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Quantification Of Atropine In Rat Plasma By Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

An analytical method based on liquid chromatography coupled with electrospray ionization (ESI) ion trap tandem mass spectrometry detection has been developed and validated for the determination of atropine in rat plasma using anisodamine as the internal standard in selected reaction mornitoring (SRM) mode. The standard curve was linear in the range of 5 to 5000 ng/mL. The assay was specific, accurate, precise and reproducible. The method was employed in a pharmacokinetic study after a single oral gavage dose of 50 mg/kg atropine to six rats.

KEYWORDS

Atropine;
LC-MS-MS;
Quantification;
Plasma;
Pharmacokinetics.

INTRODUCTION

Atropine (DL-hyoscyamine), a tropane alkaloid of medicinal interest, was found in plants of the Solanaceae family[1,2], such as atropa, datura, duboisia and hyoscyamus. Atropine has been widely utilized in clinic for many years as anticholinergic agents in premedication of anesthesia[3]. Currently, atropine is used for its antispasmodic activity on the gastrointestinal tract, as a preanesthesic agent and in ophthalmic solutions.

A great variety of methods have been developed for the quantitative determination of atropine in plants and pharmaceutical samples[4]. However, only a few papers described the methods used for quantification of atropine in biological materials including radioimmunoassay[5], high performance liquid chromatography (HPLC)[6], gas chromatography (GC)[7], gas chromatography tandem mass spectrometry (GC-MS)[8]. Those methods have various limits such as low sensitivity and specificity, time-consuming, large amount of samples and complex sample clean-up procedure. So the methodological improvements for the rapid and quantitative analyses of atropine in biological fluids are still desirable.

The LC–MS technique takes the advantage of

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the efficient separability of LC and the high selectivity and sensitivity of MS/MS and become one of the powerful analytical tools nowadays. Here a more simply, rapid and sensitive LC–MS method with electrospray ionization (ESI) was presented to determine atropine in rat plasma using one-step protein precipitation, compared with the existing methods. It was successfully applied to a pharmacokinetic study after an oral administration of atropine to six rats.

EXPERIMENTAL

Chemicals

Atropine was purchased from Sigma (St. Louis, MO, USA). Anisodamine hydrobromine was purcha sed from National Institute for the control of pharm aceutical and biological product (P.R.China). Methanol was of HPLC grade (Fisher Chemical Co., Inc, CA, USA); water was deionized and double distilled; ammonium acetate, formic acid and all other reagents were of analytical reagent grade.

Instrumentation

A LCQ Duo quadrupole ion trap mass spectrometer (ThermoFinnigan, Corp, San Jose, USA) equi pped with a modern TSP4000 HPLC pump and a TSP AS3000 autosampler were used for LC-MS-MS analysis in positive ion detection mode. Data acquisition was performed with Xcalibur version 1.2 software (Finnigan). A Laboratory refrigerated centrifuge (2K15C, Sigma, USA) was used to centrifuge extracted plasma samples.

LC-MS-MS analysis

The MS/MS analyses were performed under automatic gain control conditions, using a typical source spray voltage of 4.5 kV, a capillary voltage of 13V and a heated capillary temperature of 175°C. Nitrogen was used as the sheath gas (40 arbitrary units), the CID energy was 30%. The other parameters, including the voltages of octapole offset and tube lens offset, were also optimised for maximum abundance of the ions of interest by the automatic tune procedure of the instrument.

The chromatographic separation was performed on a reversed-phase column (ZORBAX Eclipse XDB-C18, 2.1×150 mm I.D., particle size 5 μm, Agilent), which was connected with a guard column (cartridge 4.6×12.5 mm, 5μm Agilent). The mobile phase was consisted of methanol and 2 mmol/l ammonium acetate solution (adjusted pH to 3.5 with formic acid) (70:30, v/v), which was eluted at a flow rate of 0.2 ml/min. The temperature of the column was set at 40°C .

