SHIMADZU
Excellence in Science

Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Rapid, Sensitive and Direct Quantitation of Tiotropium at sub-pg/mL in Plasma using Shimadzu LCMS-8060

Avinash Gaikwad[1], Chaitanya krishna Atmakuri[1], Yogesh Arote[1], Jitendra Kelkar[2], Pratap Rasam[2]
[1]ADC - Shimadzu Analytical (India) Pvt. Ltd., [2]Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Rapid, simple, and most sensitive method with LLOQ of 0.2 pg/mL
- Low plasma volumes in sample extraction extends the life of mass spectrometer
- Single step sample extraction method increased sample productivity

1. Introduction

Tiotropium is an inhaled long-acting anti-cholinergic for the maintenance treatment of COPD (chronic obstructive pulmonary disease) [1,2]. It is chemically described as (1α, 2β, 4β, 5α, 7β) -7- [(Hydroxydi-2-thienylacetyl) oxy] – 9.9 – dimethyl - 3 - oxa - 9 - azoniatricyclo [3.3.1.0] nonane bromide monohydrate. It is a synthetic, non-chiral, quaternary ammonium compound [3]. Structure of tiotropium and tiotropium D3 bromide is provided in Fig. 1 and Fig. 2.

The long duration of action with tiotropium is owing to prolonged, competitive binding to M (3) muscarinic receptors. Tiotropium is administered in the form of dry powder inhalation which results in very low systemic bioavailability. This translates into significant challenges to develop a sensitive and reproducible bioanalytical method that can reliably measure plasma levels of tiotropium at very low expected levels. The required LLOQ for most inhalation dosed medications is typically in the range of pg/mL to sub pg/mL. Several analytical methods have been developed to determine tiotropium in biological samples using HPLC with tandem mass spectrometric detection (LC–MS/MS), with the lowest reported LLOQ of 0.500 pg/ml [4–5]. These methods fall short of the ideal target sensitivity required by the intended low dose studies, and this motivated us for the current study. The main aim of this work is to develop a LC-MS/MS method at sub-picogram level (LLOQ - 0.2pg/mL) to support regulatory studies.

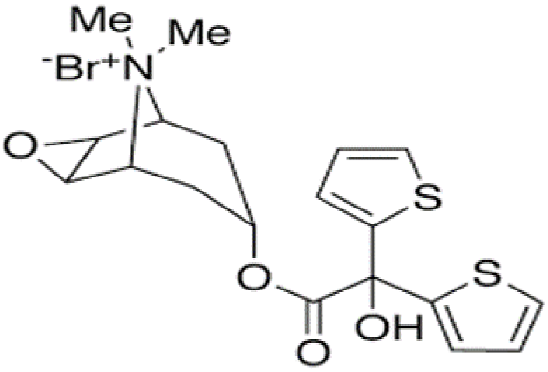


Fig. 1 Structure of Tiotropium

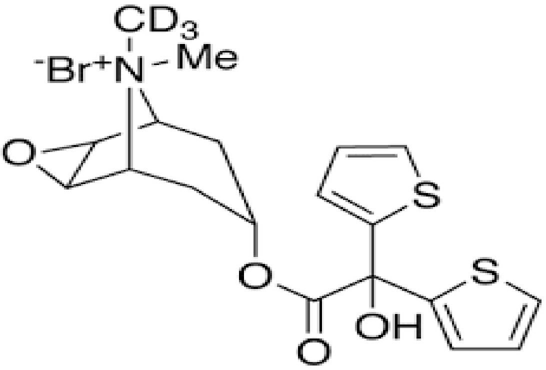


Fig.2 Structure of Tiotropium-D3 Bromide

2. Salient Features

- Quantitative method for estimation of tiotropium in human plasma was developed and validated as per the US major guidelines
- Effective throughput for quantitative assessment is increased by use of a single step extraction procedure
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission, respectively. This ensures sensitive and selective quantification of tiotropium at 0.2 pg/mL
- Optimum plasma volume avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer
- Customized gradient elution satisfied analyte peak shape, retention time and eliminated matrix effect
- Ready to use validated method as per the US major guidelines.

