivity and selectivity of tandem mass spectrometry (MS/MS) had led to a growing trend of developing fast analytical methods. LC-MS/MS mode with multiple-reaction monitoring (MRM) further enhances the accuracy of the method because the MRM response is due to the presence of both the analyte ion and its specific fragment.

In this paper, a fast and sensitive LC–MS/MS method for the determination of piracetam in rat plasma using one-step protein precipitation was developed and validated. The developed method was successfully applied to pharmacokinetic studies of piracetam in rats following oral administration.

Experimental

Chemicals and Reagents

Piracetam (purity > 98.0%) and oxiracetam (purity > 98.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC-grade acetonitrile was from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, OH, USA). Ultra-pure water (resistance > 18 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, MA, USA) was used to make the mobile phase. All other chemicals were analytical grade and used without further purification.

Instrumentation and Conditions

All analysis was performed with a 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on a 150 × 2.1 mm, 3.5 μm particle, Agilent Zorbax SB-Aq column at 30°C, with acetonitrile–1% formic acid in water (10:90 v/v) as mobile phase. The flow rate was 0.3 mL/min.

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Abbreviations used: ME, matrix effect; MEKC-UV, micellar electrokinetic chromatography with ultraviolet detection.

Drying gas flow and nebulizer pressure were set to 7 L/min and 30 psi. Dry gas temperature and capillary voltage of the system were adjusted to 360°C and 3000 V. LC-MS/MS was performed in MRM mode using target ions at *m/z* 142.8 → 125.8 for piracetam and *m/z* 158.9 → 141.8 for oxiracetam (IS) (Fig. 2) in positive ion electrospray ionization interface.

Calibration Standards and Quality Control Samples

Individual stock solutions of piracetam (1.0 mg/mL) and oxiracetam (internal standard, IS; 200 µg/mL) were prepared in water. Working solutions for calibration and controls were prepared from the stock solution by dilution using water. A 10 µg/mL working standard solution of IS was prepared by dilution of the IS stock solution with water. All of the solutions were stored at 4°C and were brought to room temperature before use.

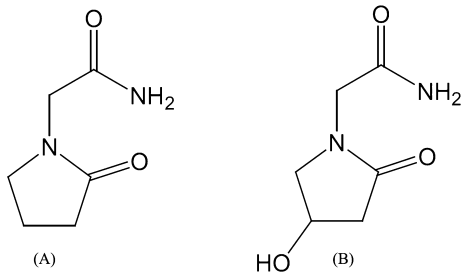

Figure 1. Structures of piracetam (A) and oxiracetam (B).

Piracetam calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 0.1–20 µg/mL for piracetam in rat plasma (concentrations 0.1, 0.2, 0.5, 2, 4, 10 and 20 µg/mL). Quality control (QC) samples were prepared by the same way as the calibration standards, with three different plasma concentrations (0.2, 2 and 16 µg/mL). The analytical standards and QC samples were stored at –20°C.

Sample Preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (10 µg/mL) was added to 0.1 mL of collected plasma sample followed by the addition of 0.2 mL of trichloroacetic acid (5%). The tubes were vortex mixed for 0.5 min. After centrifugation at 15,000 rmp for 10 min, the supernatant (10 µL) was injected into the LC-MS/MS system for analysis.

Method Validation

The selectivity of the method was evaluated by analyzing blank rat plasma and blank plasma spiked piracetam and IS. Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of piracetam to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) in the concentration range of 0.1–20 µg/mL.

To evaluate the matrix effect, blank rat plasma were protein precipitated and then spiked with the analyte at 0.2 and 16 µg/mL. The

