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Liquid chromatography–tandem mass spectrometry method for determination of phencynonate in rat blood and urine

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Abstract

A sensitive and specific high-performance liquid chromatographic assay with electrospray ionization mass spectrometry detection (LC-ESI-MS) has been developed and validated for the identification and quantification of the novel anticholinergic drug phencynonate in rat blood and urine. The sample pretreatment involves basification and iterative liquid–liquid extraction with ethyl ether–dichloromethane (2:1, v/v) solution, followed by LC separation and positive electrospray ionization mass spectrometry detection. The chromatography was on BetaBasic-18 column (150 mm × 2.1 mm i.d., 3 μm). The mobile phase was composed of methanol–water (85:15, v/v), containing 0.5‰ formic acid, which was pumped at a flow-rate of 0.2 ml/min. Thiencynonate was selected as the internal standard (IS). Simultaneous MS detection of phencynonate and IS was performed at *m*/*z* 358.4 (phencynonate), *m*/*z* 364 (thiencynonate), and the selected reaction ion monitoring (SRM) of the two compounds was at 156. Phencynonate eluted at approximately 5.25 min, thiencynonate eluted at approximately 5.10 min and no endogenous materials interfered with their measurement. Linearity was obtained over the concentration range of 1–100 ng/ml in rat blood and 1–500 ng/ml in rat urine. The lower limit of quantification (LLOQ) was reproducible at 1 ng/ml in both of rat blood and urine. The precision measured was obtained from 2.92 to 9.76% in rat blood and 4.17 to 9.76% in rat urine. Extraction recoveries were in the range of 69.57–79.49% in blood and 56.85–64.86% in urine. This method was successfully applied to the identification and quantification of phencynonate in pharmacokinetic studies.

Keywords: Phencynonate; Anticholinergic drug; Liquid chromatography–mass spectrometry; Rat blood; Quantification

1. Introduction

Phencynonate {*N*-methyl-9α-(3-azabicyclo[3,3,1] nonanyl)-2′-cyclopentyl-2′-hydroxyl- 2′-phenylacetate} is a novel anticholinergic drug developed by the Beijing Institute of Pharmacology and Toxicology in China. Pharmacological evaluation has proved that phencynonate prevents acute motion sickness with an efficacy similar to that of scopolamine [1–6]. Determination of the pharmacokinetic profile of phencynonate is important for gaining better understanding of its mechanism of action and ensuring more efficient therapeutic application. Because of the low therapeutic dose of phencynonate (2–4 mg/time for oral dose), a sensitive analytical method is needed for its determination in blood after oral administration. Liu et al. [7] has developed a gas chromatographic method with mass spectrometry to determination phencynonate in animal blood. Although the lower limit of quantification (LLOQ) of phencynonate in this method was low, the sample preparation and extraction and analytical procedure were time-consuming. No paper about LC–MS method for determining phencynonate in vivo has been published. The method reported here was validated to ensure proper quantification of phencynonate in rat blood and urine down to a concentration limit of 1 ng/ml in rat blood and urine. This method was sensitive and specific and can be applied for determining the low concentrations of phencynonate in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Phencynonate and *d*-thiencynonate {*N*-methyl-9α-(3-azabicyclo[3,3,1] nonanyl)-2′-cyclopentyl-2′-hydroxyl- 2′-thienyl-

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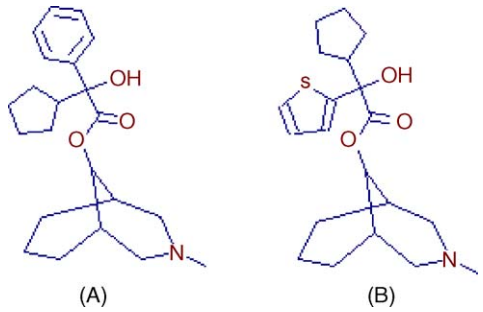

Fig. 1. The chemical structures of phencynonate (A) and the internal standard thiencynonate (B).

acetate}, the internal standard (IS), were kindly supplied by the Beijing Institute of Pharmacology and Toxicology of China. The purity of phencynonate and the IS was both more than 99% [8,9]. Their chemical structures are shown in Fig. 1. Methanol used was of HPLC grade and was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Formic acid was of HPLC grade and purchased from Dikma Reagent Company (Beijing, China). Water was triply distilled. The above solutions were filtered through 0.45-μm (organic) or 0.2-μm (water) membranes. All other reagents and chemicals were of analytical grade.

