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Development and validation of an LC/MS/MS method for the determination of L-hyoscyamine in human plasma ☆

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Abstract

A sensitive and specific LC/MS/MS method for the determination of L-hyoscyamine was developed and validated over the linearity range 20–500 pg ml^{-1} with 1.0 ml of plasma using scopolamine as the internal standard. The API III-Plus LC/MS/MS was operated under the multiple reaction monitoring mode using the atmospheric pressure chemical ionization technique. The instrument parameters were optimized to obtain 1.8 min run time with baseline separation of the internal standard from L-hyoscyamine. The between-run precision and accuracy of the calibration standards were 1.2 to 5.0% RSD and −4.5 to +2.5% relative error (RE). The within-run precision and accuracy of quality controls (60, 150 and 350 pg ml^{-1}) were 1.9–3.4% RSD and −3.3 to +5.1% RE. Stability of L-hyoscyamine in human plasma and processed samples has been established.

Keywords: LC/MS/MS; L-Hyoscyamine; Atropine; Scopolamine

1. Introduction

L-hyoscyamine is an pharmacologically active *levo* isomer of atropine (DL-hyoscyamine). It is an anticholinergic/antispasmodic agent. Hyoscyamine, atropine and scopolamine (hyoscine) are tropane alkaloids found in the potato family (solanacea) and have been utilized for many years as anticholinergic agents in premedication of anesthesia [1].

Despite the clinical use of atropine for many years, relatively little data is available on the pharmacological active component L-hyoscyamine's pharmacokinetic and pharmacological activity.

In the clinical setting, the typical dose of atropine is 1–2 mg by IV or IM injection. The peak plasma concentration is usually observed [2] within 1 h of drug administration at 4–8 ng ml^{-1} measured by modified radioimmunoassay (RIA) as first described by Wurzburger et al. [3]. Since the RIA method may have different cross reactivities to D-hyoscyamine and L-hyoscyamine from lot to lot, this could explain why the C_{max} value of atropine with the same dose varied so much from

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experiment to experiment [2,4–6]. Because the rates of absorption, distribution and elimination of L-hyoscyamine differ from those of D-hyoscyamine, simple plasma atropine measurement may not be useful for comparison of pharmacokinetic data with pharmacological effects.

To measure plasma L-hyoscyamine levels, a modified radiorecepter assay (RRA) was developed [4]. The sensitivity of the RRA method was 50 pg ml^{-1} in serum. In general, the best correlation has been found between the RRA determined concentrations (L-hyoscyamine) and various clinical effects of atropine [1]. However, RRA has its limitations. It cannot discriminate between the parent drugs and possible active metabolites, and the other drugs with anticholinergic activity can interfere with the assay.

A more sensitive and specific chromatographic method is needed for L-hyoscyamine pharmacokinetic study. The HPLC method developed by Okuda et al. [7] had a limit of detection of 8.5 ng ml^{-1} and therefore it cannot be used for measuring atropine concentration in human plasma after administering a therapeutic dose of tropane alkaloids. The GC/NPD method reported by Holstege et al. [8] had a limit of detection of 25 ng ml^{-1}, but repeated injections caused double the peak width and a reduced response due to matrix effects. The GC/MS method described by Kehe et al. [9] had a limit of quantitation of 1.0 ng ml with 1 ml sample. It did not have adequate sensitivity for the low dose clinical pharmacokinetic studies. A simple liquid chromatography/tandem mass spectrometric (LC/MS/MS) method was developed and validated to facilitate the pharmacokinetic investigation of L-hyoscyamine. This method utilized a liquid/liquid plasma extraction, and provided a limit of quantitation of 20 pg ml^{-1} with 1.0 ml of heparinized human plasma.

2. Experimental

2.1. Materials and reagents

L-Hyoscyamine sulfate was from Schwartz Pharma and the internal standard (I.S.) scopolamine (hyoscine) was from Research Biologicals International (Natick, MA, USA). Ammonium acetate and ammonium hydroxide were from Mallinckrodt (Paris, KY, USA). Acetonitrile, methanol, methylene chloride and water were HPLC grade and were from Fisher (Fair Lawn, NJ, USA). Heparinized control human plasma was from Worldwide Biologicals (Cincinnati, OH, USA)

2.2. Standard and quality controls

Three primary L-hyoscyamine stock solutions were prepared from separate weighings. The primary stock solutions were verified by a spectrometer sanning from 200 to 400 nm and two stock solutions were chosen to prepare standards and quality control samples (QCs). The primary and subsequent working stocks were prepared in methanol and stored at 4 °C during the validation.

Working standards were prepared fresh daily and a validation curve was obtained by addition of 100 μl of working stock solution to 1.0 ml of plasma. The concentrations of calibration standards were 20, 40, 60, 100, 200, 300, 400 and 500 pg ml^{-1}. Three quality controls were prepared at the beginning of the study of 60, 150, 350 pg ml^{-1} and stored at −20 °C with the clinical samples.