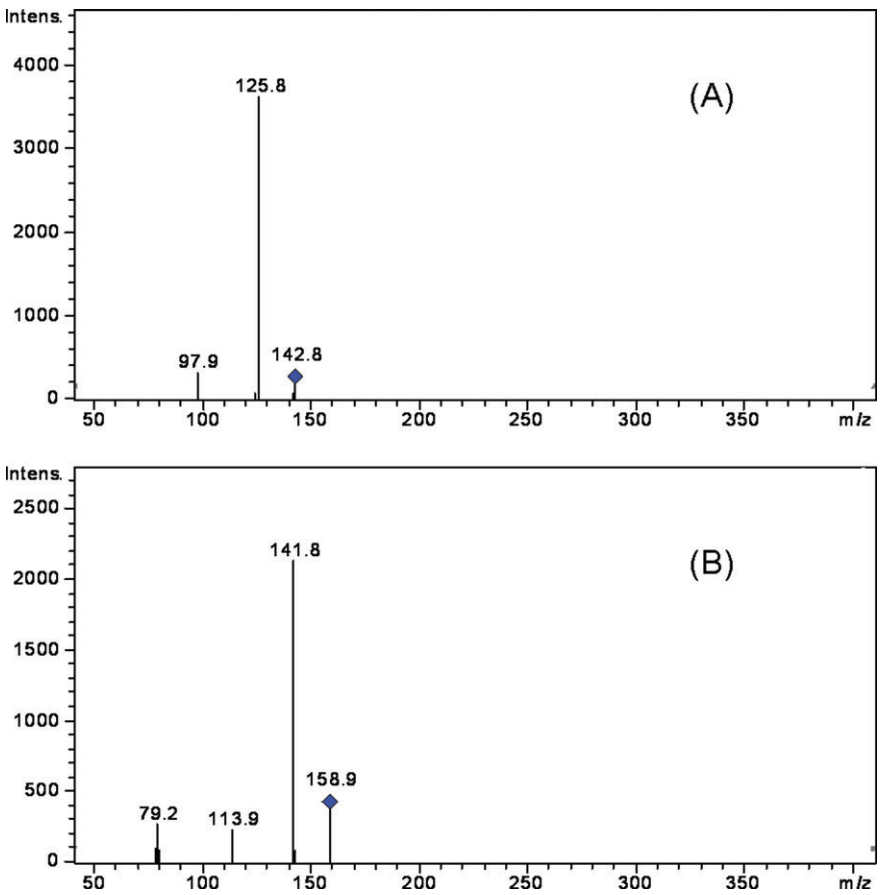

Figure 2. MS/MS product-ion spectrum of piracetam and oxiracetam (IS) with $[M+H]^+$ at *m/z* 142.8 and 158.9 as the precursor ion, respectively.

corresponding peak areas were then compared with those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect (ME). The matrix effect of IS was evaluated at the working concentration (1 μg/mL) in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in five replicates (0.2, 2, and 16 μg/mL) on three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE).

The recoveries of piracetam at three QC levels ($n = 5$) were determined by comparing the peak area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

The stabilities of piracetam in rat plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 0.2, 2 and 16 μg/mL, which were exposed to different conditions. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. The freeze–thaw stability was evaluated after three complete freeze–thaw cycles (–20 to 25°C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at –20°C for 30 days.

Pharmacokinetic Study

Male Sprague–Dawley rats (200–250 g) obtained from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China) were used to study the pharmacokinetics of piracetam. All six rats were housed at Wenzhou Medical College Laboratory Animal Research Center. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed under controlled conditions (25 ± 1°C, RH 55 ± 10%) with a natural light–dark cycle. They were allowed to adapt to the housing environment for at least 1 week before the study. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes just before oral administration of piracetam (50 mg/kg) and 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after dosing. The samples were immediately centrifuged at 2500 ***g*** for 5 min. The plasma obtained (100 μL) was stored at –20°C until analysis. Plasma piracetam concentration vs time data for each rat was analyzed by DAS software (Version 2.0, Medical College of Wenzhou, China).

Results and Discussion

Method Development

Electrospray ionization (ESI) source was used in this work since piracetam is very polar compound. The MS detector parameters were initially assessed by infusion of a standard solution directly into the ESI source. The analyte responded better to positive ESI mode, and the full-scan mass spectrum presented $[M + H]^+$ at *m/z* 142.8 as the main ion and some fragment ions at *m/z* 125.8 and 97.9. In order to optimize MS-MS conditions, the daughter ion spectrum of the $[M + H]^+$ ion was recorded by ramping the capillary voltage and the fragmentation energy. The daughter ion spectrum is shown in Fig. 2. The most abundant fragment was detected at *m/z* 125.8 with the capillary voltage of 3000 V and the fragmentation energy of 0.28 V. Therefore, the *m/z* 142.8 → 125.8 transition was selected for further LC-MS/MS analysis in MRM mode.

Piracetam is a polar compound of low molecular mass, and is not easy to chromatograph, while the addition of pH modifiers often results in good retention of polar compounds on reversed-phase columns. Various mobile phases and columns were investigated to reduce the ion suppression induced by endogenous substances. Because of similar structures for piracetam and IS, it was essential to have a chromatographic separation of the drugs so as to minimize any interference during quantitation. Chromatographic analysis of piracetam and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short run time. Thus, separation was attempted using various combinations of methanol–acetonitrile, acidic buffers and additives like formic acid on different reversed-phase columns [Zorbax XDB-C_8 (150 × 2.1 mm, 3.5 μm), Zorbax SB-C_{18} (150 × 2.1 mm, 3.5 μm), Hypersil ODS C_{18} (150 × 2.1 mm, 3.5 μm), Zorbax SB-Aq (150 × 2.1 mm, 3.5 μm)]. The best result in terms of reproducibility, good separation and peak shape were obtained on a Zorbax SB-Aq (150 × 2.1 mm, 3.5 μm) column with acetonitrile–1% formic acid in water (10: 90 v/v) as mobile phase and hence this was selected for further study.

