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Short communication

Determination of picamilon concentration in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of picamilon concentration in human plasma. Picamilon was extracted from human plasma by protein precipitation. High performance liquid chromatography separation was performed on a Venusil ASB C_{18} column with a mobile phase consisting of methanol –10 mM ammonium acetate–formic acid (55:45:01, v/v/v) at a flow rate of 0.65 ml/min. Acquisition of mass spectrometric data was performed in selected reaction monitoring mode, using the transitions of m/z 209.0 → m/z (78.0 + 106.0) for picamilon and m/z 152.0 → m/z (93.0 + 110.0) for paracetamol (internal standard). The method was linear in the concentration range of 1.00–5000 ng/ml for the analyte. The lower limit of quantification was 1.00 ng/ml. The intra- and inter-assay precision were below 13.5%, and the accuracy was between 99.6% and 101.6%. The method was successfully applied to characterize the pharmacokinetic profiles of picamilon in healthy volunteers. This validated LC–MS/MS method was selective and rapid, and is suitable for the pharmacokinetic study of picamilon in humans.

1. Introduction

Picamilon is a derivative of γ-aminobutyric acid (GABA) and nicotinic acid developed in 1970 by the All-Union Vitamin Research Institute [1]. Because it can dramatically increase blood flow and circulation within the brain, picamilon is used as a nootropic [2]. Also used for the treatment of depression, anxiety, and migraine [3], picamilon works by crossing the blood–brain barrier, after which it is hydrolyzed into GABA and nicotinic acid [4]. In addition, picamilon has extremely low toxicity and shows no allergenic or carcinogenic properties [1]. An animal study has shown that picamilon is rapidly absorbed (T_{max} = 0.23 h) and penetrates well through the blood–brain barrier in mice [5]. At oral administration, the drug bioavailability in mice was 21.9% and in rats between 53% and 78.9%.

Although picamilon has been used in humans for more than 30 years, to the best of our knowledge, no study has been reported concerning the determination of picamilon concentration in biological samples and pharmacokinetics studies in humans. Thus, the purpose of this study was to develop a rapid, sensitive, and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method of determining picamilon concentration in human plasma. The method is applied to a pharmacokinetic study of picamilon tablets in healthy Chinese volunteers.

2. Material and methods

2.1. Materials

Picamilon (99.4% purity) was kindly provided by Tri-Lion Pharmaceutical Co. Ltd. (Harbin, China). Paracetamol (100% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate and formic acid (HPLC grade) were purchased from Tedia (Fairfield, OH, USA). Deionized water was obtained from a Millipore Milli-Q Gradient Water Purification System (Molsheim, France). Drug-free human plasma was supplied by the Shanghai Shuguang Hospital (Shanghai, China).

2.2. Instruments

The Agilent 1100 liquid chromatography system used was equipped with a G1311A quaternary pump, a G1322A vacuum degasser, a G1316A thermostated column oven, and a G1367A autosampler (Agilent, Waldbronn, Germany). Mass spectrometric detection was performed on a Thermo Electron Finnigan TSQ Quantum Ultra triple quadrupole instrument (San Jose, CA, USA). The

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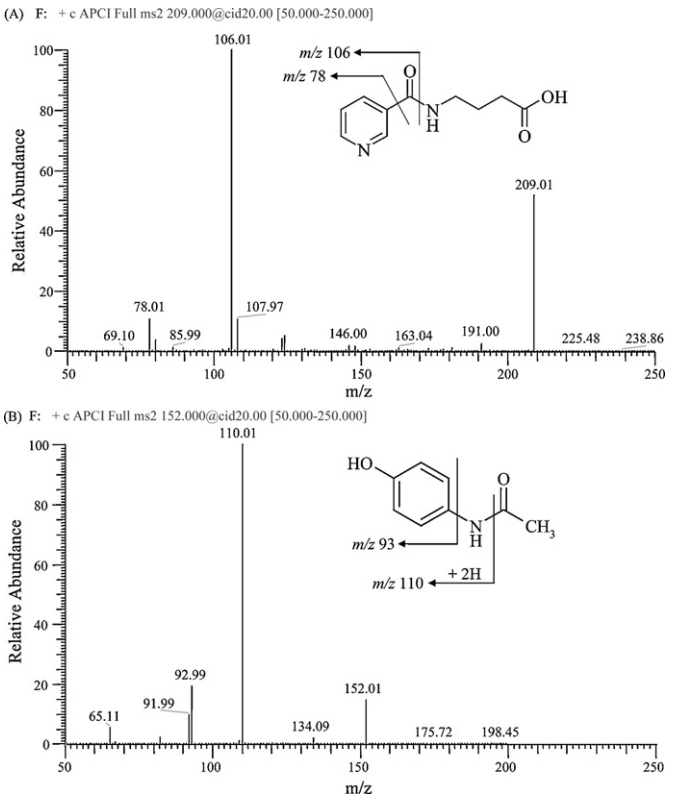