2.3. Instrument

The HPLC system consisted of an LDC 3500 HPLC pump (River Beach, FL, USA) and a Waters 717 autosampler (Milford, MA, USA). A BDS C18 guard column, 10 mm × 2 mm, 5 μm i.d., and a analytical column, BDS C18, 50 mm × 3 mm, 3 μm i.d., were from Keystone (Belleffonte, PA, USA). The HPLC system was operated isocratically at 0.5 ml min^{-1} at room temperature. The mobile phase consisted of acetonitrile, methanol and 10 mM ammonium acetate (625:375:150; v/v/v).

The mass detector was a Perkin Elmer Sciex API III-Plus triple quadrupole mass spectrometer (Thornhill, Canada) equipped with an atmospheric pressure chemical ionization (APCI) interface. Heated nebulizer temperature was 400 °C;

auxiliary nitrogen flow was 1.2 l min^{-1}; nebulizer pressure was 80 psi; curtain gas flow was 1.2 l min^{-1} and interface heater temperature was 55 °C. Ions monitored (multiple reaction monitoring, MRM) for L-hyoscyamine were m/z 290.2 (parent) to m/z 124 (product) and for scopolamine (I.S.) were m/z 304.2 (parent) to m/z 138 (product). Argon was used as the collision gas at 270×10^{12} atoms cm^2 and the electron multiplier was set at 3000 V. Declustering potential was 35 V, collision energy was 35 eV, Q1 resolution (RE1) was 118.6, and Q3 resolution (RE3) was 119.

2.4. Data treatment

Chromatograms were measured using a MacQuan 1.3 data system (PE Sciex) quantitatively and subsequently transferred into the Vax/VMS oracle data base. A weighted $1/y$ linear regression was used to determine slopes, intercepts and correlation coefficients, where y = ratio of the compound peak area to the I.S. peak area. The resulting parameters were used to calculate L-hyoscyamine concentration from the equation:

concentration = (y – intercept)/slope

2.5. Extraction procedure

To 1.0 ml heparinized plasma sample, 100 µl of I.S. methanol solution (5 ng ml^{-1}) was added. After vortexing briefly, 50 µl of 1 N ammonium hydroxide was added. After mixing, 5 ml of methylene chloride was added to extract the L-hyoscyamine and the I.S. from the plasma sample by shaking for 5 min on a horizontal shaker. The organic phase was separated from the aqueous phase by centrifugation at 2500 rev min^{-1} for 5 min. After aspirating off the top plasma layer to waste, the methylene chloride extract was evaporated to dryness under a stream of nitrogen gas in a 40 °C water bath. The residue was reconstituted

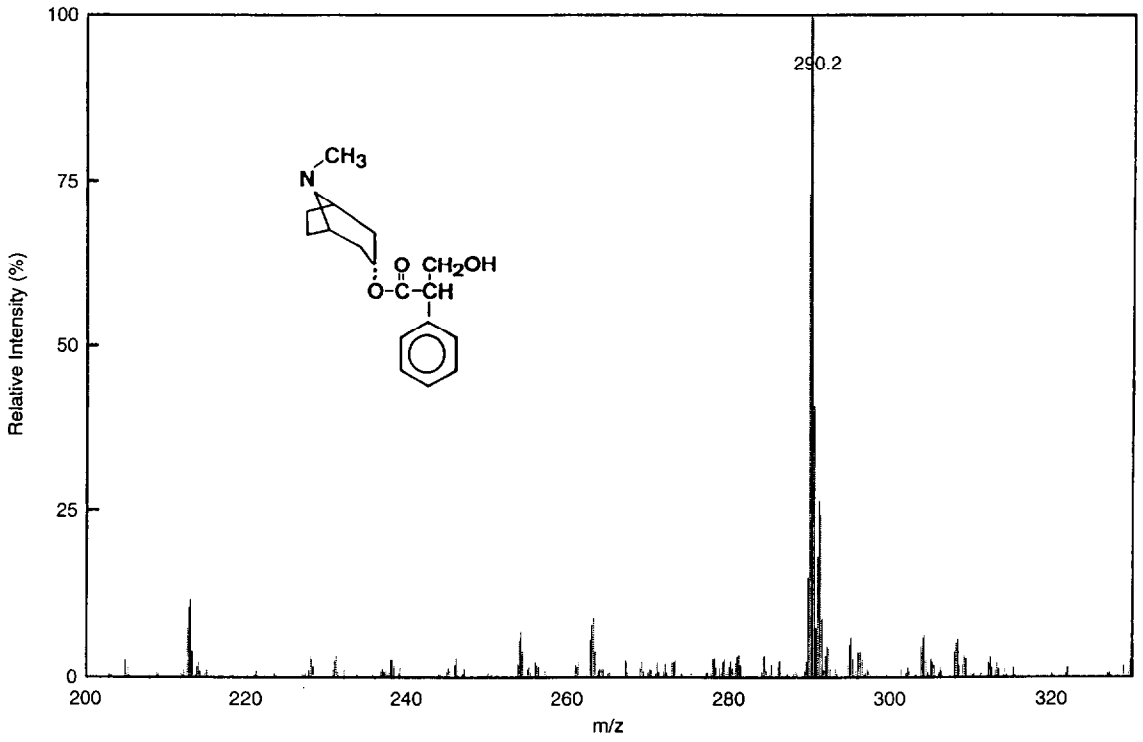

Fig. 1. Structure and APCI mass spectrum of L-hyoscyamine, illustrating the base peak ion m/z 290.2 as the protonated molecular ion.

