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Determination of thiencynonate by liquid chromatographic–mass spectrometry and its application to pharmacokinetics in rats

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Abstract

A sensitive and specific high-performance liquid chromatography–tandem mass spectrometry method (LC/ESI/MS) was developed and validated for the identification and quantification of the novel lead compound of anticholinergic drug thiencynonate in rat plasma. The analytes were determined using positive electrospray ionization mass spectrometry in the selected reaction ion monitoring (SRM). The chromatography separation was on BetaBasic-18 column (150 mm × 2.1 mm i.d., 3 μm). The mobile phase was composed of methanol–water (70:30, v/v), containing 0.5‰ formic acid, which was pumped at a flow rate of 0.2 ml/min. Phencynonate was selected as the internal standard (IS). Simultaneous MS detection of thiencynonate and IS was performed at *m/z* 364.4 (thiencynonate), *m/z* 358 (phencynonate), and the SRM of the two compounds were both at 156. Thiencynonate eluted at approximately 2.8 min, phencynonate eluted at approximately 2.9 min and no endogenous materials interfered with their measurement. Linearity was obtained over the concentration range of 1–100 ng/ml in rat plasma. The lower limit of quantification (LLOQ) was reproducible at 1 ng/ml in rat plasma. The precision measured was obtained from 2.47 to 9.28% in rat plasma. Extraction recoveries were in the range of 67.63–76.76% in plasma. This method was successfully applied to the identification and quantification of thiencynonate in pharmacokinetic studies.

Keywords: Thiencynonate; Anticholinergic drug; Lead compound; Liquid chromatography–mass spectrometry; Quantification; Pharmacokinetics

1. Introduction

Thiencynonate{*N*-methyl-9α-(3-azabicyclo[3,3,1]nonanyl)-2′-cyclopentyl-2′-hydroxyl-2′-thienylacetate} is the lead compound of a novel anticholinergic agent, which was synthesized by the Beijing Institute of Pharmacology and Toxicology in China. Thiencynonate is similar in structure to phencynonate which is a novel anticholinergic agent [1–6]. Pharmacological evaluation proved that thiencynonate has potent effects of sedative and hypnotics in coordination with sub-threshold dose of pentobarbital, inhibiting tracheobronchial contractile response of guinea pig and salivation induced oxotremorine [5–7]. Determination of the pharmacokinetic profile of thiencynonate is important for going better understanding of its pharmaceutical action and ensuring more efficient therapeutic application. Because of the low therapeutic dose of thiencynonate, a sensitive analytical method is needed for its determination in plasma after administration. No paper about the method of determining thiencynonate in vivo or in vitro has been published. This method reported here was firstly validated to ensure proper quantification of thiencynonate in rat plasma down to a concentration limit of 1 ng/ml. The method was sensitive and specific and can be applied for determining the low concentrations of thiencynonate in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Thiencynonate and phencynonate {*N*-methyl-9α-(3-azabicyclo[3,3,1]nonanyl)-2′-cyclopentyl-2′-hydroxyl-2′-phenylacetate}, the internal standard (IS), were kindly supplied by the

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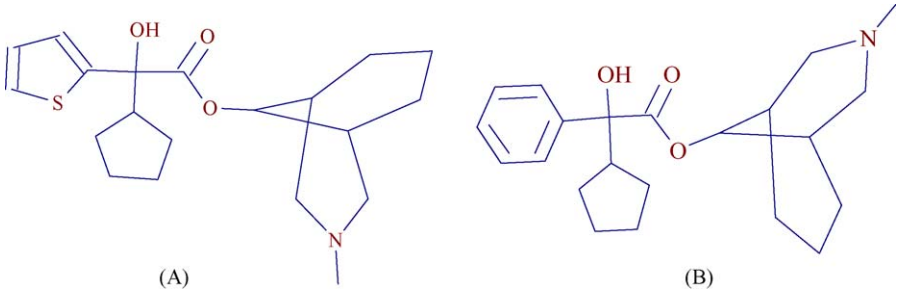

Fig. 1. The chemical structures of thiencynonate (A) and the internal standard phencynonate (B).

