Journal of Chromatography B, 878 (2010) 981–986

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of the quaternary ammonium compound trospium in human plasma by LC–MS/MS: Application to a pharmacokinetic study

Kishore Kumar Hotha [a,b], D. Vijaya Bharathi [a,*], Sanagapati Sirish Kumar [a], Y. Narasimha Reddy [a], Pankaj K. Chatki [a], L.K. Ravindranath [c], K.N. Jayaveera [b]

[a] Bioanalytical Department, Integrated Product Development, Dr. Reddy's Laboratories Ltd, Bachupalli, Hyderabad 500 072, India
[b] Department of Chemistry, JNT University, Anantapur, Andhra Pradesh 515 001, India
[c] Department of Chemistry, SK University, Anantapur, Andhra Pradesh 515 001, India

ARTICLE INFO

Article history:
Received 4 November 2009
Accepted 24 February 2010
Available online 3 March 2010

Keywords:
Quaternary compound
Trospium
Human plasma
LC–MS/MS
Pharmacokinetics

ABSTRACT

A highly sensitive, specific and evaporation free SPE extraction, LC–MS/MS method has been developed for the estimation of trospium in human plasma using trospium-d8 as an internal standard (IS). The analyte was separated using isocratic mobile phase on reverse phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective [M^+] cations, m/z 392–164 for trospium and m/z 400–172 for the IS. The total run time was 3.50 min and the elution of trospium and trospium-d8 (IS) occurred at 2.8 min. The developed method was validated in human plasma with a lower limit of quantification of 0.05 ng/mL. A linear response function was established for the range of concentrations 0.05–10 ng/mL ($r > 0.998$) for trospium in human plasma. The intra- and inter-day precision values for trospium met the acceptance as per FDA guidelines. Trospium was stable in the battery of stability studies viz., bench-top, auto-sampler, dry extracts and freeze/thaw cycles. The developed assay method was applied to an oral pharmacokinetic study in humans.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Trospium chloride (Fig. 1) (CAS no. 10405-02-4) (spiro [8-azoniabicyclo[3,2,1]octane-8,1′-pyrrolidinium]-3-[(hydroxydiphenyl-acetyl)-oxy]chloride(1α, 3β, 5α)-(9Cl) is an antimuscarinic agent indicated for the treatment of OAB with symptoms of urge urinary incontinence, urgency, and urinary frequency. Trospium has 3 chemical and pharmacokinetic properties unique among antimuscarinic agents: it is a positively charged quaternary ammonium compound with minimal central nervous system penetration; it is not metabolized by the cytochrome P450 system, resulting in a lower tendency for drug interactions; and it is excreted mainly unchanged in the urine as the active parent compound, providing local activity to achieve early onset of clinical effect and prolonged efficacy [1–5].

Administration of SANCTURA XR immediately after a high (50%) fat-content meal reduced the oral bioavailability of trospium chloride by 35% for AUC (0–T last) and by 60% for C_{max}. Other pharmacokinetic parameters such as T_{max} and $t_{1/2}$ were unchanged in the presence of food. It is therefore recommended that SANCTURA XR be taken on an empty stomach at least 1 h before a meal [5]. Hence to characterize pharmacokinetic parameters under fed conditions lower LLOQ is required.

Few methods were reported earlier, using fluorescence detection with an LLOQ of 1 ng/mL [6]. An LC–MS method for quantification of trospium in human plasma was published by Zhi-rong et al. (2007) in Chinese language, with an LLOQ of 0.2 ng/mL [7]. Here in we are presenting a highly sensitive and rugged LC–MS/MS method which has overcome the draw backs of the previously reported methods viz., usage of single step evaporation free extraction method which has enabled sensitive and reproducible lower LLOQ of 0.05 ng/mL which is 4-fold less than the previously reported LLOQ for trospium by Zhi-rong et al. (2007). In the present method trospium-d8 was used as IS to track the analyte response which is most suitable IS when compared to tramadol (IS) used by Zhi-rong et al. (2007). The method can be applied to pharmacokinetic studies following the administration of single dose of trospium 60 mg capsules.