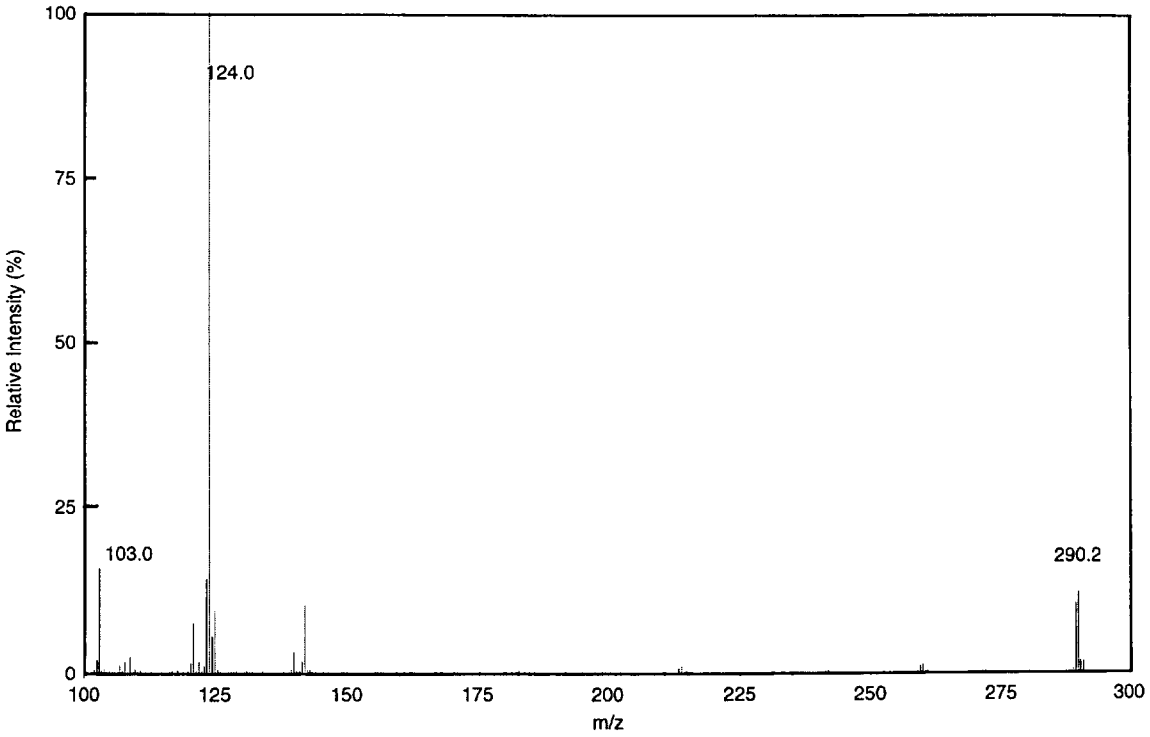

Fig. 2. Mass spectrum of product ions of m/z 290.2 (L-hyoscyamine). The base peak of m/z 124 indicated the cleavage between the tropine ring and the oxygen at the center of L-hyoscyamine.

in 100 μl of mobile phase. 20 μl was injected onto the LC/MS/MS system. Note: the entire procedure should be done under yellow light.

3. Results and discussion

LC/MS/MS for the detection of L-hyoscyamine in human plasma was investigated. Liquid/liquid extraction characteristics of L-hyoscyamine were determined by APCI LC/MS/MS. The target drug (L-hyoscyamine) eluted at 1.2 min and the I.S. (scopolamine) eluted at 0.8 min. The baseline separation of these two compounds was achieved with the short analytical column (50 mm × 3 mm). Fig. 1 displays the full scan mass spectrum of L-hyoscyamine standard, where the base peak was protonated molecular ion $(MH)^+$ at m/z 290.2. The product ion mass spectrum (Fig. 2) had a base peak at m/z 124 which indicated the cleavage of the drug next to the oxygen at the center. The Q1 mass spectrum (Fig. 3) of the I.S. (scopolamine) displayed a similar pattern to L-hyoscyamine, the predominant ion observed being m/z 304.4, which was the protonated I.S. The product ion mass spectrum (Fig. 4) had a base peak m/z 138 as expected.

MRM spectra were used to determine L-hyoscyamine and the I.S. levels. MRM spectra selectively filter out ions not related to the target analytes and produce a very clean total ion chromatogram (TIC) due to the great selectivity and sensitivity of this operational mode.