2.2. Instrumentation

An Agilent HPLC system (Series 1100, Agilent technology, Palo Alto, CA, USA) was used for analysis, which included a HP 1100 G1312A binary pump, G1379A vacuum degasser, G1313A ALS and G1315B diode-array detector. The chromatography was performed on a BetaBasic-18 column (150 mm × 2.1 mm i.d., 3 μm; Thermo Electron, CA, USA) at ambient temperature. A C_{18} guard column (13 mm × 4.6 mm i.d., Upchurch Scientific) was used to protect the analytical column. The mobile phase was composed of methanol and water (85:15, v/v), containing 0.5‰ formic acid at pH 6, which was pumped at a flow-rate of 0.2 ml/min. The sample injection volume was 10 μl and the run time of samples was 7 min. The effluent was on-line transferred to ESI-MS system without splitting.

Mass spectrometric measurements were performed on a LCQ Deca XP ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ion (ESI) source working in positive ion mode. The instrument was connected to the LC system outlet. Nitrogen was used as a sheath gas and ultra-high purity helium as the dampening gas in the ion-trap. MS detection of phencynonate and the IS was performed at *m/z* 358.40 (phencynonate) and *m/z* 364 (IS), and their selected reaction ion monitoring (SRM) was at *m/z* 156, which was used for quantification. ESI was operated at the sheath flow-rate of 35 psi; capillary temperature of 320 °C; capillary voltage of 18 V and skimmer voltage of 70 V. The collision-induced dissociation energy for the two compounds was 36%. The transitions of 358.4 → 156 and 358.6 → 342.2 for the analyte and 364 → 156 for the IS were monitored using an isolation width of 1.0 Da. The product ion *m/z* 156 was monitored because it had the most abundant and stable ion for both the analyte and the IS. The divert valve was programmed to waste the first 1 min and last 0.5 min. The LC system and mass spectrometer were controlled using the Thermo Finnigan Chemstation software (version 1.3). Data were processed using the IS method of plotting peak area ratios versus relative analyte/IS concentration with a weighting factor 1.

2.3. Materials

Male Sprague–Dawley rats, weighing 250 ± 20 g, were obtained from Animals Center of Capital University of Medical Sciences (CPUMS, Beijing, China). Pooled drug-free blood and urine were obtained from the healthy rats, after aliquoting, both blood and urine controls were stored at −20 °C and then thawed at room temperature for use in calibration curves and quality control samples.

2.4. Sample preparation

Stock solutions were prepared by dissolving 10 mg of phencynonate and 10 mg of the IS in 10 ml of HPLC-grade methanol. Working standard solution was prepared by serial diluting of the stock solution using the methanol. Quality control (QC) samples were also prepared in the same way, using a separately weighed stock solution. All solutions were stored at 4 °C before use.

2.5. Method validation

Calibration samples in blood were prepared by mixing solutions of phencynonate and IS with rat blank blood at a volume ratio of 100:100:100 (μl) to form a concentration series of 1, 2, 5, 10, 25, 50 and 100 ng/ml phencynonate and 50 ng/ml IS. Calibration samples in urine were prepared by mixing solutions of phencynonate and IS with rat blank urine at a volume ratio of 500:100:100 (μl) to form a concentration series of 1, 2, 10, 50, 100, 200 and 500 ng/ml phencynonate and 10 ng/ml IS. Precision and accuracy of the assay were determined by performing replicate analyses of QC samples against calibration standards. The precision and accuracy of the method were calculated as the relative standard deviation (R.S.D.) and the percentage deviation of observed concentration from theoretical concentration, respectively. The extraction recovery was determined by calculating the ratio of the amount of extracted compound from drug-free blood or urine spiked with known amounts of phencynonate to the amount of compound added at the same concentrations to mobile phase solution. The stability of the sample was also investigated by measuring QC samples: (1) allowed to stand at ambient temperature for at least 24 h before extraction; (2) allowed to stand at ambient temperature in the autosampler for at least 24 h after extraction; and (3) subjected to three freeze-thaw cycles for at least 7 days. An aliquot of the blank blood (100 μl) was also analyzed according to the method. The lower limit of detection (LLOD) was considered as 3 times the signal/noise ratio (S/N) and the LLOQ was 10 times the S/N ratio. The specificity of the method was characterized by assessing to accurate quantification of phencynonate in the presence of endogenous compound and the metabolites, confirmed by the analysis of blank and spiked quality control samples.