Table 1 Method Validation Summary

Calibration curve range	0.20 pg/mL to 200.00 pg/mL		
Intraday precision and accuracy (For LLOQ QC)	Accuracy (% Nominal)	112.96	
	Precision (% RSD)	10.03	
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	102.88 to 109.00	
	Precision % RSD)	1.35 to 4.34	
Global precision and accuracy (For LLOQ QC)	Accuracy (% Nominal)	106.92	
	Precision % RSD)	18.63	
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	102.84 to 105.35	
	Precision (% RSD)	6.35 to 10.40	
Global % recovery	Recovery (%)	57.61	
	Precision % RSD)	8.66	
Matrix effect	IS normalized Matrix Factor	0.86	
Bench top stability in matrix (6.0 hrs.)	% Change	LQC=-1.33	HQC=-12.57
	Accuracy (% Nominal)	LQC=103.25	HQC=87.86
	Precision (% RSD)	LQC=3.25	HQC=-12.14
Auto sampler stability in matrix (30.0 hrs.)	% Change	LQC=-8.15	HQC=-12.69
	Accuracy (% Nominal)	LQC=96.11	HQC=87.74
	Precision (%RSD)	LQC=-3.89	HQC=-12.26
Freeze thaw stability in matrix (Third Cycle)	% Change	LQC=-0.88	HQC=-1.06
	Accuracy (% Nominal)	LQC=103.72	HQC=99.42
	Precision (%RSD)	LQC=3.72	HQC=-0.58

3. Experimental

3.1. Sample preparation and analytical conditions

To a 450 µL aliquot of human plasma, 50 µL tiotropium – D3 and 400 µL of buffer was added followed by SPE purification. Samples were loaded on the preconditioned C-18 SPE cartridge. The loaded samples were washed with water, 5% methanol and eluted in 50% methanol in water. The eluent was directly injected on LCMS for analysis.

3.2. Instrument parameters on LCMS-8060

Refer Table 2 for analytical conditions and instrument parameters and Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-packTM Velox C18 column 100 x 2.1 mm, 2.7 µm (P/N: 227-32015-03)
Mobile Phase	A: 0.1% formic acid in water B: 0.1% formic acid in Acetonitrile
Flow Rate	0.2 mL
Oven Temp	50 ˚C
Injection	40 µL
Parameter	**MS**
Interface	ESI
Interface temp and Voltage	1 KV and 400 ˚C
MS Mode	MRM, Positive
Heat Block Temp	500 ˚C
DL Temp	200 ˚C
CID Gas	400
Nebulizing Gas	3
Drying Gas	10

Table 3 MRM transition and parameters of Tiotropium on LCMS

Compound	MRM (m/z)	CE (V)
Tiotropium	391.95-152.05	-30
TiotropiumD3	395.00-155.20	-30
TiotropiumD3	395.00-173.10	-32

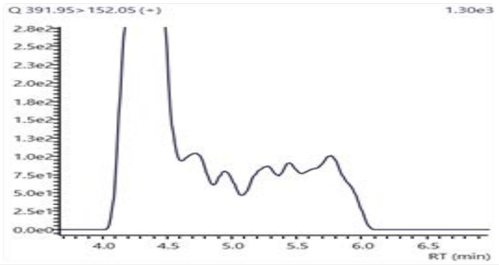
4. Result and Discussion

4.1. Method Development

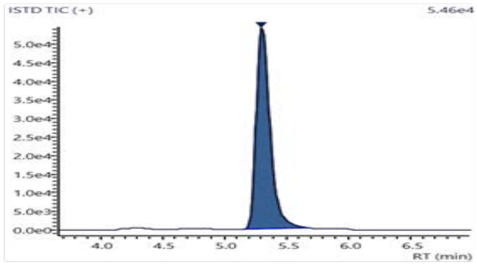
The mobile phase composition was optimized to limit chromatographic peak tailing phenomena and to achieve good resolution between the analyte and interfering matrix peaks. Finally, 0.1% formic acid in water and 0.1% formic acid in acetonitrile (80:20, v/v) was used as a mobile phase to provide acceptable peak shapes, low background noise, shorter retention times, and good resolution. Representative chromatograms of extracted plasma blank, extracted Tiotropium D3, and extracted LLOQ are shown in Figure 3. For both Tiotropium and Tiotropium D3, the total analysis run time was 7.0 minutes and the analyte retention time was 5.4 minutes.