Quantitation of L-hyoscyamine was achieved by preparing calibration curves of peak area ratio (L-hyoscyamine/I.S.) against concentration of fortified L-hyoscyamine. The calibration consisted of eight calibrators ranging between 20 pg ml^{-1} and

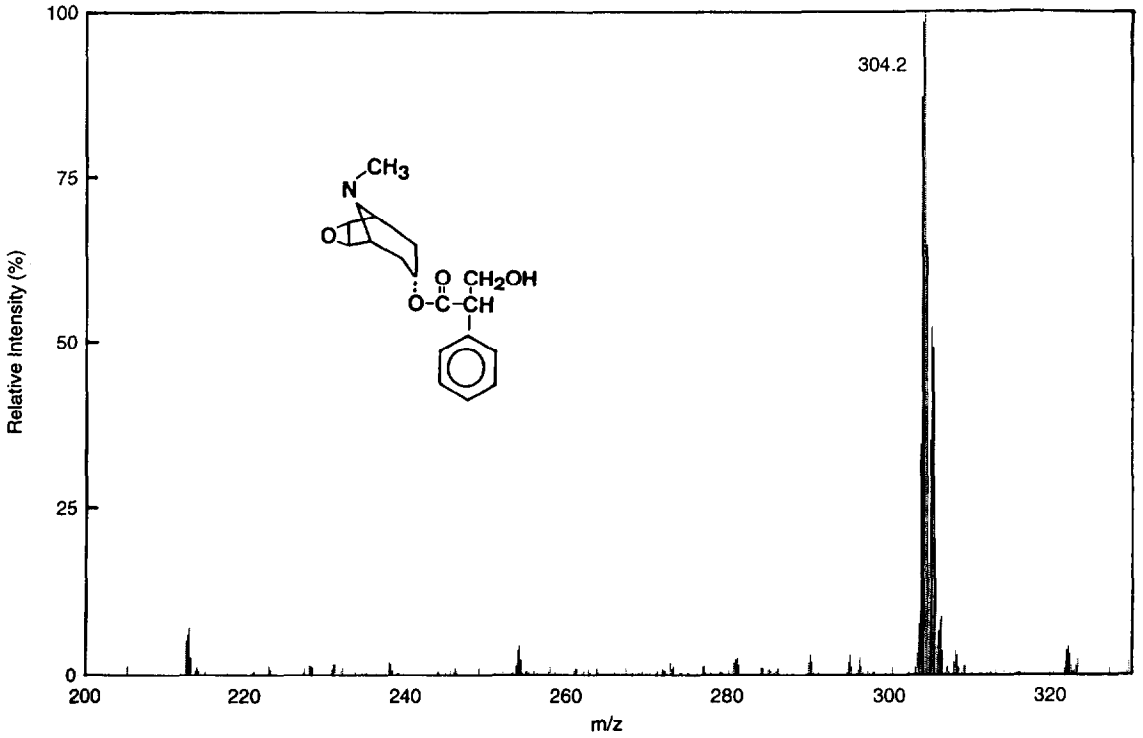

Fig. 3. Structure and Q1 mass spectrum of scopolamine (I.S.) using APCI. The observed base peak of m/z 304.2 was the protonated molecular ion.

500 pg ml^{-1}. The resulting calibration curve was linear and gave correlation coefficients consistently greater than 0.998.

The chromatogram of L-hyoscyamine standard solution and I.S. solution is shown in Fig. 5. Fig. 6 shows the chromatogram of the plasma sample with L-hyoscyamine and I.S. at the limit of quantitation (LOQ) of the linearity range. Excellent baseline separation of L-hyoscyamine and the I.S. was achieved although it was not required. Fig. 7 shows the chromatogram of blank control plasma.

During the method development, eight lots of commercially obtained control plasma were screened and no interference was observed. Figs. 8 and 9 show chromatograms of QCs at 150 pg ml^{-1} and 60 pg ml^{-1}.

In order to increase the sample throughput, high percent organic mobile phase was used in conjunction with a very short HPLC analytical column. With a run time of 1.8 min per sample, 250 unknown samples were analyzed per instrument per day.

The method described here is suitable for pharmacokinetic studies of L-hyoscyamine and atropine administration but will not differentiate D- and L-hyoscyamine. However, in case separate measurement of D- and L-hyoscyamine is required, it can be achieved by optimizing the HPLC condition with the solvent gradient.

3.1. Extraction

A simple liquid/liquid extraction procedure was developed to extract L-hyoscyamine and I.S. from plasma. The recoveries of L-hyoscyamine from human heparinized plasma were close to 100% using methylene chloride as an extraction solvent. The recoveries at 20, 100 and 500 pg ml^{-1} were averaged at 110% ($n = 6$) for L-hyoscyamine. The I.S. (scopolamine) recovery was 93% ($n = 6$).

