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ORIGINAL RESEARCH

Quantitative analysis of phenibut in rat brain tissue extracts by liquid chromatography–tandem mass spectrometry

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ABSTRACT: Phenibut (3-phenyl-4-aminobutyric acid) is a γ-aminobutyric acid mimetic drug, which is used clinically as a mood elevator and tranquilizer. In the present work, a rapid, selective and sensitive liquid chromatography–tandem mass spectrometry method for quantification of phenibut in biological matrices has been developed. The method is based on protein precipitation with acidic acetonitrile followed by isocratic chromatographic separation using acetonitrile–formic acid (0.1% in water; 8:92, v/v) mobile phase on a reversed-phase column. Detection of the analyte was performed by electrospray ionization mass spectrometry in multiple reaction monitoring mode with the precursor-to-product ion transition *m/z* 180.3 → *m/z* 117.2. The calibration curve was linear over the concentration range 50–2000 ng/mL. The lower limit of quantification for phenibut in rat brain extracts was 50 ng/mL. Acceptable precision and accuracy were obtained over the whole concentration range. The validated method was successfully applied in a pharmacological study to analyze phenibut concentration in rat brain tissue extract samples. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: phenibut; LC-MS-MS; brain tissue extracts

INTRODUCTION

Phenibut (3-phenyl-4-aminobutyric acid) is a psychotropic drug with anxiolytic and nootropic properties and is widely used as a mood elevator and tranquilizer (Lapin, 2001; Khaunina and Lapin, 1989; Sytinsky and Soldatenkov, 1978). Owing to its high tranquilizing and cognition-enhancing activities, phenibut was included in the medical kits for the Soviet space flights (Neumyvakin *et al.*, 1978). Structurally, phenibut is a γ-aminobutyric acid (GABA)-mimetic compound similar to baclofen (3-*para*-chlorophenyl-4-aminobutyric acid), another clinically used GABA receptor agonist that acts on metabotropic GABA(B) receptors (Bowery, 2006). There is recent growing interest in the therapeutic value of phenibut as a GABA(B) receptor modulator (Ziablitseva and Pavlova, 2007; Perfilova *et al.*, 2006; Talalaenko *et al.*, 2006). Phenibut is believed to provide better penetration through the blood–brain barrier than GABA (Lapin, 2001).

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Abbreviations used: GABA, γ-aminobutyric acid; MRM, multiple reaction monitoring.

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The capillary electrophoresis technique has previously been applied for the quantification of baclofen in human plasma (Kowalski *et al.*, 2004). Currently, a combination of high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS-MS) has been developed as a powerful modern analytical technique that merges the resolving power of HPLC with the detection specificity and potentially low detection limits of tandem MS. Several HPLC-MS methods have been developed for the determination of GABA in biological fluids by combining chromatography with atmospheric pressure chemical ionization mass spectrometry, capillary liquid chromatography and MS-MS, as well as LC-MS-MS (Ma *et al.*, 1999; Song *et al.*, 2005; Bourcier *et al.*, 2006). The only published study of the brain-penetrating properties of phenibut did not describe technical details and failed to quantify the concentration of the drug in adult rat brains (Khaunina and Maslova, 1968). Only some traces of phenibut were found in brain homogenates 20 min after intraperitoneal administration of the phenibut at a pharmacologically relevant dose of 200 mg/kg (Khaunina and Maslova, 1968). As a result, detailed studies of bioavailability and pharmacokinetics of phenibut have been hindered by the lack of reliable analytical procedures for the analysis of the drug in biological matrices.

In the present study, a simple and sensitive HPLC-MS-MS method for the quantification of phenibut

in rat brain tissue extracts is described. The sample preparation consisted of precipitation of proteins with acetonitrile and centrifugation of the resulting mixture. The obtained supernatant was used directly for analysis. This method was successfully applied for determination of phenibut in rat brain tissue extracts after intraperitoneal administration of the drug.

EXPERIMENTAL

Chemicals and reagents. Phenibut (3-phenyl-4-aminobutyric acid) was obtained from JSC Olainfarm (Olaine, Latvia). Both HPLC-grade acetonitrile and ACS-grade formic acid were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA).

LC-MS-MS instrument and conditions. All experiments were performed on Quattro Micro tandem quadrupole mass spectrometer (Micromass UK Ltd, UK) equipped with an electrospray ionization source and interfaced to Acquity UPLC system (Waters Co., USA). The data were acquired and analyzed using MassLynx version 4.1 data processing software (Micromass UK Ltd). Chromatographic analysis was performed using Acquity UPLC™ BEH Shield RP_{18} column (2.1 × 100 mm, i.d., 1.7 µm, Waters). A mixture of 0.1% formic acid in water and acetonitrile (92:8, v/v) was used as the mobile phase at a flow rate of 0.2 mL/min. The column and autosampler tray temperatures were 30 and 10°C, respectively. The full analytical run time was 5 min. MS data were recorded from 0.5 to 2.5 min using flow switching by divert/injection valve. MS detection was performed with the ESI source operated in positive ion mode, using multiple reaction monitoring (MRM). The ion source and desolvation temperatures were held at 120 and 300°C, respectively, and the cone voltage was 20 V. The molecular ion of phenibut was fragmented at collision energies of 20 eV, using argon as the collision gas, and ion transition 180.3 → 117.2 was used for quantification.