Fig. 1. Product ion mass spectra of $[M+H]^+$ ion of picamilon (A) and paracetamol (B).

Agilent ChemStation and Finnigan Xcalibur software packages were used to control the LC–MS/MS system, as well as for data acquisition and processing.

2.3. LC–MS/MS conditions

Chromatographic separation was achieved on a Venusil ASB C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Agela Technologies Inc, Newark, DE, USA) protected by a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). A mixture of methanol −10 mM ammonium acetate–formic acid (55:45:0.1, v/v/v) was used as the mobile phase at a flow rate of 0.65 ml/min. The column temperature was maintained at 25 °C.

The mass spectrometer was operated in the positive ion mode using an atmospheric pressure chemical ionization (APCI) source. The corona discharge current was set at 4.0 μA. Nitrogen was used as sheath gas (40 Arb) and auxiliary gas (10 Arb) to assist with nebulization and desolvation. The vaporizer temperature and heated capillary temperature were maintained at 420 °C and 320 °C, respectively. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 1.2 mTorr. Quantification was performed using selected reaction monitoring (SRM) of the transitions *m/z* 209.0 → (78.0 + 106.0) for picamilon and *m/z* 152.0 → (93.0 + 110.0) for paracetamol (internal standard) (Fig. 1), with a dwell time of 200 ms per transition. Optimized collision energy of 20 eV was used for the analyte and the internal standard. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

2.4. Preparation of calibration standards and quality control samples

A stock solution of picamilon with a concentration of 1.00 mg/ml was prepared by dissolving the accurately weighed reference compound in methanol. A series of standard working solutions with picamilon concentrations in the range of 1.00–5000 ng/ml were obtained by further dilution of the stock solution with methanol. Calibration curves were prepared by spiking 100 μl of the appropriate standard solution into 100 μl blank human plasma. Quality control (QC) samples were similarly prepared at concentrations of 2.50, 300, and 4000 ng/ml in human plasma, by a separate weighing of the reference compound. A 625 ng/ml internal standard (IS) working solution was prepared by diluting a stock solution of paracetamol with methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use. The analytical standards and QC samples were stored at −20 °C.

2.5. Sample preparation

Frozen plasma samples from the participants in the human study were thawed to room temperature prior to preparation. After vortexing, a 50 μl aliquot of the IS solution (paracetamol, 625 ng/ml), 100 μl methanol and 250 μl acetonitrile were added to 100 μl of plasma sample. The mixture was vigorously vortexed for 1 min and centrifuged at 11,000 × *g* for 5 min. Then, the supernatant was separated and evaporated to dryness at 40 °C under a stream of nitrogen in a TurboVap evaporator (Zymark Corp., Hopkinton, MA, USA). The residue was reconstituted in 200 μl of the mobile phase and then vortex-mixed. A 20 μl aliquot of the resulting solution was injected into the LC–MS/MS system for analysis.

2.6. Method validation

To ensure its accuracy, selectivity, reproducibility, and specificity, the method was validated as follows. Selectivity was performed by analyzing the blank plasma from six different sources to test interference at the retention times of the analyte and the IS. The linearity of the method was determined by plotting the peak area ratios of the analyte to the IS against the concentrations of picamilon in human plasma in duplicate on three consecutive validation days. The precision and accuracy of the method were assessed by the determination of QC samples at three concentration levels (2.50, 300, and 4000 ng/ml), in six replicates, on three validation days. The intra- and inter-day precisions were required to be below 15%, and the accuracy within ±15% [6,7]. The lower limit of quantification (LLOQ), was determined in six replicates on three consecutive validation days. The precision should be equal to or less than 20% and accuracy between 80% and 120% of nominal concentrations [6]. The recovery of picamilon was determined by comparing the mean peak areas of the regularly pretreated QC samples at three concentration levels (six samples each) to those of spike-after-extraction samples. To evaluate the matrix effect (ME), six different lots of blank plasma were extracted and then spiked with QC samples and the IS. The corresponding peak area ratios of the analyte to the IS in plasma spiked post-extraction (A) were then compared with those of the solution standards in the mobile phase (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the matrix factor (MF). The variability in matrix factors, as measured by the coefficient of variation, should be less than 15% [8]. The stabilities of picamilon in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at LQC and HQC level, which were exposed to different conditions (time and temperature).