2. Experimental

2.1. Chemicals and reagents

Trospium was obtained from Dr Reddy's Laboratories Limited, Hyderabad, India. Trospium-d8 (I.S) was obtained from Vardha Biotech, Mumbai, India. All the compounds were found to be >98.5%

* Corresponding author. Tel.: +91 40 44346850; fax: +91 40 44346285.
E-mail address: vijayabd@drreddys.com (D.V. Bharathi).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.02.028

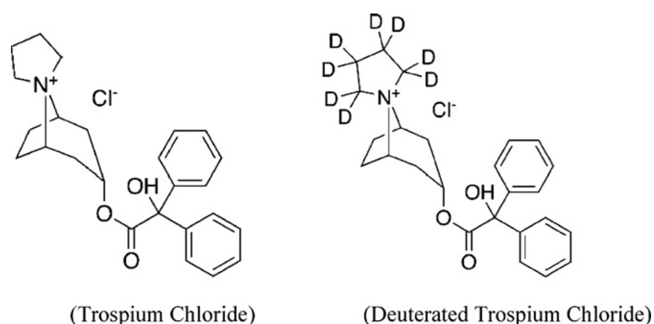

Fig. 1. Structural representation of trospium and deuterated trospium (d8).

purity determined by chromatographic (HPLC, LC–MS/MS) analysis. Chemical structures are presented in Fig. 1. HPLC grade of acetonitrile; analytical grade ammonium acetate was purchased from Merck Specialties Pvt. Ltd, Mumbai, India. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control K2 EDTA human plasma was purchased from registered blood bank, Secunderabad, India.

2.2. Instrumentation and chromatographic conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1322A), isopump (G1310A) along with auto-sampler (G13167B) was used to inject 10 μL aliquots of the processed samples on a HyPurity C_{18} column (4.6 mm × 50 mm, 5 μm, Thermo Electron Corporation, Cheshire, UK), which was kept at room temperature (24 ± 2 °C). The isocratic mobile phase, a mixture of 2 mM ammonium acetate and acetonitrile mixture (20:80, v/v) was delivered at 0.50 mL/min into the mass spectrometer's electrospray ionization chamber.

Quantitation was achieved by MS–MS detection in positive ion mode for both trospium and IS, using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray^{TM} interface at 450 °C. The ion spray voltage was set at 5500 V. The common parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 35 psi, 25 psi, 45 psi and 5 psi, respectively. The compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 100 V, 41 V, 10 V, 15 V and 65 V, 25 V, 10 V, 15 V for IS. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, the transition pairs of trospium at the m/z 392.4 amu precursor ion to the m/z 164.2 amu, 400.5 amu precursor ion to the m/z 172.2 amu product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst Software (Version 1.4.2).

2.3. Standard solutions

Primary stock solutions of standard and quality control (QC) samples were prepared by weighing separately. The primary stock solution (1.00 mg/mL) of trospium and IS were prepared in methanol and stored at −20 °C, which were found to be stable for 1 month (data not shown). Appropriate dilutions were made in methanol to produce working stock solutions of 991.4, 893.4, 740.9, 496.4, 148.9, 50.6, 27.8, 11.1 and 5.5 ng/mL on the day of analysis and these stocks were used to prepare calibration curve (CC). Another set of working stock solutions were made in methanol (from primary stock) at 828.0, 490.3,16.7 and 5.5 ng/mL for preparation of QC samples accordingly. Working stock solutions were stored at approximately 5 °C for a week (data not shown). A working IS solution (100 ng/mL) was also prepared in methanol. Calibration samples were prepared by spiking 490 μL of control human plasma with the appropriate amount of analytes (10 μL) and IS (50 μL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations and 200 μL volumes were aliquoted into different tubes and depending on the nature of experiment samples were stored at −80 ± 10 °C until analysis.

2.4. Sample preparation

To an aliquot of 200 μL human plasma sample, IS solution (50 μL) was added; 200 μL of 1% ortho phosphoric acid and vortex mixed for 30 s on a cyclomixer (Remi Instruments, Mumbai, India). This sample mixture was loaded on pre-conditioned (1 mL methanol followed by 1 mL water) Oasis HLB cartridges (1 cc, 30 mg) and washed with 1 mL water followed by 1 mL 15% methanol in water and finally eluted with 1 mL of mobile phase. From the eluate 20 μL was directly injected onto LC–MS/MS system.