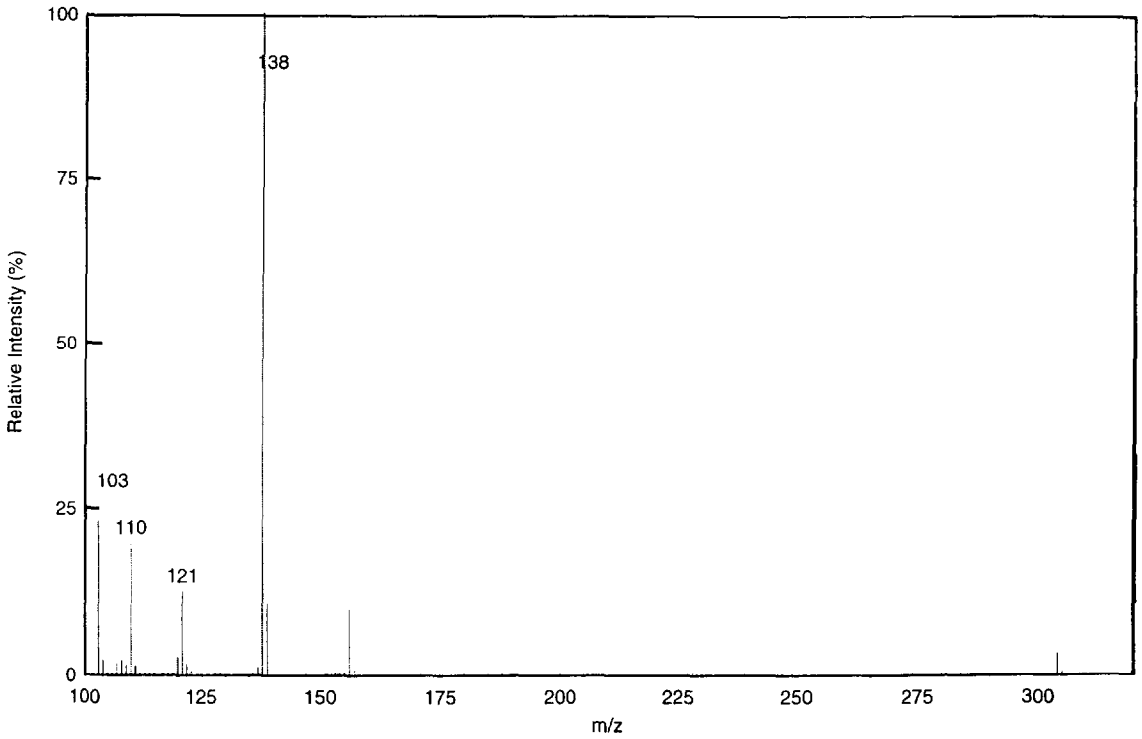

Fig. 4. Product ion mass spectrum of m/z 304.2 (scopolamine). The observed base peak was m/z 138 which indicated a similar fragmentation pattern to L-hyoscyamine.

3.2. Performance characteristics

Five batches of validation samples were run on three separate days. Consistently good correlation coefficients ($r > 0.998$) were observed throughout the validation process. Table 1 shows interday precision and accuracy data for each calibration standard.

The limit of quantitation (LOQ) was originally validated at 50 pg ml^{-1}, which was substantially more sensitive and specific than the many literature methods. However, the pharmacokinetic studies planned had an estimated C_{max} value of 0.5 ng ml^{-1}, therefore the lower LOQ became necessary. The final validated LOQ was 20 mg ml^{-1} with a signal-to-noise ratio equal to 20.

Table 2 shows the inter- and intraday precision and accuracy of three QC levels. The precision and accuracy data show that this LC/MS/MS method is consistent and reliable with relatively low error and %RDS for the standards and QCs over the entire concentration range. The standard

Table 1
Interday precision and accuracy of L-hyoscyamine standards

	L-Hyoscyamine (pg ml^{-1})							
	20	40	60	100	200	300	400	500
Mean	20	41	61	101	191	296	413	499
RSD%	5.00	2.44	1.64	3.96	2.09	1.94	2.03	1.20
R.E.%	0.00	+2.50	+1.67	+1.00	−4.50	−1.35	+3.25	−0.20
n	4	5	5	5	5	5	5	5