2.6. Extraction procedure

Blood samples (0.1 ml) were spiked with 100 μl each of phencynonate and 100 μl of IS stock solution (50 ng/ml). Then 100 μl of 0.2 mol/l NaOH and 2 ml of mixed solvent (ethyl ether–ichloromethane 2:1,v/v) were added. The combined samples were adjusted to pH 10, vortex -mixed for 1 min and centrifuged at 3000 × *g* for 10 min. The upper organic phase was extracted twice and combined. Then 0.2 ml of 0.1 mol/l HCl was added to the combined organic potions and the pH was adjusted to 2. The mixed system was vortex-mixed and centrifuged as above, and the upper organic layer was discarded. Then, 0.3 ml of 0.2 mol/l NaOH and 2 ml of the mixed solvent were added to the lower aqueous phase and the pH was adjusted to 10, which was vortex-mixed, centrifuged and separated twice. And the upper organic potions were combined and evaporated to dryness under nitrogen at 40 °C, the residue was dissolved in 100 μl of the LC mobile phase and transferred to HPLC autosampler vials, and aliquots (10 μl) of were injected into the LC–MS system.

Urine samples (0.5 ml) were spiked with 100 μl each of phencynonate and IS stock solution. The following procedure was the same as above.

2.7. Pharmacokinetic studies

The LC–MS method was successfully applied to pharmacokinetic studies of phencynonate in Sprague–Dawley rats. Rats were fasted for 12 h before the test, with water available ad libitum. Blood was collected from the orbital vein of the rats before and after receiving a single oral dose of phencynonate (0.7 mg/kg). Approximately 0.1 ml blood was collected in heparinized tubes before drug administration and post-dose at 1, 2, 5, 10, 30, 60, 120, 240 and 480 min, respectively. Other rats were housed in metabolic cages with access to food and water after phencynonate administration for collecting urine at 0–4 h, 4–8 h, 8–12 h, 12–24 h and 24–48 h post-dose. All blood and urine samples were sealed and stored at −20 °C until analysis. The blood and urine samples were extracted as above.

3. Results and discussion

3.1. Method development

Sample preparation plays a key role for determination of drugs in biological samples. After several trials, a reverse phase liquid–liquid extraction was found to be suitable for the determination of phencynonate in rat urine and blood. The method was adopted and proved to be reliable for sample preparation in this experiment. Methanol rather than acetonitrile was selected as protein-precipitation solvent for compatibility with the mobile phase to produce symmetric peak shapes for the analytes and IS. This procedure produced a clean chromatogram for urine and blood sample.

A BetaBasic-18 column was used for the chromatographic separation and a C_{18} guard column was used to protect the analytical column. Other chromatographic conditions, especially the composition of mobile phase, were optimized through several trails to obtain good resolution and symmetric peak shapes, as well as a short run time. It was found that a mixture of methanol and water (85:15, v/v) with 0.5% formic acid could achieve these goal and was finally adopted as the mobile phase for the chromatographic separation. An internal standard is necessary for the determination of analytes in biological samples. In initial stage of the experiment, several compounds were compared and screened, and thiencynonate, an analog of phencynonate, was found to be optimal for a IS.

For the quantification of phencynonate in animal blood and urine, some parameters related to mass spectrometric detection were investigated. ESI was adopted to quantify phencynonate in rat blood and urine due to its lower levels of background noise. The capillary temperature, vaporizer temperature and flow-rate were optimized to obtain protonated molecules of the analytes. The fragment energy was optimized to achieve maximum response of the fragment ion peaks. Selected reaction ion monitoring in positive mode was used for the quantitation of both phencynonate and thiencynonate at *m*/*z* 156. Two detection channels were adopted, channel 1 (MSD 1) for internal standard and channel 2 (MSD 2) for phencynonate.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The retention times were 5.25 min for phencynonate and 5.10 min for IS. The quasi-molecular ions for quantitative determination of the analytes were at *m*/*z* 156 for both phencynonate and for IS. Fig. 2 shows that no endogenous substances in rat blood and urine interfered with the analytes or IS.