Data acquisition and analysis

Data acquisition and analysis were performed using the Xcalibr 1.2 software (Finnigan, San Jose, CA, USA). Post-acquisition quantitative analyses were performed using LCQuan software (Finnigan, San Jose, CA, USA). The calibration curves were constructed from the peak area ratios of each analyte to the IS versus plasma concentrations using a $1/x^2$ weighted linear least-squares regression model. Concentrations of each analyte in quality control samples (QCs) or unknown samples were subsequently interpolated from these calibration curves.

Preparation of calibration standards and quality control (QC) samples

The stock solution of atropine (5 mg/ml) was prepared in methanol and serially diluted to produce a 50 μg/ml stock solution, which was serially diluted with methanol to give working solutions at concentration of 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml. A 500 ng/ml solution of the internal standard, anisodamine, was similarly prepared in methanol. All the solutions were stored at 4°C and were brought to room temperature before use.

Calibration standards and QC samples of atropine were prepared by spiking 100 μl of the working solutions and 10 μl of anisodamine to l00 μl of drug-free rat plasma. Calibration standards were prepared at concentrations of 5, 10, 50, 100, 500, 1000 and 5000 ng/ml of atropine in plasma, while QC samples were prepared at 20, 200, and 2000 μg/ml, and then treated as indicated below.

Sample treatment

Atropine was determined after plasma protein precipitation, which was performed by adding 900

μl of methanol to 100 μl of plasma following the addition of 10 μl of anisodamine (IS). Subsequently, the mixture was vortex-mixed for 30 s, followed by centrifugation for 10 min at 4000 × g, and 20 μl of clear supernatant was directly injected onto the LC-MS/MS system.

Method validation

Plasma samples were quantified using the ratio of the peak area of atropine to that of IS as the assay parameter. Peak area ratios were plotted against concentrations and atropine concentrations were calculated using a weighted ($1/x^2$) least squares linear regression. During prestudy validation, plasma standard curves were prepared and assayed in triplicate on three separate days.

The linearity of the standard curves was evaluated through least-squares linear regression analysis of peak area ratios of atropine/IS versus atropine concentrations in spiked plasma samples. Accuracy and precision were also assessed by determining QC samples at three concentration levels (see TABLE 1, six samples each) on three different validation days. The accuracy was expressed by (mean observed concentration—spiked concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.). During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns. Absolute recoveries of atropine at three QC levels and IS were determined by assaying the samples as described above and comparing the peak areas of both atropine and IS with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma. The stability of atropine in the reconstituted solution obtained by protein precipitation procedure was assessed by placing QC samples at three concentrations under ambient conditions for 24 h.

Application of the method

Adult wistar rats (200 ± 20g, Hubei Experimental Animal Research Center, China) were fasted for 24 h and administered atropine (dissolved in water) by a single oral gavage dose of 50 mg/kg to six rats, respectively. Blood samples (0.3 ml) were collected into heparinized tubes from each rat by the puncture of the retro-orbital sinus. This was performed at 0.083, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 6.0, 9.0 and 12h after administration. Blood was shaked 1 min and processed for plasma by centrifugation at 3000 × g for 10 min. Plasma samples were frozen and maintained at -70°C refrigerator until analysis.

RESULTS

Mass spectrometry

The typical mass spectra of atropine and anisodamine are showed in figure 1. Each MS spectrum revealed a base peak at m/z 290 and 306, respectively, corresponding to the molecular ion $[M+H]^+$. The MS/MS spectra were operated by choosing the two molecular ions as the parent ions. Some characteristic m/z peaks and those possiable fragmentation pathway were showed in figure 1II. The main daughter ions are m/z 124 for atropine (Figure 1IIA) and m/z 140 for anisodamine (Figure 1IIB). The quantitation of atropine was based on selected reaction monitoring (SRM). The fragment transitions for the SRM were m/z 290→124 for atropine, and m/z 306→140 for anisodamine.