2.5. Method validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [8].

The specificity of the method was determined by analyzing six different batches of human plasma as is, to demonstrate the lack of chromatographic interference from endogenous plasma components. Sets of spiked standards and QC samples (n = 6 at each concentration) were prepared and analyzed on four different occasions to evaluate linearity, precision and accuracy. Precision and accuracy was also assessed at the lowest concentration of the standards (55 pg/mL), representing the lower limit of quantification (LLOQ) for the assay.

The recovery of trospium and IS was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations. Recovery was determined at low, medium and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 5 ng/mL. The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations [9,10]. Matrix effect was determined at same concentration of analyte and IS as in recovery experiment.

The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 66 h (in auto-sampler) after the initial injection. The peak areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the biomatrix after 8 h exposure (bench-top) was determined at two concentrations in six replicates. Freezer stability of the analytes in biomatrix was assessed by analyzing the QC samples stored at −80 ± 10 °C for at least 30 days. The stability of analytes in biomatrix following repeated three freeze–thaw cycles (stored at −80 ± 10 °C between cycles) was assessed using QC samples spiked with analytes. Samples were processed as described under Section 2.4. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% R.S.D.) and precision (i.e., 15% R.S.D.) [8].

2.6. Pharmacokinetic study

A pharmacokinetic study was performed in healthy male subjects. The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples

were obtained following oral administration of 60 mg of trospium hydrochloride capsules into polypropylene tubes containing K2 EDTA solution as anti-coagulant at pre-dose 1.0, 1.5, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72 and 96 h. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 × g for 5 min and stored frozen at −80 ± 10 °C until analysis.

An aliquot of 200 μL of thawed plasma samples were spiked with IS and processed as mentioned in sample preparation section (Section 2.4). Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration; (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data of trospium was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA).

3. Results

3.1. Method development

3.1.1. Sample pre-treatment

Different methods of sample pre-treatment were investigated. Protein precipitation using acidified acetonitrile or methanol gave strong interferences. Liquid–liquid extraction with various organic solvents such as hexane, methyl tert-butyl ether, diethyl ether and ethyl acetate and their mixtures resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analytes (data not shown). Subsequently, SPE was investigated as samples pre-treatment technique. Hydrophilic–lipophilic balance, cation exchange and anion exchange cartridges were used for optimizing the extraction procedure. Cation exchange cartridges were investigated as per Oasis® SPE protocol and also with several dilution, conditioning, washing and elution reagents and it resulted in good recovery but had strong matrix interferences, whereas hydrophilic–lipophilic balance cartridges, Oasis HLB cartridges (1 cc, 30 mg) with several dilution, conditioning, washing and elution reagents gave consistent results in terms of recovery of trospium and its IS and also gave cleaner plasma blank samples. The SPEs were pre-conditioned (1 mL methanol followed by 1 mL water) and sample mixture was loaded and were washed with 1 mL water followed by 1 mL 15% methanol in water and finally eluted with 1 mL of mobile phase. From this eluate was directly injected into the LC–MS/MS system.

3.1.2. Liquid chromatography

In pursuit of symmetric peak shape and retention time of ~2.8 min, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH

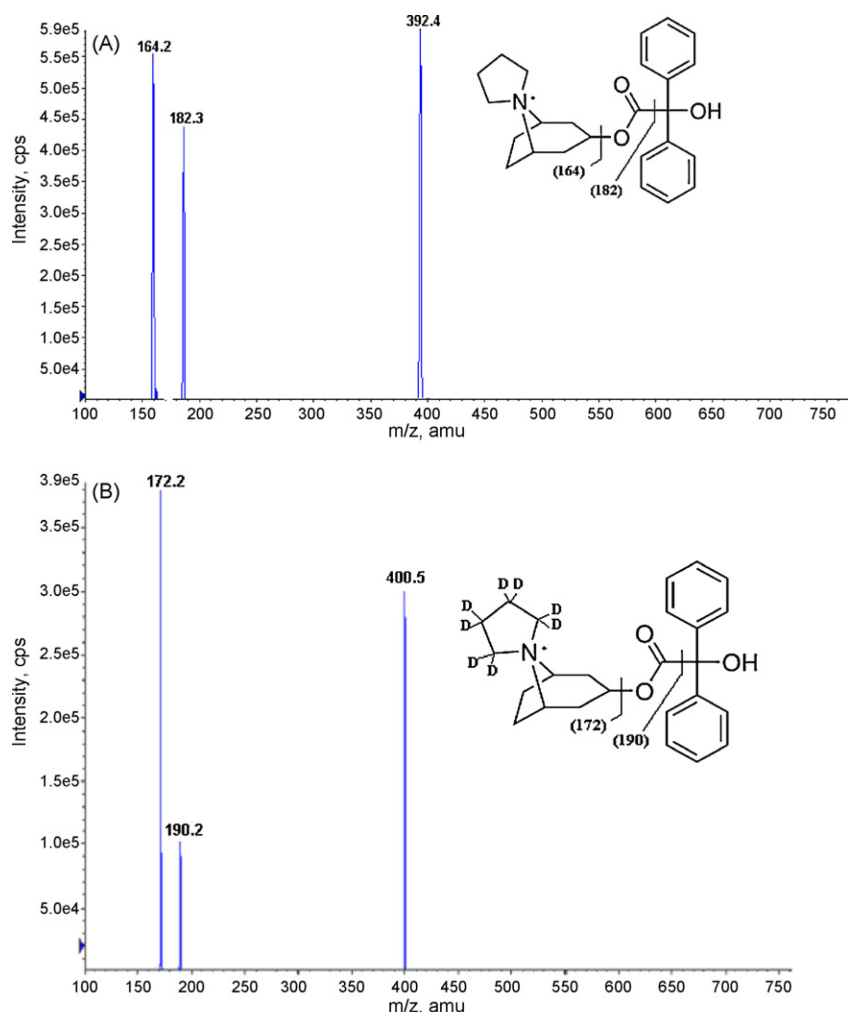

Fig. 2. Fragmentation pattern of trospium and deuterated trospium.

range of 4.0–7.0, along with altered flow-rates (in the range of 0.3–1.0 mL/min) were tested for complete chromatographic resolution of trospium and IS (data not shown). The resolution of peaks was achieved with 2 mM ammonium acetate and acetonitrile mixture (20:80, v/v) with a flow rate of 0.5 mL/min, on a HyPurity C_{18} column and was found to be suitable for the determination of electrospray response for trospium and IS.

3.1.3. Mass spectrometry

In order to optimize ESI conditions for trospium and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for trospium and IS revealed peaks at m/z 392.4 and 400.5 amu, respectively as cation, [M^+]. Following detailed optimization of mass spectrometry conditions (provided in Section 2.2) m/z 392.4 amu precursor ion to the m/z 164.2 amu was used for quantification for trospium. Similarly, for IS m/z 400.5 amu precursor ion to the m/z 172.2 amu was used for quantification purpose. Fragmentation pattern of trospium and deuterated trospium are presented in Fig. 2.

3.2. Specificity and selectivity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with trospium at LLOQ are shown in Fig. 3, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention time of trospium and IS was 2.8 min. The total chromatographic run time was 3.5 min.

3.3. Recovery

Recovery was found to be $75.35 \pm 3.78\%$, $72.53 \pm 1.89\%$ and $70.29 \pm 2.87\%$ at LQC, MQC and HQC, respectively for trospium. The