Beijing institute of Pharmacology and Toxicology of China. The purity of thiencynonate and the IS was both more than 99% [8–10]. Their chemical structures are shown in Fig. 1. Methanol (HPLC grade) was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Formic acid (HPLC grade) was purchased from Dikma Reagent Company (Beijing, China). Water was triply distilled. All other reagents and chemicals were of the highest quality available.

2.2. Instrumentation

The LC/ESI/MS system consisted of a HPLC system (Series 1100, Agilent technology, Palo Alto, CA, USA) including a HP 1100 binary pump, a vacuum degasser, and an autosampler and coupled to Finngian LCQ Deca XP ion-trap spectrometer equipped with electrospray source (Thermo Finnigan, San jose CA, USA). The LC/ESI/MS system was controlled by Xcalibur® (version 1.3) software. The LC separation was performed on a BetaMax Acid C_{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 (70:30, v/v), containing 0.5‰ formic acid, 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 6 min. The effluent was on-line transferred to ESI–MS system without splitting.

The compound-dependent parameters were also tuned for the target compound to achieve the highest instrument response. A positive ion mode was selected to get higher sensitivity than that in negative mode. Nitrogen was used as a sheath gas and an aux/sweep gas in the ion trap. MS detection of thiencynonate and the IS was performed at *m/z* 364.40 (thiencynonate) and *m/z* 358 (IS), and their selected reaction ion monitoring (SRM) was both 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 *m/z* 364.2 → 156 for the analyte and *m/z* 358.4 → 156 for the IS were monitored using an isolation width of 1.0 Da. The divert valve was programmed to waste the first 1 min and last 1 min.

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 plasma was obtained from the healthy rats, after aliquoting, plasma controls were stored at −20 °C and then thawed at room temperature for use in calibration curves and quality control (QC) samples.

2.4. Preparation of sample

Stock solutions of thiencynonate and the IS were prepared by dissolving 10 mg in 10 ml of HPLC grade methanol, respectively. The working solutions were prepared by serial diluting of the stock solution with methanol. Quality control samples were also prepared in the same way, using a separately weighed stock solution. All solutions were stored at 4 °C prior to use.

2.5. Method validation

Calibration samples in plasma were prepared by mixing solutions of thiencynonate and IS with rat blank plasma at a volume ratio of 100:100:100 (μl) to form a concentration series of 1, 5, 10, 25, 50 and 100 ng/ml thiencynonate and 50 ng/ml IS. Three QC samples prepared at nominal concentration of 1.6, 5, 25 and 100 ng/ml validated the method presented here to determine the intra-day and inter-day precision and accuracy. Quantification was based on the IS method of plotting peak areas ratios of analyte/IS versus the concentration of the samples with a weighting factor 1. 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. The extraction recovery was determined by calculating the ratio of the amount of extracted compound from blank plasma spiked with known amounts of thiencynonate 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 5 days. An aliquot of the blank plasma (100 μl) was also analyzed according to the method. The lower limit of detection (LLOD) was defined as the lowest concen-

tration at which the analytical assay can reliably differentiate analyte LC peaks from background levels (S/N > 3). The lower limit of quantification (LLOQ) was 10 times the S/N ratio. The specificity of the assay to quantify thiencynonate accurately in the presence of endogenous compounds and its metabolites was confirmed through the analysis of blanks and spiked quality control samples, respectively.

2.6. Extraction procedure

Plasma samples (0.1 ml) were spiked with 100 μl each of thiencynonate and 100 μl of IS stock solution. Then 100 μl of 0.2 mol/l NaOH and 2 ml of mixed solvent (ethyl ether-dichloromethane 2:1, v/v) were added. The combined samples were adjusted to pH 9, vortex-mixed for 1 min and centrifuged at 3000 × *g* for 10 min. The upper organic layer was transferred to another tube. The plasma sample was extracted twice and the organic layer was combined. The mixture was dried under a flow of nitrogen gas at 40 °C. The residue was reconstituted in 100 μl of the LC mobile phase and transferred to HPLC autosampler vials, a volume of 10 μl of was introduced into the LC–MS system.