3.3. Linearity

To evaluate the linearity of the LC-ESI-MS method, the calibration curves of blood and urine were determined in triplicate on three separate days. The r^2 values for the three calibration curves were >0.9992 in rat blood and >0.9976 in rat urine. The mean equations for the calibration curves for phencynonate were $y = -(0.0355 \pm 0.0306) + (0.0338 \pm 0.0071)x$ in blood and $y = -(1.5973 \pm 0.6183) + (0.2014 \pm 0.034)x$ in urine. The assay proved to be linear and acceptable. Good linearity was observed over the concentration ranges of 1–100 ng/ml for blood and 1–500 ng/ml for urine.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed in both blood and urine by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standard series. The within-day and between-day precision and accuracy of the method are presented in Table 1. The data indicate that the precision and accuracy of the method are acceptable.

Sensitivity was evaluated by determining the LLOQ, which is defined as the lowest concentration that can be reliably and reproducibly measured in at least five replicates. To determine

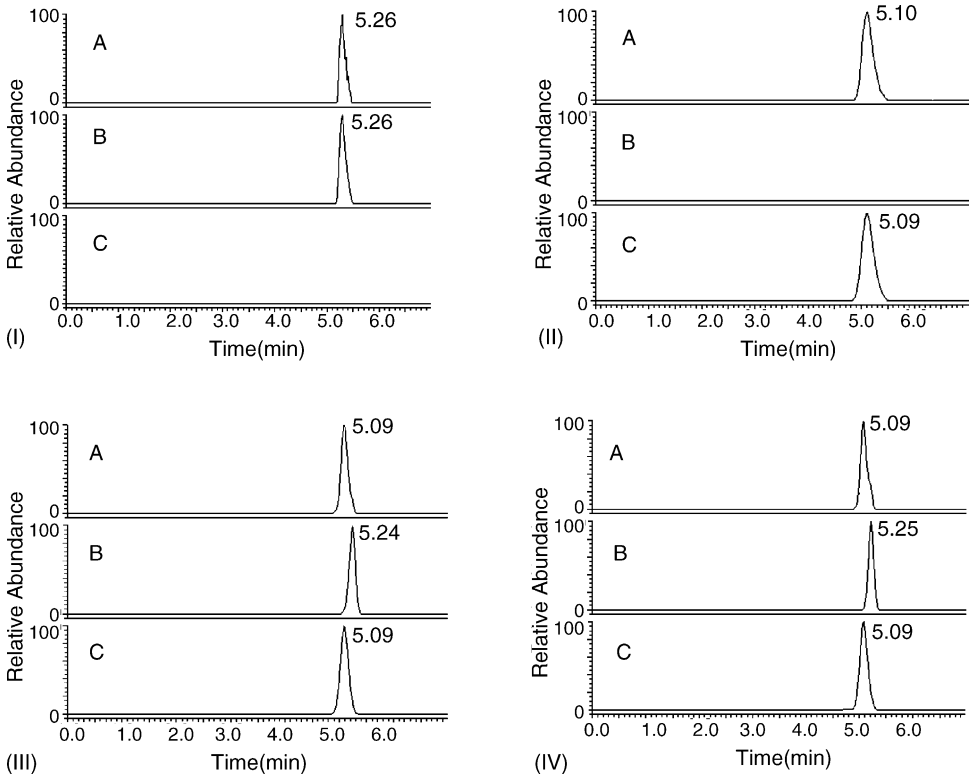

Fig. 2. LC–MS chromatogram of phencynonate solution (I), IS solution (II), phencynonate and IS isolated from rat blood (III) and phencynonate and IS isolated from rat urine (IV). (A) Total ion current. (B) SRM of m/z 358.40 → 156 for phencynonate. (C) SRM of m/z 364 → 156 for IS.

the LLOQ, pooled blood and urine samples were spiked to contain 1 ng/ml phencynonate and were analyzed on five different days. The phencynonate peak had to be distinct from noise peaks and for verification of LLOQ; the peak area in chromatograms for spiked blood and urine samples containing 1 ng/ml phencynonate was compared with the noise signal. The LLOQ had to have precision of ≤20% and a signal/noise ratio ≥10.