2.7. Applications to pharmacokinetic study

The developed LC–MS/MS method was applied to determine the plasma concentrations of picamilon in 30 (15 males and 15 females) healthy Chinese subjects enrolled in a clinical pharmacokinetic study. The pharmacokinetic study was approved by the Ethical Committee of the Second Affiliated Hospital of Liaoning

University of Traditional Medicine. The 30 volunteers were randomized into three groups. Each group was orally administered picamilon tablets (Tri-Lion Pharmaceutical Co. Ltd., Harbin, China) of 50, 100, or 200 mg, respectively. Venous blood samples were collected in heparinized tubes before dosing and 0.33, 0.67, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 h post-dosing, and centrifuged at 2000 × *g* (4 °C) for 10 min to separate plasma. The plasma samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of the LC–MS/MS conditions

To the best of our knowledge, no LC–MS/MS method has been previously reported for the determination of picamilon concentrations in human plasma. Revealing the pharmacokinetic profile of picamilon requires a rugged, sensitive, and specific LC–MS/MS method with a short run time. To accomplish this aim, different options were evaluated to determine the optimal detection, extraction, and chromatographic conditions.

In positive mode, picamilon formed predominantly a protonated precursor ion $[M+H]^+$ at *m/z* 209.0. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were evaluated to obtain the better response of analyte. It was found that the signal intensities in the APCI mode were approximately twice as high as those in the ESI mode. Two product ions, *m/z* 106.0 and *m/z* 78.0, of picamilon were monitored, which gave better sensitivity and selectivity. As a result, the transitions *m/z* 209.0 → (106.0 + 78.0) were chosen for use in SRM.

During method development, three reversed-phase columns, including Zorbax XDB C_{18} (150 mm × 4.6 mm, 5 μm; Agilent), Venusil ASB C_{18} (150 mm × 4.6mm, 5 μm; Agela), and Luna C_{18} (150 mm × 4.6 mm, 5 μm; Phenomenex), were tested. Using the same mobile phase system, ASB C_{18} achieved better retention of picamilon than the other two columns; thus, it was chosen for further optimization. A broad and tailing peak was observed when methanol–water–formic acid (55:45:0.1, v/v/v) was used as the mobile phase. The mobile phase was then optimized by adding 10 mM ammonium acetate, and the peak shape of picamilon was sharp and symmetric.

Paracetamol was chosen as the internal standard due to its similarity to picamilon in terms of extractability, chromatographic behavior, and ionization property.

3.2. Preparation of plasma samples

In the early stage of the method development, a liquid–liquid extraction (LLE) method was employed to separate picamilon from plasma samples. However, the recovery of the analyte was low (<30%) when either ethyl acetate or ethyl ether–dichloromethane (3:2, v/v) was used as the extraction solvent. Finally, protein precipitation was used in a plasma sample preparation to obtain a high recovery, and acetonitrile was selected as the precipitant. Following this preparation procedure can both save time and simplify the operating process.

3.3. Method validation

3.3.1. Selectivity

Fig. 2 shows the typical chromatograms of a blank and a spiked plasma sample with picamilon at LLOQ and IS, and a plasma sample from a healthy volunteer 3.0 h after oral administration of 200 mg picamilon tablets. No interfering peaks from endogenous compounds were observed at the retention times of the analyte and the IS. The retention time for picamilon and the IS were 3.75 and 3.64 min, respectively.