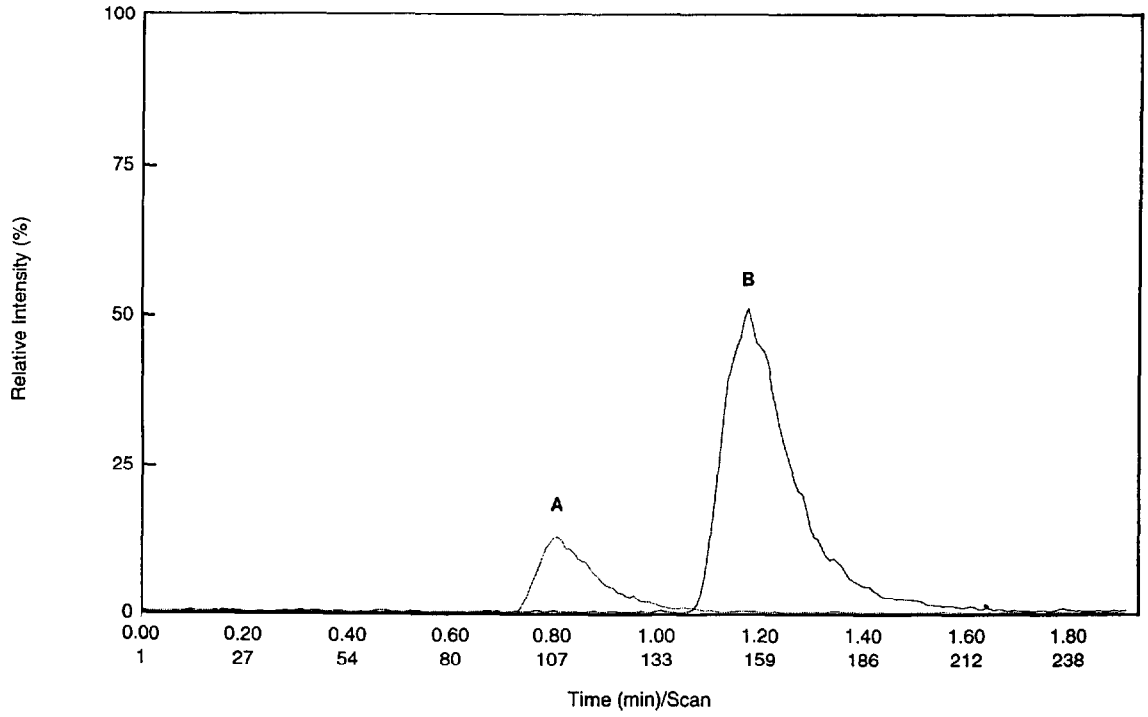

Fig. 5. MRM ion chromatograms of I.S. (A) and L-hyoscyamine solutions (B). The MRM channels for L-hyoscyamine and the I.S. were m/z 290.2 → m/z 124 and m/z 304.2 → m/z 138. Dwell time 150 ms. See text for HPLC conditions.

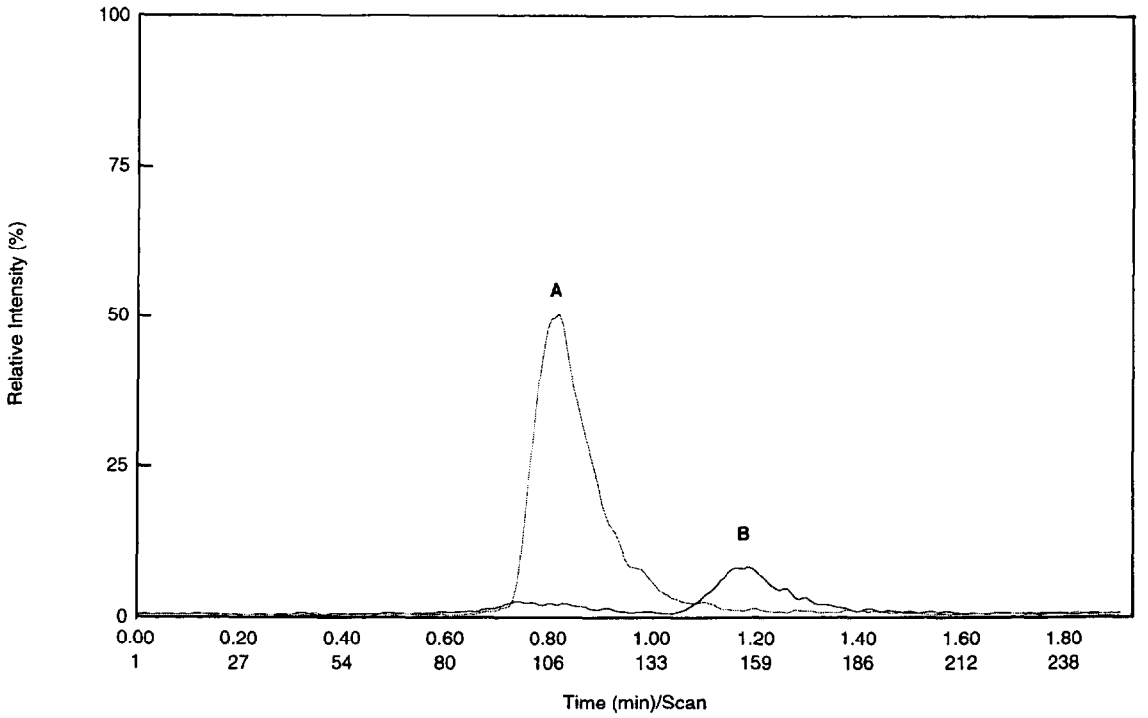

Fig. 6. MRM ion chromatogram of plasma sample with I.S. (A) and L-hyoscyamine (B) at the limit of quantitation of 20 pg ml^{-1}. The actual amount of L-hyoscyamine injected into the column was 4 pg with a signal-to-noise ratio equal to 30.