Chromatography

To achieve maximum peak response and symmetric chromatographic peaks, the mobile phase containing varying percentages of organic phase, pH modifiers and column temperature was tested. As a result, methanol and 2 mmol/l ammonium acetate solution (aqueous, 70:30, v/v, adjusted pH to 3.5 with formic acid) was applied through a RP-C18 column (ZORBAX Eclipse XDB-C18, 2.1×150 mm I.D., particle size 5 μm Agilent). The presence of a low amount of ammonia acetate and formic acid in the mobile phase can improve the mass response of anisodamine and anisodamine in positive ion mode, and the shape of chromatograms. The elution rate was 0.2 ml/min. The temperature of the column was set at 40°C. figure 2 shows plasma blank and spiked blank as well as a real plasma sample. Typical retention times for atropine and the internal standard were 2.01 and 1.99 min, respectively.

Assay specificity

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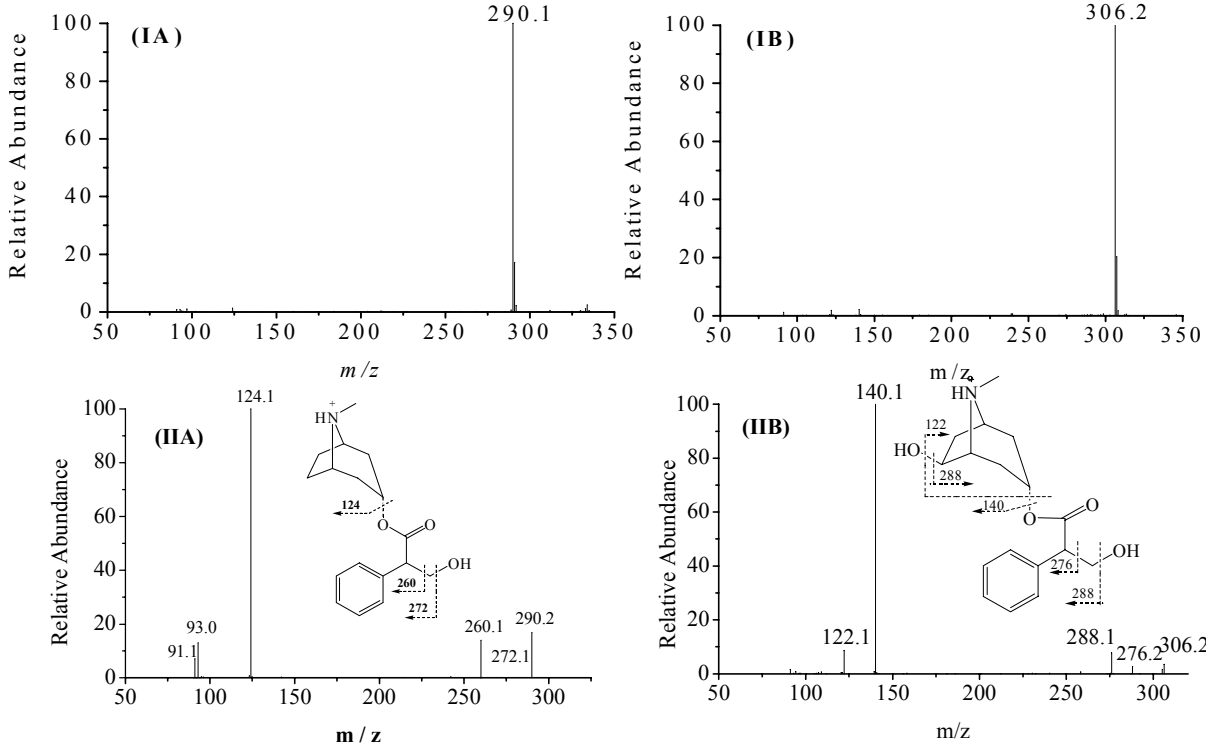

Figure 1: Full scan mass spectra of atropine (IA) and atropine (IB) in positive mode; Full scan product ion mass spectra of the molecular ions of atropine (IIA) and atropine (IIB)