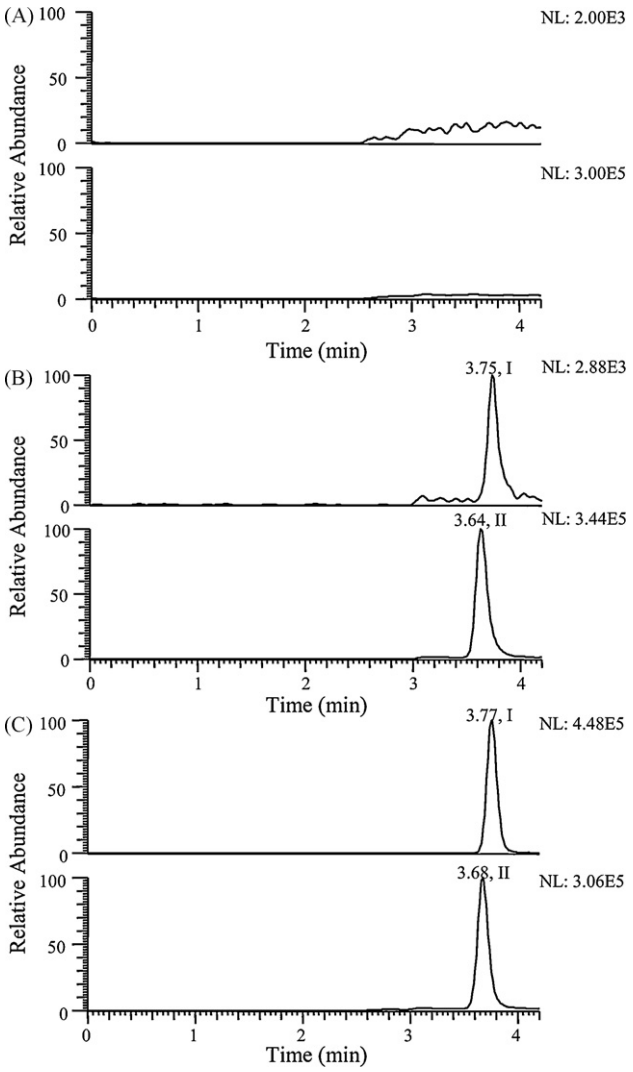

Fig. 2. Representative SRM chromatograms of picamilon (I) and paracetamol (IS, II) in human plasma. (A) Blank plasma sample; (B) Plasma sample spiked with picamilon at 1.00 ng/ml and paracetamol (IS) at 312.5 ng/ml; (C) A plasma sample collected 3.0 h after an oral dose of 200 mg picamilon. Peaks I and II refer to picamilon and paracetamol, respectively.

3.3.2. Linearity of calibration curves and lower limit of quantification

The calibration curve was linear over the range of picamilon concentrations from 1.00 to 5000 ng/ml in human plasma with a coefficient of correlation (r^2) >0.99. The mean (±standard deviation) regression equation from replicate calibration curves from three different validation days was:

$$y = (0.00365 \pm 0.00165) + (0.00279 \pm 0.00015)\,x,$$

$$r^2 = 0.9937 \pm 0.0009$$

where *y* represents the peak area ratio of analyte to IS and *x* represents the plasma concentration of picamilon.

The lower limit of quantification (LLOQ) of this assay was 1.00 ng/ml for picamilon. The precision and accuracy data of LLOQ are shown in Table 1. Under the present LLOQ, picamilon could be determined in plasma samples until 10 h after a single oral dose of 50 mg picamilon. This result is sensitive enough to investigate the pharmacokinetic behaviors of picamilon.

Table 1
Precision and accuracy of the LC–MS/MS method to determine picamilon in human plasma (n = 3 days, six replicates for each day).

Concentration (ng/ml)		RSD (%)		RE (%)
Added	Found	Intra-day	Inter-day	
1.00	1.01 ± 0.05	5.4	2.7	1.5
2.50	2.53 ± 0.23	8.1	13.5	1.2
300	299 ± 19	5.5	10.4	−0.4
4000	4065 ± 240	5.6	7.9	1.6

Table 2
Main pharmacokinetic parameters in healthy Chinese volunteers following single oral dose of 50, 100, or 200 mg of picamilon (n = 10).

Parameters	50 mg	100 mg	200 mg
C_{max} (ng/ml)	667 ± 317	1551 ± 550	2971 ± 848
T_{max} (h)	1.00 ± 0.46	0.64 ± 0.19	0.67 ± 0.22
AUC_{0-10} (ng·h/ml)	1100 ± 239	2221 ± 533	4259 ± 751
$AUC_{0-\infty}$ (ng·h/ml)	1106 ± 239	2230 ± 533	4284 ± 732
$t_{1/2}$ (h)	1.46 ± 0.40	1.46 ± 0.21	1.46 ± 0.48
CL/F (l/h)	47.4 ± 11.6	47.0 ± 10.4	47.8 ± 7.39

3.3.3. Precision and accuracy

Table 1 summarizes the intra- and inter-day precision and accuracy values for the QC samples. In this assay, the intra- and inter-day precisions were measured to be below 8.1% and 13.5%, respectively, with relative errors from −0.4% to 1.6%. These results indicate that the present method has good precision, accuracy, and reproducibility.