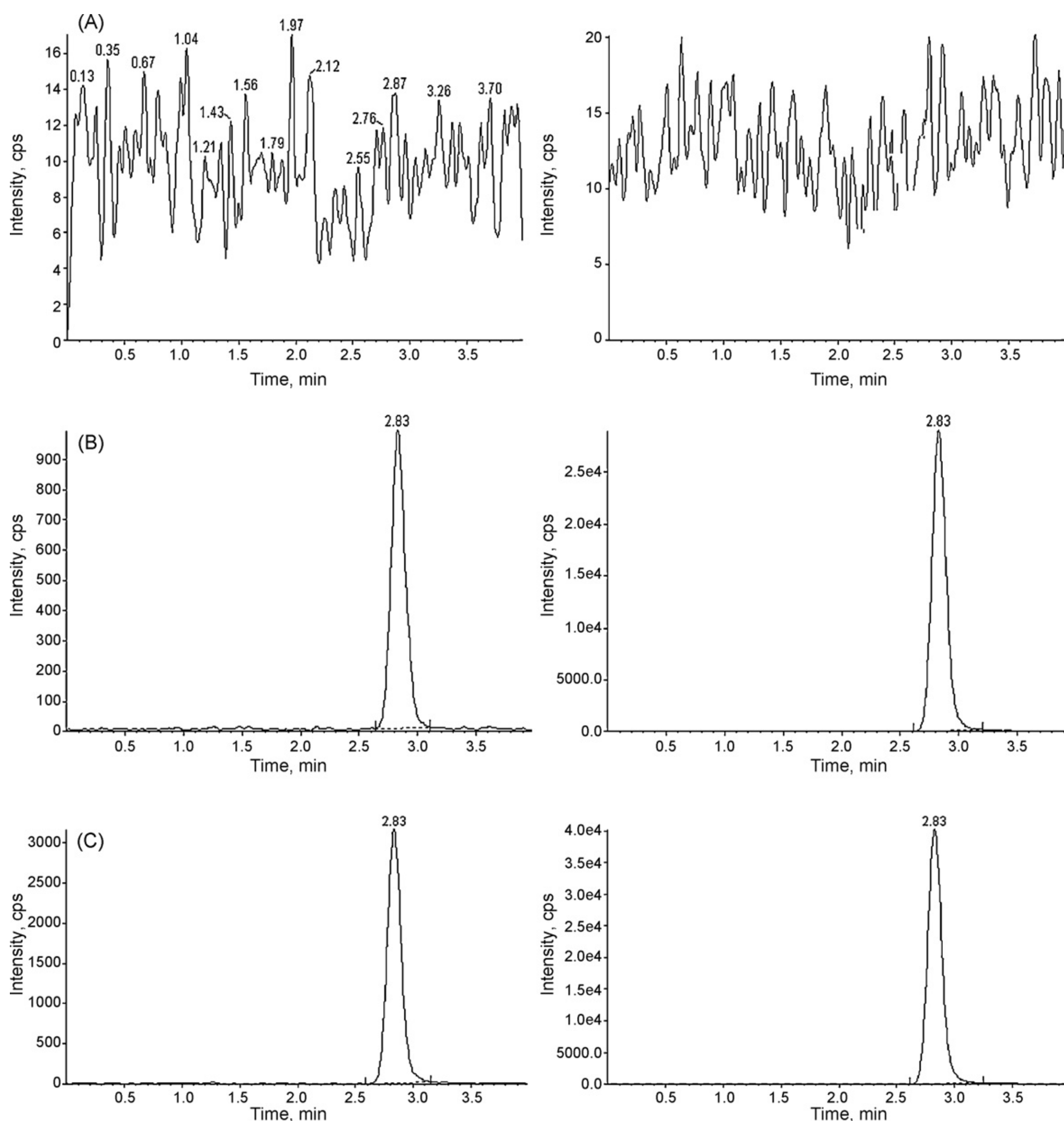

Fig. 3. Typical MRM chromatograms of trospium (left panel) and IS (right panel) in (A) human blank plasma; (B) human plasma spiked with trospium at LLOQ (0.05 ng/mL) and IS (C) a 5.0 h (6.3 ng/mL) plasma sample showing trospium peak obtained following oral dose of trospium capsule to healthy volunteer along with IS under fasted conditions.

Table 1
Intra- and inter-day precision of determination of trospium in human plasma.

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL)			
		Mean	SD	RSD	Accuracy (%)
Intra-day variation (six replicates at each concentration)					
0.055		0.053	0.004	7.03	96.83
0.167		0.167	0.008	4.98	100.1
4.900		5.056	0.176	3.46	103.2
8.280		8.728	0.148	1.74	105.4
Inter-day variation (eighteen replicates at each concentration)					
0.055		0.055	0.006	10.85	100.0
0.167		0.159	0.011	6.92	95.21
4.900		4.936	0.396	8.02	100.7
8.280		8.388	0.584	6.96	101.3

mean recovery for trospium was found to be 72.72 ± 3.49%. The recovery of IS was 69.58 ± 4.90%.