2.7. Pharmacokinetic studies

The LC/MS/MS procedure was successfully applied to investigate the plasma concentration–time profile of thiencynonate in Sprague–Dawley rats. Rats were fasted for 12 h before the test, with water available ad libitum. Plasma was collected from the orbital vein of the rats before and after receiving a single oral dose of thiencynonate (0.35 mg/kg). Approximately 0.1 ml plasma was collected in heparinized tubes before drug administration and post-dosing at 2, 5, 10, 20, 30, 60, 120, 240, 480 and 720 min, respectively. All plasma samples were sealed and stored at −20 °C until analysis. The plasma samples were extracted as Section 2.6. The pharmacokinetic parameters of thiencynonate in rats were calculated by 3P87 program of pharmacokinetics (Beijing, China).

3. Results and discussion

3.1. Method development

Sample preparation plays a key role for determination of drugs in biological samples. Liquid–liquid extraction was necessary and important because the method cannot only purify but also concentrate the samples. After several trials, a basification and liquid–liquid extraction was found to be suitable for the determination of thiencynonate in rat plasma. 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 plasma sample. A BetaMax Acid C_{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 (70:30, v/v) with 0.5‰ formic acid could achieve these goals 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 phencynonate, an analog of thiencynonate, was found to be optimal for a IS. For the quantification of thiencynonate in animal plasma, some parameters related to mass spectrometric detection were investigated. ESI was adopted to quantify thiencynonate in rat plasma 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 thiencynonate and phencynonate at *m/z* 156. Two detection channels were adopted, channel 1 (MSD 1) for internal standard and channel 2 (MSD 2) for thiencynonate.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The retention times were 2.84 min for thiencynonate and 2.94 min for IS. The quasi-molecular ions for quantitative determination of the analytes were at *m/z* 156 for both thiencynonate and for IS. Fig. 2 includes five parts: a LC/MS/MS chromatogram of a rat plasma single blank extracts (I), showing lack of interference of plasma components; a rat plasma single blank extract (spiked with IS) (II); a rat plasma single blank extract (spiked with thiencynonate) (III), (II) and (III) demonstrate lack of cross-talk; a chromatogram of a rat plasma double extract (to demonstrate selectivity) (IV), showing the thiencynonate and IS SRM channels; and a rat plasma extract spiked at the LLOQ (1 ng/ml) (V).

3.3. Linearity

To evaluate the linearity of the LC–ESI–MS method, the calibration curves of plasma were determined in triplicate on three separate days. The coefficient of correlation (r^2) for the three calibration curves were >0.9973 and 0.9968 in rat plasma. Calibration graphs were constructed using a linear regression of test compound/IS peak area ratio (y) to nominal plasma concentration of the test compound (x, ng/ml). The mean equations for the calibration curves for thiencynonate were $y = -(0.1314 \pm 0.1730) + (0.0343 \pm 0.0225)x$ in the ranges of 5–100 ng/ml and $y = -(0.01169 \pm 0.00155) + (0.0201 \pm 0.00374)x$ in the ranges of 0.8–5 ng/ml. The assay proved to be linear and acceptable.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed in plasma 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 intra-day and inter-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 the LLOQ, blank plasma samples were spiked to contain 1 ng/ml thiencynonate and were analyzed on five different days. The thiencynonate peak had to be distinct from noise peaks and for verification of LLOQ; the peak area in chromatograms for spiked plasma sam-