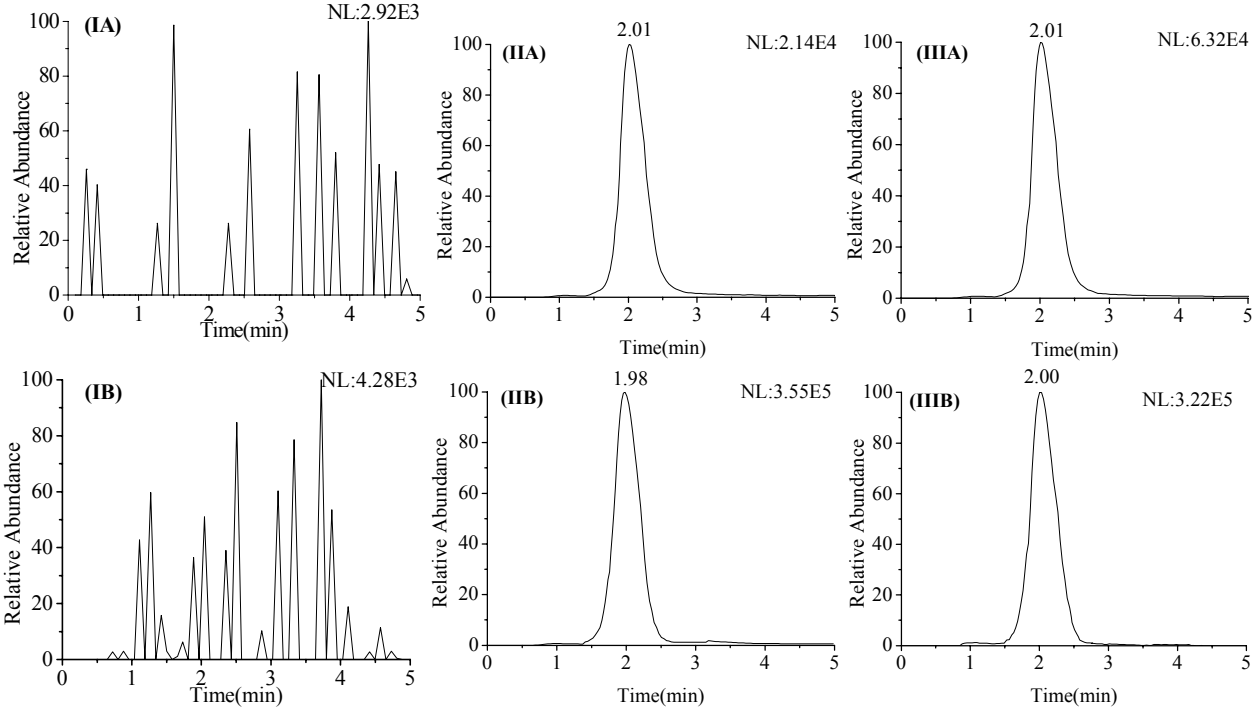

Figure 2: SRM chromatograms (A: m/z 290→124; B: m/z 320→140) for (I) drug-free plasma, (II) plasma spiked with 5 ng/mL of atropine and 5μg/mL anisodamine and (III) a plasma sample (14.8ng/mL) 9 h after the oral gavage administration to a rat with 50 mg/kg of atropine

The LC–MS/MS method has high specificity because only ions derived from the analytes of interest are monitored. SRM chromatograms of atropine and anisodamine for a drug-free plasma sample, a plasma sample spiked with 5 ng/ml of atropine (the LLQ) and 500 ng/ml of anisodamine and a plasma sample (14.8 ng/ml) 9 h after oral gavage administration of 50 mg/kg of atropine spiked with 500 ng/ml of anisodamine are shown in figure 2. No significant peaks interfering with atropine or the internal standard were observed in the drug-free rat plasma.

Validation

The calibration graph for atropine was obtained by SRM of increasing amounts of atropine spiked in blank serum samples with a constant level of atropine as IS. A linear calibration graph was constructed using least-squares regression of quantities versus peak area ratio. A good linear response over the range of 5 ng/ml to 5000 ng/ml was demonstrated in rat plasma. Typical stanard curve was y=0.0265207+ 0.0090222x, R=0.9997 W:1/x^2, where y is the ratio of the peak area of atropine to IS', x is the analyte concentration.