Since tiotropium has a quaternary ammonium in its structure, positive-ion monitoring mode was adopted for LC-MS analysis. Additionally, tiotropium is ready to form tiotropium cations in the mobile phase, providing abundant positive ions in the ESI method. Therefore, we chose the ESI mode for tiotropium measurements. ESI produced an abundant molecular ion at $[M]^+$ m/z 391.95 for tiotropium in SIM mode. Tiotropium produced two intense product ions at m/z 152.05 and m/z 170.2 in MRM mode, however the product ion m/z 152.05 was more abundant than m/z 170.2. Thus, the transition m/z 391.95 → m/z 152.05 was used to quantify tiotropium. Internal standard tiotropium D3 produced two intense product ion m/z 155.2 and m/z 173.10 and both were selected for summation of peak area response.

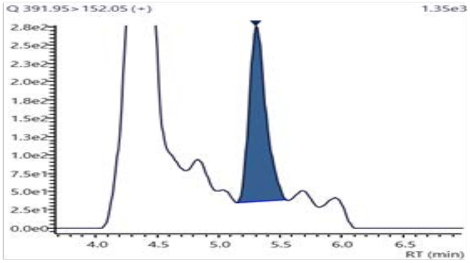
Various sample extraction techniques such as liquid-liquid Extraction, protein precipitation and SPE have been attempted to separate the analyte of interest from the matrix component. With the SPE technique outlined above, gave promising results compared to published methods.



Extracted Blank



Extracted IS



Extracted LLOQ

Fig. 3 Chromatograms of Tiotropium

4.2. Method Validation

The method was validated for linearity, accuracy, precision, selectivity, matrix effect, recovery, and stability according to the US major guidelines (for summary of results refer table 1). Linearity was assessed by weighted linear regression (1/x2) of analyte-internal standard peak area ratios. Accuracy and precision were determined using QC samples at 0.20, 0.60, 10.00 and 150.00 pg/mL in plasma. Intra- and inter-day precisions were found less than 15%, and the accuracy within±15% (refer Tables 5 & 6). Precision at LLOQ level was found <20% and accuracy within ±20%. Lower limit of quantification sample showed signal-to-noise ratio of 20:1.

Selectivity

Selectivity of the method was assessed in different lots of blank human plasma. Interference from blank plasma was assessed for both tiotropium and tiotropium D3. No significant interference was observed at the retention time and MRM transition of tiotropium and tiotropium D3 as shown in Table 4 below.

Table 4 Selectivity

Tiotropium			
Plasma lot no.	Area in blank matrix	LLOQ area	% Interference
V3071	159	1,362	11.67
V1889	267	1,416	18.86
V1789	208	1,912	10.88
V1166	0	1,371	0.00
V3074	374	2,051	18.24
V3077	168	1,267	13.26

Table 4 Selectivity (continued)

Tiotropium-D3			
Plasma lot no.	Area in blank matrix	Area	% Interference
V3071	205	93,692	0.22
V1889	243	86,611	0.28
V1789	638	89,217	0.72
V1166	690	89,838	0.77
V3074	574	91,397	0.63
V3077	864	89,865	0.96

Linearity

Calibration curve was found linear from 0.20-200.00 pg/mL (refer figure 4) The goodness of fit was consistently greater than 0.99 during validation. Signal to noise ratio (s/n) at LLOQ level was found more than 20:1, across 6 PA batches. Representative chromatogram is shown in Fig. 3.

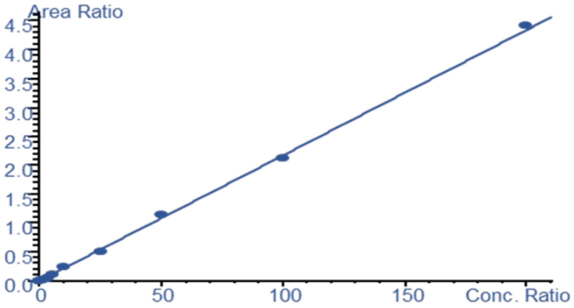


Fig. 4 Calibration curve

Intra-day and Inter-day accuracy and precision

Intraday precision and accuracy were evaluated by processing 6 replicates of LLOQQC, LQC, MQC and HQC in a single P&A batch. Quantitative data is summarized in Table 5.