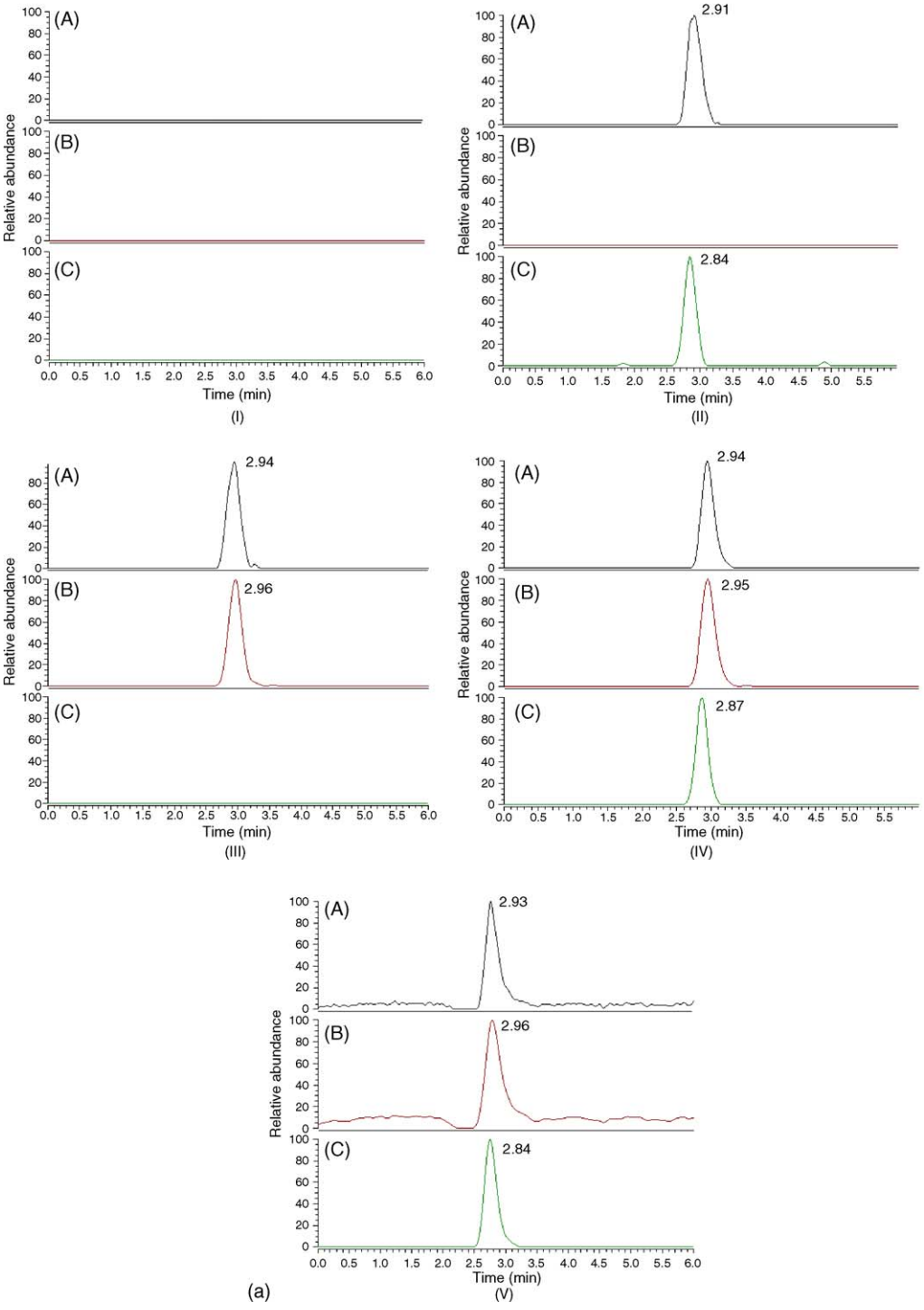

Fig. 2. (a) LC/MS Chromatogram of rat blank plasma (I); blank plasma (spiked with IS 50 ng/ml) (II); blank plasma (spiked with thiencynonate 10 ng/ml) (III); blank plasma (spiked with thiencynonate 10 ng/ml and IS 50 ng/ml) (IV); and a rat plasma extract spiked at the LLOQ (1 ng/ml) (V). (A) Total ion current. (B) SRM of m/z 358.4 → 156 for IS. (C) SRM of m/z 364.2 → 156 for thiencynonate. (b) LC–MS chromatogram of thiencynonate and IS solution (I); thiencynonate and IS isolated from rat plasma (II). (A) Total ion current. (B) SRM of m/z 358.4 → 156 for IS. (C) SRM of m/z 364.2 → 156 for thiencynonate.