An efficient clean-up for bio-samples to remove protein and potential interferences prior to LC-MS/MS analysis was an important point in the studies. The simple and effective protein precipitation was employed in our work. Trichloroacetic acid (5%) was chosen as the protein precipitation solvent because it exhibited better effect than methanol or acetonitrile, which could provide acceptable recoveries and avoid the matrix effects. Oxiracetam was chosen as internal standard because of its chromatographic, MS and extraction behaviors.

Selectivity and Matrix Effect

Figure 3 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with piracetam and IS, and a plasma sample. No interfering endogenous substances were observed at the retention times of the analyte and IS.

The MEs for piracetam at concentrations of 0.2 and 16 μg/mL were measured to be 95.8 and 96.5% ($n = 3$), respectively. The ME for IS (1 μg/mL) was 94.4% ($n = 3$). As a result, the ME from plasma was negligible in this method.

Calibration Curve and Sensitivity

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 0.1–20 μg/mL for piracetam in rat plasma. A typical equation of the calibration curve was: $y = 0.203855x + 0.299692$, $r^2 = 0.9977$, where y represents the ratios of piracetam peak area to that of IS and x represents the plasma concentration. The LLOQ for the determination of piracetam in plasma was 0.1 μg/mL. The precision and accuracy at LLOQ were 12.7 and 11.6%, respectively.

Precision and Accuracy

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision was 8% or less and the inter-day precision was 9% or less at each QC level (0.2, 2, and 16 μg/mL). The accuracy of the method ranged from 94.6 to 103.2% at each QC level.

Assay performance data are presented in Table 1. The above results demonstrate that the values are within the acceptable range and the method is accurate and precise.

Recovery

Mean recoveries of piracetam were 90.2, 97.3 and 95.6% ($n = 5$) at concentrations of 0.2, 2 and 16 μg/mL, respectively. The recovery of the IS was 94.6% ($n = 5$).