Brain tissue extract preparation. Male Wistar rats (Laboratory Animals Breeding Facility, Riga Stradins University, Riga, Latvia) weighing 250–280 g were housed under standard conditions (21–23°C, 12 h light–dark cycle) with unlimited access to standard food and water. The experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies, and were approved by Latvian Animal Protection Ethical Committee, the Food and Veterinary Service, Riga, Latvia.

Phenibut was dissolved in saline and administered intraperitoneally at a dose of 100 mg/kg daily for 3 days (n = 8). Control rats (n = 3) received an adequate volume of saline. One hour after the last administration rats were decapitated, and the brains were excised and homogenized at 1500 rpm for 1 min with a glass–PTFE homogenizer in ice-cold distilled water at w/v ratio of 1:5. The homogenate was spun at 14,000***g*** for 10 min at 4°C. The supernatant was then decanted, and the pellet was homogenized in the same volume of distilled water as before. The obtained homogenate was spun once more at 14,000***g*** for 10 min at 4°C. The supernatants were combined and stored frozen (–80°C) until analyzed.

Sample preparation. A standard stock solution of phenibut (1 mg/mL) was prepared in water–methanol (1:1, v/v). Working solutions for calibration standards and controls were prepared by dilution of the stock solution with water to cover the concentration range from 0.5 to 20 µg/mL. Spiked samples for method validation were prepared by adding of 10 µL of the working standard solution to 100 µL of brain tissue extract to obtain phenibut concentration levels from 50 to 2000 ng/mL.

To prepare samples for analysis, 100 µL of brain tissue extract were diluted with 0.5 mL of 0.1% formic acid solution in acetonitrile. The samples were mixed thoroughly, sonicated for 10 min and centrifuged at 10,000***g*** for 15 min. The supernatant was collected, and a 5 µL aliquot was injected into the LC-MS-MS system for analysis.

Both precision and accuracy of the method were determined by analysis of spiked samples of rat brain tissue extracts at four concentration levels (n = 5 at each concentration) on three separate days.

Accuracy was calculated by comparing the mean experimental concentrations of phenibut in assayed spiked brain tissue extract samples with their nominal values, and percentage values were used as the index. Relative standard deviation (RSD) of the found concentration values was used as an index of precision.

RESULTS AND DISCUSSION

Mass spectrometry

The detector parameters were initially assessed by infusion of a standard solution directly into the ESI source. The analyte responded best to positive ESI mode, and the full-scan mass spectrum presented $[M + H]^+$ at *m/z* 180 as the main ion and some fragment ions at *m/z* 163, *m/z* 145 and *m/z* 117. In order to optimize MS-MS conditions, the daughter ion spectrum of the $[M + H]^+$ ion was recorded by ramping the cone voltage and the collision energy. The daughter ion spectrum is shown in Fig. 1. The most abundant fragment was detected at *m/z* 117, resulting from loss of ammonia and formic acid. Therefore, the *m/z* 180 → *m/z* 117 transition was selected for further HPLC-MS-MS analysis in MRM mode.

Chromatography

A simple one-step protein precipitation technique was found to be appropriate for the sample preparation. Thus, a volume of 100 µL of homogenized brain tissue extract was mixed with 500 µL of acidic acetonitrile. Precipitate was easily separated by centrifugation of the samples.

Various combinations of acetonitrile, methanol, formic acid and ammonium acetate were investigated in an

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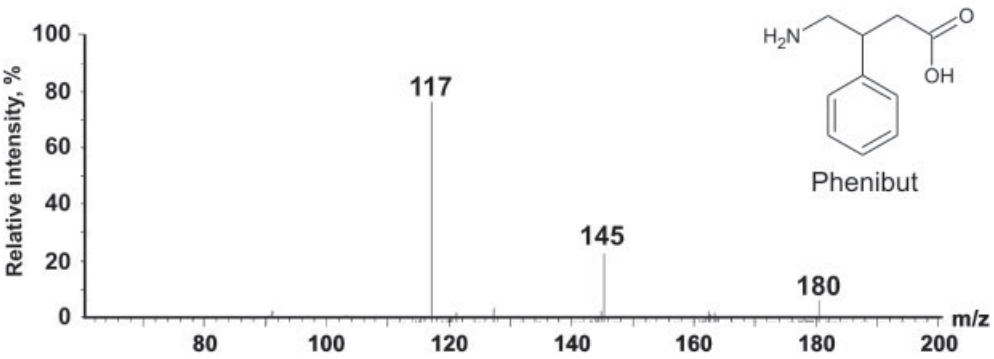

Figure 1. Product ion mass spectrum for molecular ion $[M+H]^+$ of phenibut.