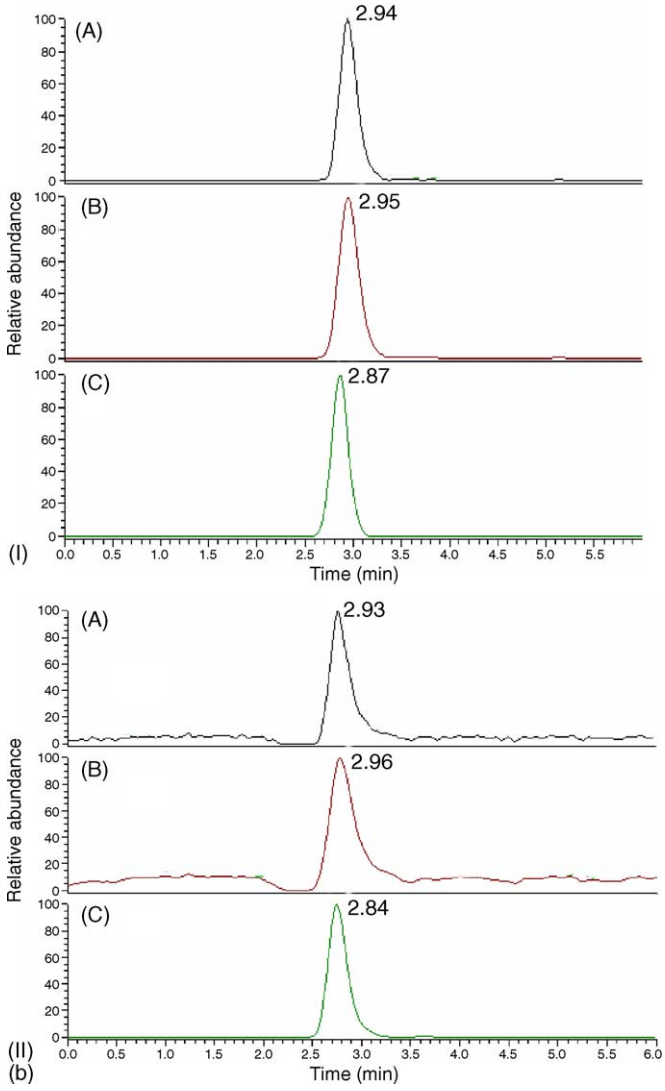

Fig. 2. (*Continued*).

ples containing 1 ng/ml thiencynonate was compared with the noise signal. The LLOQ had to have precision of ≤14% and a signal/noise ratio ≥10. Although the matrix effects are common within the retention time from 1.0 to 2.0 in LC–MS/MS analysis, the ratio of the response values for thiencynonate and IS in matrix (extraction from plasma) versus in solution are 93.2 and 93.8 and the evaluation results of matrix effect in our test are affected weakly.

Table 1
Precision and accuracy of the LC/MS analysis of thiencynonate

Matrix	Theoretical concentration (ng/ml)	*n*	Experimental concentration (ng/ml)	Precision (R.S.D.,%)	Accuracy percent error (%)
Intra-day					
	1.6	5	1.66 ± 0.23	13.93	3.75
	5	5	5.07 ± 0.43	8.42	1.40
	25	5	25.3 ± 1.23	4.85	1.12
	100	5	101.8 ± 9.45	9.28	1.82
Inter-day					
	1.6	5	1.66 ± 0.15	9.16	3.75
	5	5	4.85 ± 0.39	6.74	−3.00
	25	5	25.9 ± 1.55	5.99	3.52
	100	5	104.5 ± 2.58	2.47	4.50