3.4. Matrix effect

In this study, the matrix effect was evaluated by analyzing LLOQ sample. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained for trospium was +0.96 (CV: 9.98%, $n = 6$), at LLOQ level, whereas on IS it was found to be +0.98 (CV: 3.89%, $n = 6$) at tested concentration of 5 ng/mL.

3.5. Calibration curve

The plasma calibration curve was constructed using calibration standards of 0.055 to 9.91 ng/mL. Calibration curve was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 4$) was found to be ≥0.998 respectively. The lowest concentration with the R.S.D. < 20% was taken as LLOQ [8] and was found to be 0.055 ng/mL. The % accuracy observed for the mean of back-calculated concentration for four linearities was within 94.17–105.02. The precision (% CV) values ranged from 0.98 to 4.68.

3.6. Precision and accuracy

The accuracy, intra- and inter-assay precision which was determined by analyzing six replicates of QC samples at four concentrations on two different days are shown in Table 1.

3.7. Stability

The predicted concentrations for each analyte at LQC and HQC samples deviated within ±15% of the nominal concentrations in a batter of stability tests viz., in-injector (66 h), bench-top (8 h), repeated three freeze/thaw cycles and at −80 ± 10 °C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

3.8. Pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from 24 healthy human volunteers following oral administration of 60 mg of trospium hydrochloride XR capsules manufactured by Dr. Reddy's Laboratories Limited as a part of pharmacokinetic study. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of trospium in humans. Fig. 4A depicts the mean plasma concentration vs. time profile of trospium in these volunteers under fasted conditions. Following the oral administration of 60 mg of trospium capsules to volunteers under fasting conditions, the mean maximum plasma concentrations (C_{max}), 6.82 ng/mL, were attained at ∼5.5 h (T_{max}), while the AUC (0-T) was 66 ng × h/mL.

Fig. 4B depicts the mean plasma concentration vs. time profile of trospium in these volunteers under fed conditions. Following the oral administration of 60 mg of trospium capsules to volunteers, the mean maximum plasma concentrations (C_{max}), 0.42 ng/mL, were attained at ∼6.50 h (T_{max}), while the AUC (0-T) was found to be 9.09 ng × h/mL.

4. Discussion

To the best of our knowledge, we have developed for the first time fully validated LC–MS/MS method for the determination of trospium, which provides the highest sensitivity (0.05 ng/mL) using a simple SPE extraction procedure which did not involve reconstitution or drying step to achieve the desired sensitivity. Usually drying and reconstitution step is used to obtain lower sensitivity but in the present method directly SPE eluate gives sensitivity as low as 0.05 ng/mL, further we can still go lower by adding drying and reconstitution step to the current method. A good internal standard should track the analyte during extraction and any inconsistent response due to matrix effect. This is also established with almost the same recovery of IS compared to the analyte. The most appropriate IS for typical cations are none other than deuterated compounds and hence trospium-d8 was used as IS. Results obtained by usage of d8 internal standard were consistent and reproducible which was evident by incurred sample analysis conducted on this study. The use of only 10 μL of the final eluate gave an on-column loading of 0.22 pg/injection for trospium. This minimizes matrix interference and suppression of analyte peak and helps to extend the life of the column. Hence the sensitivity further

Table 2
Stability data – quality controls in human plasma.

Nominal conc. (ng/mL)	Stability	Mean ± S.D.[a] $n = 6$ (ng/mL)	Accuracy (%)[b]	Precision (% CV)
0.167	0 h (for all)	0.168 ± 0.0106	100.6	6.30
	3rd freeze–thaw	0.180 ± 0.0075	107.8	4.16
	10 h (bench-top)	0.172 ± 0.0097	103.0	5.65
	66 h (in-injector)	0.174 ± 0.0107	104.2	6.16
	30 days at −80 °C	0.162 ± 0.0086	96.99	5.31
8.280	0 h (for all)	8.317 ± 0.3176	100.4	3.82
	3rd freeze–thaw	9.042 ± 0.1781	109.2	1.97
	10 h (bench-top)	8.287 ± 0.2296	100.1	2.77
	66 h (in-injector)	8.270 ± 0.1075	99.88	1.30
	30 days at −80 °C	8.069 ± 0.1815	97.45	2.25