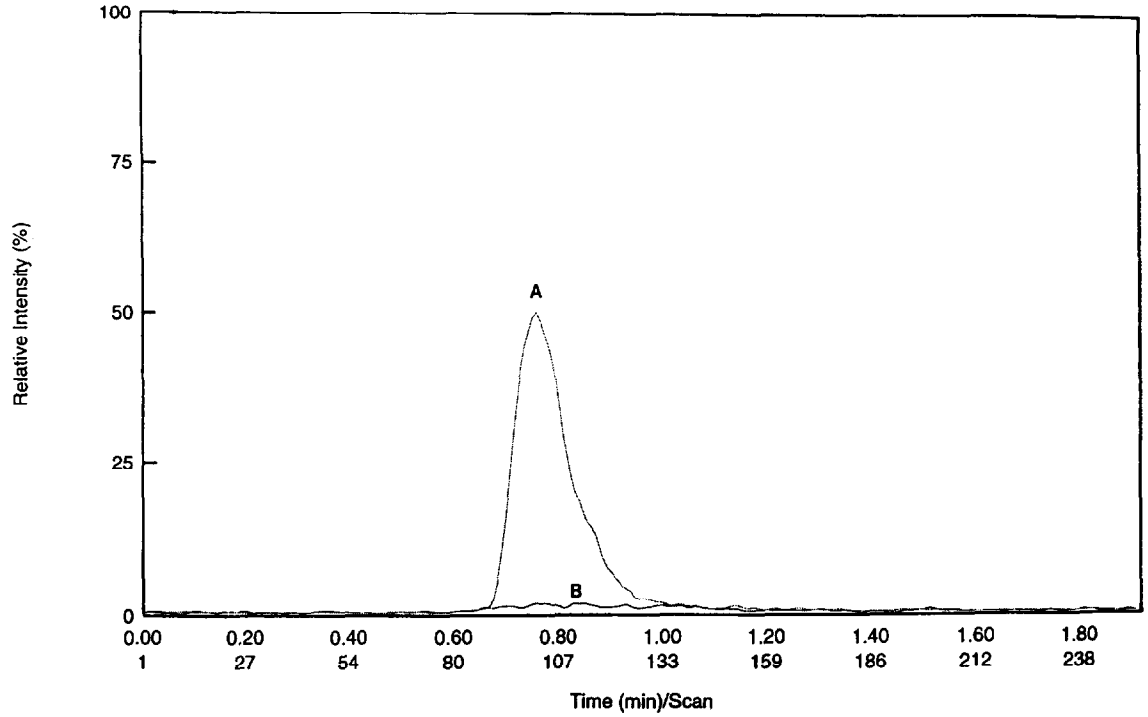

Fig. 7. A typical MRM ion chromatogram of blank plasma (B) with I.S. (A). No interference was observed at the retention time of L-hyoscyamine (1.2 min).

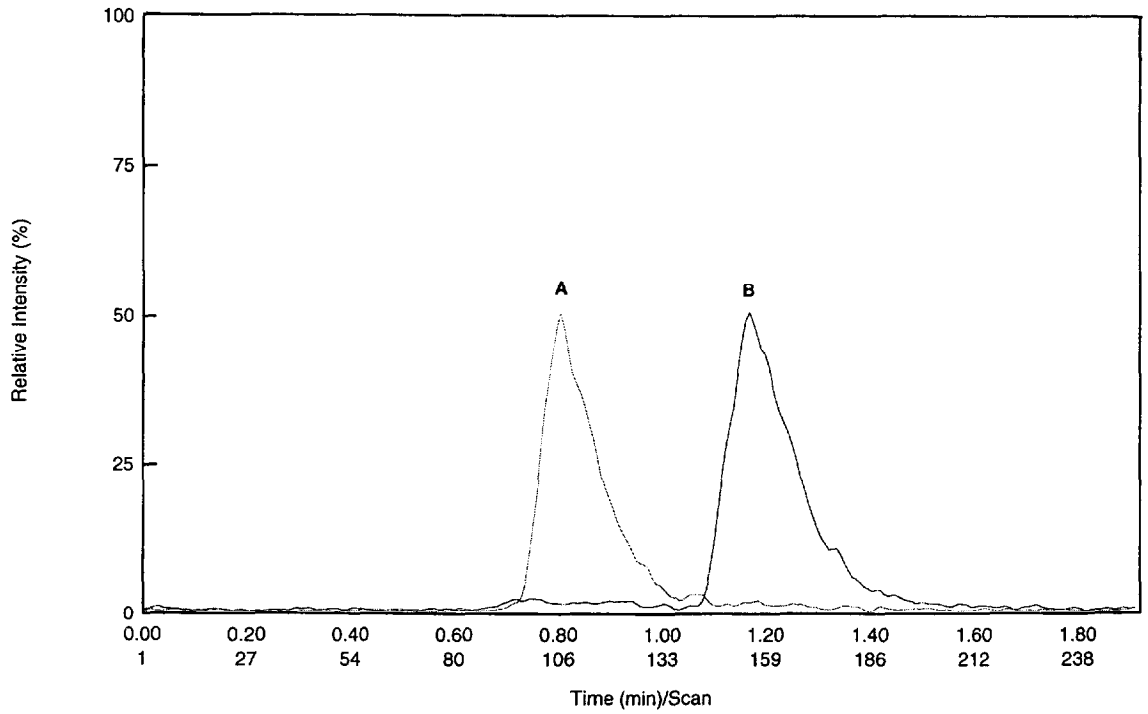

Fig. 8. A typical MRM ion chromatogram of 150 pg ml^{-1} QC sample (B) with I.S. (A).