Table 5 Intra-day precision and accuracy

QC (n=6)	**LLOQQC**	**LQC**	**MQC**	**HQC**
Nominal concentration (pg/mL)	0.20	0.60	10.00	150.00
Precision Accuracy Batch	0.22	0.68	10.22	153.93
	0.23	0.6	10.56	151.71
	0.21	0.68	10.10	156.62
	0.24	0.66	10.47	152.21
	0.26	0.65	10.50	156.48
	0.19	0.65	10.49	154.98
Mean	0.22	0.65	10.39	154.32
Standard deviation	0.02	0.03	0.18	2.09
% CV	10.03	4.34	1.78	1.35
% Nominal	112.96	109.00	103.89	102.88

Global precision and accuracy

Precision and accuracy experiments were evaluated at LLOQ, LQC, MQC and HQC level in 5 batches. Excellent accuracy and repeatability were observed with %RSD less than 15 % and % accuracy within ±15% at LQC, MQC and HQC level. At LLOQQC level, the %RSD was found less than 20 % and % accuracy within ±20%. Global precision and accuracy results are presented in Table 6.

Table 6 Global precision and accuracy

QC level (n=30)	**Mean Conc. (pg/mL)**	**%RSD**	**% Accuracy**
LLOQ QC (0.20 pg/mL)	0.21	18.63	106.92
LQC (0.60 pg/mL)	0.63	10.40	105.35
MQC (10.00 pg/mL)	10.31	6.48	103.13
HQC (150.00 pg/mL)	154.26	6.35	102.84

Matrix factor

Matrix factor was evaluated by comparing mean peak area ratio of QC sample in presence of matrix compared with mean peak area ratio of QC samples in absence of matrix. Post-extracted QC samples were prepared by extracting blank plasma from six different human plasma lots, followed by reconstitution with aqueous LQC and HQC samples. Post-extracted quality control samples and neat solution of quality control samples at LQC and HQC levels were analyzed on LCMS system. The IS normalized matrix factor at LQC and HQC levels were found 0.85 and 0.87 respectively, which is within acceptance criteria.

Recovery

Recovery experiments were conducted to evaluate precision, reproducibility and consistency of the analyte(s) at LQC, MQC and HQC level. Global recovery for tiotropium was found 57.61 % with precision less than 15%. Recovery of tiotropium was found to be precise, consistent, and reproducible at all QC levels.

Table 7: Recovery

QC level	Recovery
LQC	52.09
MQC	58.95
HQC	61.79
Mean	**57.61**
SD	**4.99**
% CV	**8.66**

Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

5. Conclusion

LCMS-8060, along with special sample preparation method, optimized chromatography provides a very selective and sensitive method for bioanalysis of Tiotropium in human plasma. Ultra-high speed and high-separation analysis was achieved on Nexera^{TM} X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.2 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

1. https://www.drugbank.ca/drugs/DB01409 (accessed Jan 02, 2020)
2. https://en.wikipedia.org/wiki/Tiotropium_bromide (accessed Jan 02, 2020)
3. https://www.rxlist.com/spiriva-drug.htm#description (accessed Jan 02, 2020)
4. Jiang Wang, Yao Jiang, Yingwu Wang, Hao Li, J Paul Fawcett, Jingkai Gu, Highly sensitive assay for tiotropium, a quaternary ammonium, in human plasma by high-performance liquid chromatography/tandem mass spectrometry, Rapid Communications in Mass Spectrometry. 21(11) 2007 1755-1758.
5. https://issx.confex.com/issx/intl11/webprogram/Paper35910.html (accessed Jan 02, 2020).

06-SAIP-LC-057-EN First Edition: May. 2023

Shimadzu Corporation
www.shimadzu.com/an/

Shimadzu Analytical (India) Pvt.Ltd.
www.shimadzu.in

Related Products Some products may be updated to newer models.

LCMS-8060

High Performance Liquid Chromatograph Triple Quadr…

Related Solutions

- Small Molecule Pharmaceutical
- Life Science

Price Inquiry

Product Inquiry

Technical Service / Support Inquiry

Other Inquiry