The extraction recoveries of atropine at three QC levels mentioned above, calculated by (mean response of each concentration extracted from rat plasma/corresponding mean response of each concentration in mobile phase without extracted), were 72.6%, 70.8% and 70.8%, respectively (n=6). The extraction recovery of IS was 75.3% (R.S.D=1.7%).

The assay performance for the determination of atropine is shown in TABLE 1. Accuracy and precision of the method were assessed by determining QC samples at three concentration levels of 20, 200 and 2000 ng/ml using six separate rat plasma samples, respectively. The relative standard deviation (R.S.D) was used to report the precision (See TABLE 1).The accuracies for all tested concentrations were within 5% of nominal and both the within-and between-run precisions were acceptable.

TABLE 1: Precision and accuracy for atropane in rat plasma (n=6)

Nominal plasma concentration (μg/ml)	Mean measured concentration (μg/ml)	Accuracy (%)	Intra-run R.S.D (%)	Inter-run R.S.D (%)
20	20.575	2.88	2.14	1.28
200	197.304	- 1.35	3.78	2.77
2000	2022.135	1.11	3.54	0.73

TABLE 2 : Stability data for atropine

Stability test	Initial concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Stability after three freeze–thaw cycles	20.575	99.0	1.7
	197.304	102.7	3.2
	2022.135	100.2	5.3
Stability in rat plasma at ambient temperature for 24 h	20.575	101.5	2.3
	197.304	98.0	3.5
	2022.135	99.3	4.2
Stability after protein precipitation at ambient temperature for 8 h	20.575	98.4	3.8
	197.304	100.5	4.0
	2022.135	99.1	5.8

A number of stability experiments were performed and the results are summarized in TABLE 2. No signifcant changes in the atropine concentrations were measured after three freeze–thaw cycles and storage for 24 h at ambient temperature. The analyte was stable in the supernatant after protein precipitation in the autosampler at ambient temperature for at least 8 h.

Application of the method

The presented method was successfully applied to quantify atropine in the plasma of six rats for 12 h following a single 50 mg/kg oral gavage dose. The

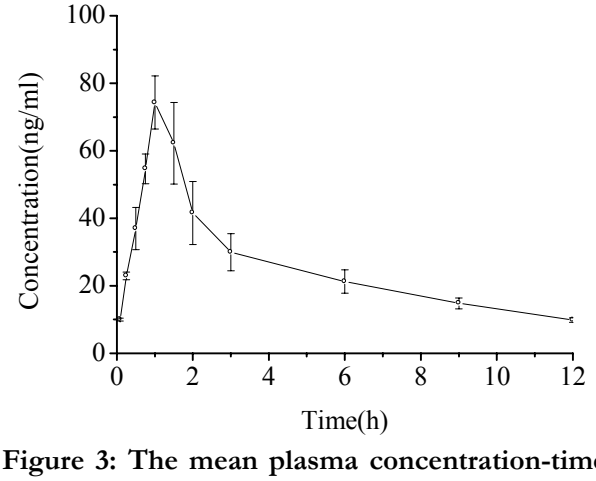

Figure 3: The mean plasma concentration-time profile of atropine after an oral dose of 50 mg/kg to rats (n = 6).

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concentration versus time profile is shown in figure 3.

CONCLUSION AND DISCUSSION

A sensitive LC-MS/MS method for the quantification of atropine in rat plasma was developed and validated. The method satisfied the requirements of high sensitivity, selectivity and high throughput for pharmacokinetic studies.

A number of extraction method of atropine from biological matrices had been reported, such as a ODS-18 solid-phase extraction cartridge and several liquid-liquid extraction solvents (ethyl acetate, dichloromethane and chloroform). Acording to the solubility of atropine, fairly water soluble, at least at acidic pH, We use methanol to proceed the plasma samples because atropine has good solubility in methanol as in water, and methanol is also used as protein precipitation reagent. In the sample prepared procedure, atropine in plasma was extracted in the methanol and water mixture solution, meanwhile the protein was precipitated by the adding methanol. Recovery of this extraction method was studied and the result showed it acceptable. It is simple and rapid.

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