3.3.4. Recovery

The extraction recoveries of picamilon obtained from plasma (n = 6) were 99.3 ± 6.8%, 99.8 ± 7.6% and 102 ± 3.5% at concentrations of 2.50, 300, and 4000 ng/ml, respectively. Mean recovery for the IS was 105 ± 1.1%.

3.3.5. Matrix effect

The peak area ratios (analyte/IS) in extracted blank matrix spiked with solutions of 2.50 and 4000 ng/ml were in the range of 104–107% compared with those in the same nominal solutions prepared with the mobile phase, and the CV values from six lots of plasma were less than 4.9%. These results indicate that ion suppression or enhancement from the plasma matrix was negligible under the current conditions [8].

3.3.6. Stability

The tests of picamilon stability were designed to cover anticipated conditions of handling typical clinical samples. Samples of picamilon in human plasma were stable at room temperature for at least 2 h (RSD ≤ 5.1%, RE in the range of −10.7% to −3.1%), at −20 °C for 31 days (RSD ≤ 13.1%, RE in the range of −0.8% to −0.6%), and for three freeze-thaw cycles (RSD ≤6.5%, RE in the range of −0.1% to −0.8%). Picamilon was stable in the reconstituted solvent when extracts were stored at room temperature for at least 24 h (RSD ≤ 10.8%, RE in the range of −2.3% to −5.2%).

3.3.7. Application of the method to pharmacokinetic study of healthy volunteers

Following the single oral administration of picamilon tablets at doses of 50, 100, or 200 mg to healthy Chinese volunteers, the plasma concentrations of picamilon were successfully determined by the LC–MS/MS method described in Section 2. The main pharmacokinetic parameters are summarized in Table 2. The mean plasma concentration versus time curves of picamilon are shown in Fig. 3.

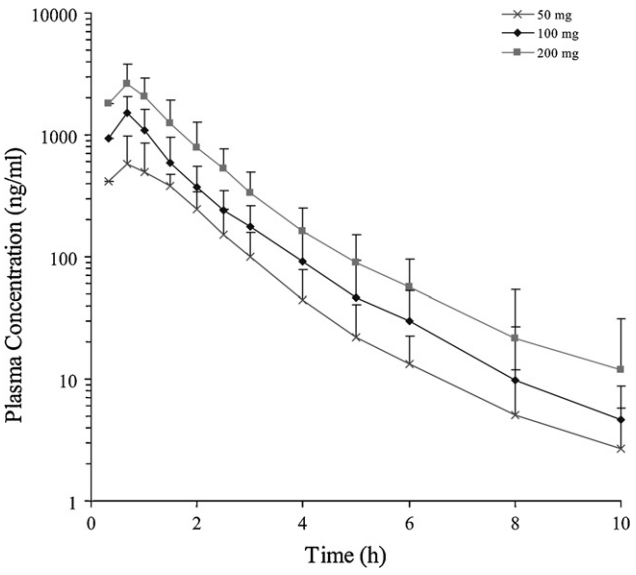

Fig. 3. Mean plasma concentration–time curves of picamilon after single oral doses of 50, 100, and 200 mg to 30 volunteers, respectively (mean ± standard deviation).

Over the range of picamilon doses studied, mean values of AUC and C_{max} increased in proportion to the doses, indicating that systemic exposure to picamilon increases linearly with the dose over the range of 50–200 mg. The values of CL/F and $t_{1/2}$ had no significant differences among the three groups.

4. Conclusion

An LC–MS/MS method was developed and validated for the quantification of picamilon in human plasma. The method offers the advantages of high selectivity and simple plasma sample preparation. It was successfully applied to the evaluation of the pharmacokinetics of picamilon in healthy Chinese volunteers, marking the first report of the pharmacokinetics of picamilon in humans. The pharmacokinetics of picamilon in healthy Chinese volunteers were linear over the range of single oral doses of 50–200 mg.

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