Table 2
Recovery of thiencynonate

Treatment	Added (ng/ml)	Recovery ± S.D. (%)	R.S.D. (%)
Plasma (*n* = 5)	1.6	65.77 ± 9.36	14.23
	5	67.63 ± 9.73	14.39
	25	69.93 ± 7.21	10.31
	100	76.76 ± 8.20	10.68

3.5. Recovery

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

In the test, the ionization suppression was assessed by injecting rat plasma extracts while infusing drug at 1.6 and 25 ng/ml QC level into the MS and comparing the response to a neat QC at the same level. It existed ionization suppression and the low recovery is partially due to ionization suppression.

3.6. Stability

The stability of stock and standard solution kept at 20 °C and frozen (−20 °C) plasma samples, as well as frozen plasma extracts, was checked. Plasma 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 5 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 thiencynonate in both matrices under frozen storage, assay processing and freeze-thaw conditions.

3.7. Pharmacokinetics of thiencynonate

The LC/MS/MS method showed satisfactory results for the determination of thiencynonate in rat plasma and was successfully used for the pharmacokinetic study of the novel compound following oral administration to rats. The plasma concentration–time profiles for thiencynonate are shown in Fig. 3. The concentration–time profile of thiencynonate was all best fitted to first order absorption two-compartment open model after administrating a single oral dose (0.35 mg/kg) in rats. The mean value of the maximum plasma concentration (C_{max}) was 23.5 ng/ml at approximate 2 min (t_{max}, the time to reach C_{max}) post-dosing. The main pharmacokinetic parameters are presented in Table 3. It shown that thiencynonate absorbed and distributed and eliminated fast and the level of the plasma concentration and distribution were higher relatively. It provided

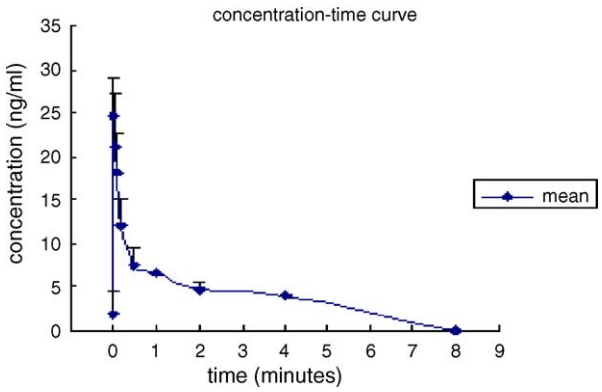

Fig. 3. Pharmacokinetic profile of thiencynonate in plasma following administration of a single oral dose (0.35 mg/kg) to rats.

Table 3
Pharmacokinetic parameters of thiencynonate after administrating a single oral dose (0.35 mg/kg) to rats

Parameter	Unit	($\bar{x} \pm s$)
$T_{1/2ka}$	Hour	0.0158 ± 0.0157
$T_{1/2\alpha}$	Hour	0.0991 ± 0.1058
$T_{1/2\beta}$	Hour	2.81 ± 0.157
V_d	(mg/kg)/(ng/ml)	0.0117 ± 0.0093
$CL_{(s)}$	mg/kg/h/(ng/ml)	0.0101 ± 0.0004
AUC	(ng/ml) h	34.8 ± 1.39
T_{peak}	Hour	0.0325 ± 0.0056
C_{max}	ng/ml	23.5 ± 8.06

$n = 6$, mean ± S.D.

important information for developing a novel anticholinergic drug and the clinical use of thiencynonate.

4. Conclusion

A LC/MS/MS method has been developed for the determination of thiencynonate in rat plasma 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 thiencynonate in rat plasma. It was successfully applied to the pharmacokinetic study of thiencynonate after oral administration. The pharmacokinetic investigation provided important information for developing a novel anticholinergic drug and the clinical use of thiencynonate.

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