[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

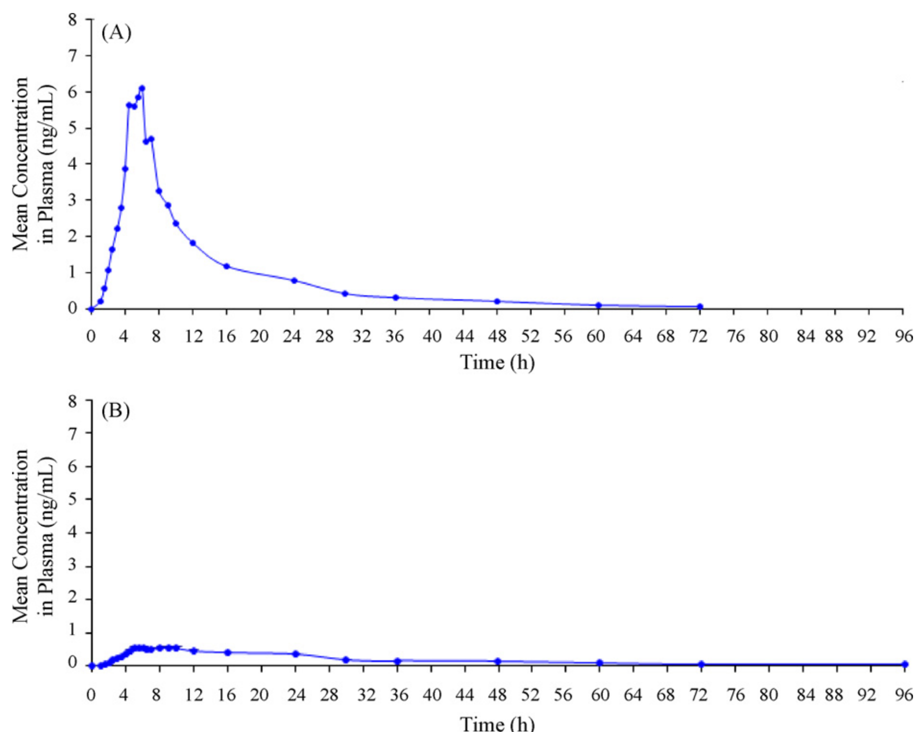

Fig. 4. Mean plasma concentration–time profile of trospium in human plasma following oral dosing of 60 mg trospium capsules to 18 subjects under (A) fasting and (B) fed conditions.

can be brought down at least five times by using the present method.

5. Conclusions

In summary, we have developed and validated a highly sensitive, specific and reproducible LC–MS/MS assay to quantify trospium in human plasma. From the results of all the validation parameters, we can conclude that the present method can be useful for pharmacokinetic studies with desired precision and accuracy.

Acknowledgements

The authors wish to thank the management of Dr. Reddy's Laboratories Limited, Hyderabad for permitting this work to be published. Co-operation extended by all colleagues of Clinical Pharmacology and Pharmacokinetics is gratefully acknowledged.

References

[1] A. Pfeiffer, T. Schmidt, T. Höller, H. Herrmann, C. Pehl, B. Wendl, H. Kaess, Eur. J. Clin. Pharmacol. 44 (1993) 219.
[2] G. Schladitz-Keil, H. Spahn, E. Mutschler, Drug Res. 36 (1986) 984.
[3] O. Doroshyenko, A. Jetter, K.P. Odenthal, U. Fuhr, Clin. Pharmacokinet. 44 (2005) 701.
[4] K. Biastre, T. Burnakis, Ann. Pharmacother. 43 (2009) 283.
[5] Prescribing information SANCTURA XR.
[6] G. Schladitz-keil, H. Spahn, E. Mutschler, J. Chromatogr. 345 (1985) 99.
[7] X. Hong-lin, D. Jin-song, T. Zhi-rong, Y. Guo-ping, Chin. Hosp. Pharm. J. 27 (2007) 451.
[8] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001, Center for Veterinary Medicine (CV), May 2001. http://www/fda.gov/cder/guidance/index.htm.
[9] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.
[10] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135.