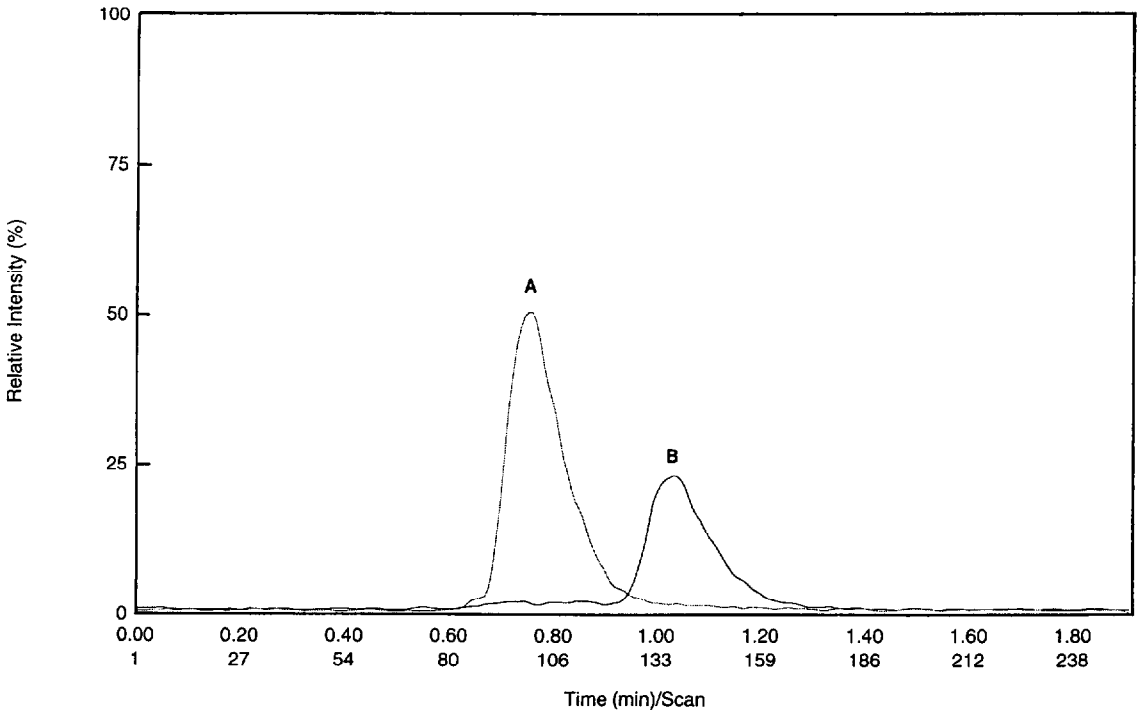

Fig. 9. A typical MRM ion chromatogram of 60 pg ml^{-1} sample (B) and I.S. (A). The actual amount of L-hyoscyamine injected into the column was 12 pg ml^{-1}.

solutions used for inter- and intraday precision and accuracy were prepared once from a single stock weighing and used over a 3-day period.

3.3. Stability

Table 3 shows the stability of sample processing (freeze–thaw, benchtop), chromatography (on-system and reinjection), and storage (refrigeration of processsed spiked samples and long term sample storage at −20 °C). No degradation of L-hyoscyamine was observed after stability tests.

Table 2
Inter- and intraday precision and accuracy of quality controls

	L-Hyoscyamine (pg ml^{-1})		
	60	150	350
Interday (n = 30)			
Mean	60	159	365
RSD%	6.67	5.10	2.77
R.E.%	0.00	+4.46	+3.05
Mean	58	157	368
RSD%	3.45	3.18	1.90
R.E.%	−3.33	+4.67	+5.14

4. Conclusions

A quantitative LC/MS/MS method for analysis of L-hyoscyamine in human plasma has been developed and validated. The method is very sensitive and specific due to excellent recovery achieved by the simple one-step liquid/liquid extraction and the inherent sensitivity of tandem mass spectrometry. With the short analytical column and high percent organic mobile phase, 250 unknown samples can be analyzed simultaneously per instrument per day. This method can also be applied to the quantitation of L-

Table 3
Stability of L-hyoscyamine in plasma control samples

	Time period	% of normal control	
		150 pg ml^{-1}	350 pg ml^{-1}
Reinjection	23 h	87%	93%
Refrigeration	72 h	98%	96%
Benchtop	5.5 h	97%	98%
Freeze–thaw (three cycles)		97%	103%
Sample storage (−20 °C)	6 months	103%	100%

hyoscyamine and atropine. As the results of development of this LC/MS/MS method, the pharmacokinetic investigation of low dose L-hyoscyamine administration is now possible.

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