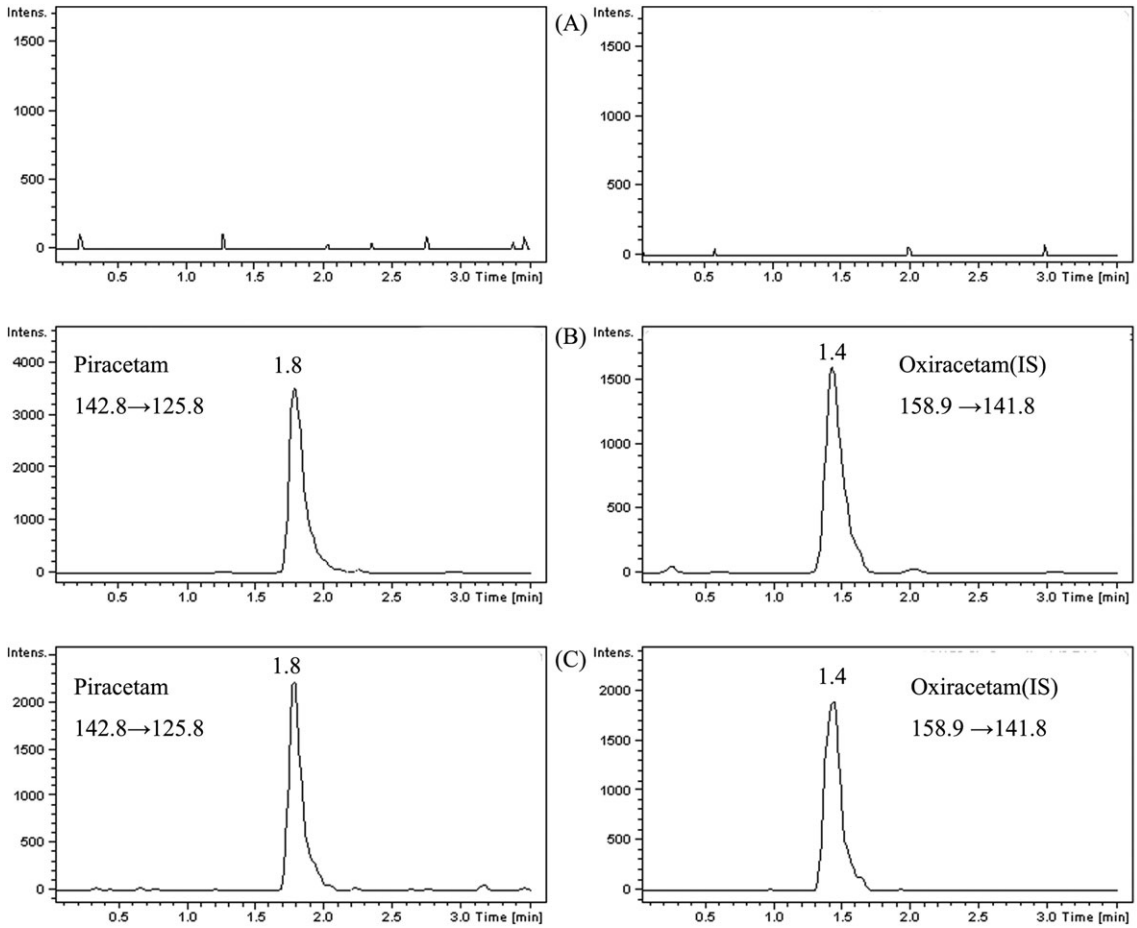

Figure 3. Representative LC–MS/MS chromatograms for piracetam and oxiracetam (IS) in rat plasma samples: (A) blank plasma sample; (B) blank plasma sample spiked with piracetam (1 μg/mL) and IS (1 μg/mL); (C) rat plasma sample 8 h after oral administration of single dosage 50 mg/kg piracetam.

Table 1. Precision and accuracy for piracetam of quality control sample in rat plasma ($n = 5$)

Concentration (μg/mL)	RSD (%)		RE (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.1	7.5	8.3	−5.4	−3.2
2	6.2	3.4	3.2	2.4
16	1.3	4.5	−2.1	1.2

Table 2. Summary of stability of piracetam under various storage conditions ($n = 3$)

Condition	Concentration (μg/mL)		RSD	RE
	Added	Found	(%)	(%)
Ambient, 2 h	0.2	0.18	2.1	−10.0
	16	14.51	1.4	−9.3
−20°C, 30 days	0.2	0.19	7.2	−5.0
	16	15.61	3.5	−2.4
Three freeze–thaw	0.2	0.21	10.6	5.0
	16	15.12	6.2	−5.5
Autosampler ambient 24 h	0.2	0.19	4.2	−5.0
	16	16.29	3.1	1.8

Stability

The stability results showed that piracetam spiked into rat plasma was stable for 2 h at room temperature, for 30 days at −20°C, and during three freeze–thaw cycles. Stability of piracetam extracts in the sample solvent on an autosampler was also observed over a 24 h period. The results of stability experiments are listed in Table 2.

Application of the Method

The method was applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve after administration of a single 50 mg/kg oral dose of piracetam is shown in Fig. 4. The main pharmacokinetic parameters from two-compart-ment model analysis are summarized in Table 3.

Conclusions

A sensitive, rapid and specific LC–MS/MS method for the determination of piracetam in rat plasma was developed and validated over the concentration range of 0.1–20 μg/mL. A very low limit of

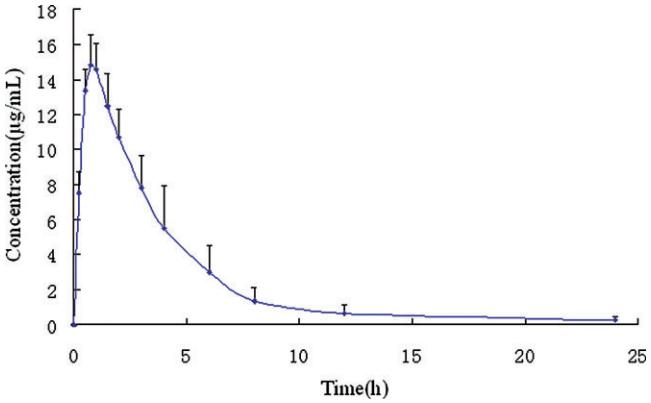

Figure 4. Mean plasma concentration (±SD) time profile after oral administration of single dosage 50 mg/kg oral piracetam on six rats.

Table 3. The main pharmacokinetic parameters after oral administration of single dosage 50 mg/kg piracetam to rats ($n = 6$)

Pharmacokinetic parameters	Mean (±SD)
$t_{1/2}$ (h)	2.64 ± 1.14
CL (L/h)	0.86 ± 0.27
t_{max} (h)	0.92 ± 0.44
C_{max} (μg/mL)	15.74 ± 2.56
$AUC_{(0-t)}$ (μg h/mL)	61.56 ± 17.76
$AUC_{(0-\infty)}$ (μg h/mL)	69.73 ± 17.93

quantitation was obtained and a simple procedure for pretreatment of plasma samples was used. Compared with previously reported analytical methods, this method showed high throughput (4 min each sample) and more sensitivity. The method was validated to meet the requirements for pharmacokinetic determination of the piracetam in rat plasma, and also could be suitable for clinical medical study.

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