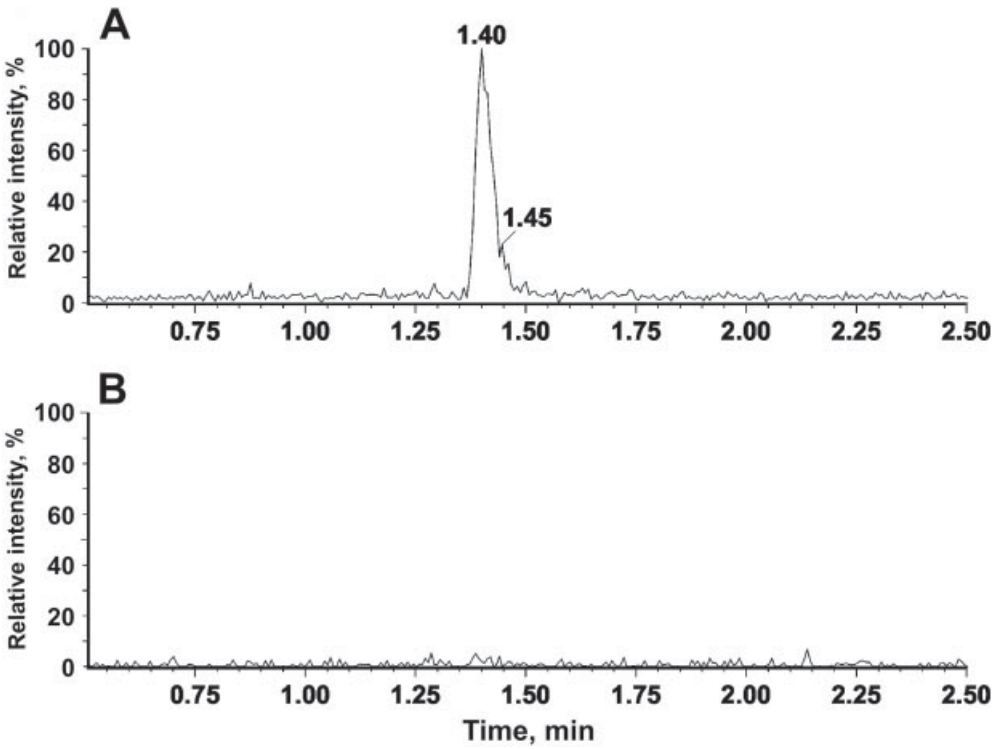

Figure 2. Representative mass chromatograms obtained from blank (B) and spiked (A) rat brain tissue extracts (concentration of phenibut 50 ng/mL).

attempt to optimize the mobile phase for sensitivity, speed and peak shape. As a result, a mixture of 0.1% formic acid and acetonitrile (92:8, v/v) was found to be the most suitable combination, and it was then applied as the mobile phase in further experiments. Under the optimized assay conditions, the analyte gave a retention time of 1.4 min. The run time of 2.5 min allows a fast analysis of phenibut in biological samples. Representative chromatograms obtained from both a blank biological sample and a brain tissue extract sample spiked with phenibut at a concentration of 50 ng/mL are shown in Fig. 2. As seen in the figure, no interfering peaks from endogenous compounds were observed at the retention times of analytes in the blank brain tissue extract sample.

The linear calibration curve was obtained over the concentration range from 50 to 2000 ng/mL of the analyte in brain tissue extracts (correlation coefficient $R > 0.99$). The lower limit of quantification (LLQ) of phenibut was found to be at 50 ng/mL, and the signal-to-noise ratio at this concentration level was approximately 25. The analysis of spiked samples has shown

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Table 1. Accuracy and precision of the method for determination of phenibut in rat brain tissue extracts (five replicates per day on three separate days)

Spiked concentration (ng/mL)	Observed concentration (ng/mL)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
50.23	46.56	92.7	5.8	20.1
200.91	229.06	114.0	7.6	8.6
803.64	830.73	103.4	4.4	1.8
2009.09	1947.95	97.0	4.6	1.8

that the intra-day precision was better than ±7.6% (RSD) and accuracy was 102 ± 9%. The inter-day precision of the procedure was ±20% at 50 ng/mL concentration and ±1.8% at 2000 ng/mL (Table 1).

Bioavailability of phenibut

The above described procedure was used to determine the concentration of phenibut in rat brain tissue extracts that were prepared 1 h after intraperitoneal administration of phenibut at a dose of 100 mg/kg daily for 3 days. Data are presented as mean ± SEM of eight measurements. We found that the concentration of phenibut in rat brain tissue extracts was 214 ± 20 ng/mL. Taking into account the dilution factor in the tissue extract preparation procedure, this corresponds to about 2 μg, or about 10 nmol, of phenibut per 1 g of rat brain tissue. This is the first study to quantify the concentration of phenibut in rat brain tissues after intraperitoneal administration of phenibut at a pharmacologically relevant dose.

CONCLUSION

A simple and reliable analytical procedure is described for the quantification of phenibut in rat brain tissue extracts by LC-MS-MS in positive electrospray ionization mode using multiple reaction monitoring. The method allows precise, accurate and fast quantification of phenibut in the concentration range from 50 to 2000 ng/mL in biological samples.

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