3.5. Recovery

The extraction recovery was determined for five replicates of rat blood and urine spiked with low, medium and high concentrations of phencynonate. The results are summarized in Table 2. The data indicate that the recovery of phencynonate from rat blood and urine was concentration-independent in the concentration range evaluated.

Table 1
Precision and accuracy of the LC–MS/MS analysis of phencynonate

Matrix	Theoretical concentration (ng/ml)	n	Experimental concentration (ng/ml)	Precision (R.S.D., %)	Accuracy percent error (%)
Blood	Within-day				
	2	5	1.954 ± 0.187	9.59	2.30
	10	5	10.49 ± 0.456	4.34	4.98
	50	5	50.11 ± 1.464	2.92	−0.22
	Between-day				
	2	5	2.301 ± 0.22	9.76	−15.05
	10	5	10.65 ± 0.550	5.13	−6.49
	50	5	49.62 ± 1.609	3.24	0.76
Urine	Within-day				
	1	5	0.996 ± 0.041	4.17	0.40
	25	5	25.69 ± 1.363	5.30	−2.76
	500	5	524.77 ± 22.46	4.28	−4.95
	Between-day				
	1	15	0.963 ± 0.042	4.36	3.70
	25	15	26.56 ± 2.592	9.76	−6.24
	500	15	532.42 ± 32.75	6.15	−6.48

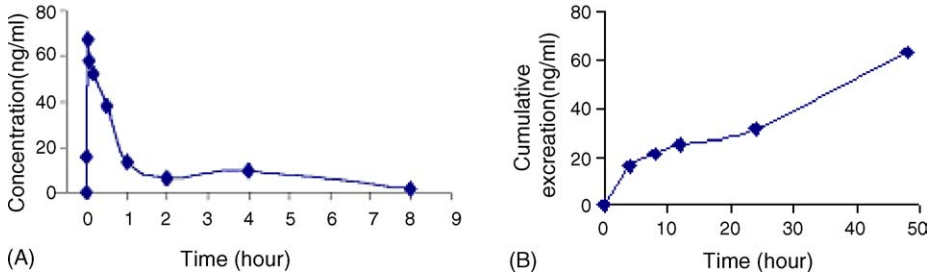

Fig. 3. Pharmacokinetic profile of phencynonate in blood (A) and urine (B) following administration of a single oral dose (0.7 mg/kg) to rats.

Table 2
Recovery of phencynonate

Treatment	Added (ng/ml)	Mean ± S.D. (%)	R.S.D. (%)
Blood	2	79.05 ± 11.43	14.45
	10	69.57 ± 8.98	12.91
	50	70.49 ± 6.56	9.31
Urine	1	56.85 ± 3.79	6.68
	25	64.86 ± 4.54	7.01
	500	62.49 ± 5.32	8.52

Note: $n = 5$ for each concentration.

3.6. Stability

The stability of stock and standard solution kept at 20 °C and frozen (−20 °C) blood and urine samples, as well as frozen blood and urine extracts, was checked. Blood QC samples were: (1) allowed to stand at ambient temperature for at least 24 h before extraction; (2) allowed to stand at ambient temperature in the autosampler for at least 24 h after extraction; and (3) subjected to three freeze-thaw cycles for at least 7 days. Analysis of these samples consistently afforded values that were nearly identical to those for freshly prepared QC samples, thus confirming the overall stability of phencynonate in both matrices under frozen storage, assay processing and freeze-thaw conditions.

3.7. Pharmacokinetics of phencynonate

The LC–MS method showed satisfactory results for the determination of phencynonate in rat blood and urine and was successfully used for the pharmacokinetic study of the novel drug following oral administration to rats. The mean blood and urine concentration–time profiles for phencynonate are shown in Fig. 3. The mean value of the maximum blood concentration (C_{max}) was 67.9 ng/ml at approximate 5 min (t_{max}, the time to reach C_{max}) post-dosing.

4. Conclusion

A LC–MS method has been developed for the determination of phencynonate in rat blood and urine by our laboratory. The performance criteria for specificity, precision, accuracy, recovery, sensitivity, linearity and stability have been assessed and were within the FDA recommended guidelines, indicating that the method can be used for determination of phencynonate in rat blood and urine. The method is currently being used to further pharmacokinetic studies.

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