



Quantification of Tiotropium in Human Plasma with a 1 pg/mL LLOQ using HPLC and LC/MS/MS

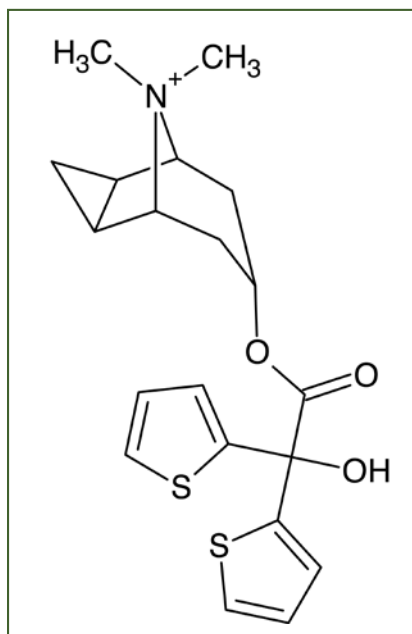
Authors

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Introduction

Tiotropium is a pharmaceutical drug used in the management of chronic obstructive pulmonary disease. Its structure consists of a tropane ring with a quaternary amine that also has an epoxide functional group and two aromatic thiophene rings. Tiotropium is an antagonist of the muscarinic receptor and although it is not a selective muscarinic antagonist, its topical application acts mainly on the M₃ muscarinic receptor located on the smooth muscle cells and submucosal glands to produce a bronchodilatory effect.

FIGURE 1: Chemical Structures of Tiotropium





Methodology

SAMPLE PREPARATION AND EXTRACTION

1. Aliquot 400 μ L of samples to corresponding wells of a 96-well deep well plate
2. Add 50.0 μ L working internal standard [600 pg/mL of Tiot-d3].
3. Use Tomtec® to transfer samples from the 96 well plate to a preconditioned Strata™-X-CW SPE plate
4. Wash with aqueous and organic solvent.
5. Elute sample with 400 μ L of elution buffer.
6. Evaporate and reconstitute.

CHROMATOGRAPHIC CONDITIONS

Column: Luna PFP 5 μ m, 2.0 x 50 mm
Mobile Phase: A: 0.1% FA in (5 mM Ammonium acetate)
B: MeOH
LC program: isocratic
Injection volume: 20 μ L
Column temperature: 30°C
Flow rate: 0.400 mL/min
AS Temperature: RT

MASS SPECTROMETER CONDITIONS

Instrument: AB Sciex API 5000™
Ionization mode: Turboionspray, Positive ion mode
Source Temperature: 500°C
SRM transitions: Tiotropium 392.1 \rightarrow 152.1
Tiotropium-d3 395.1 \rightarrow 155.1

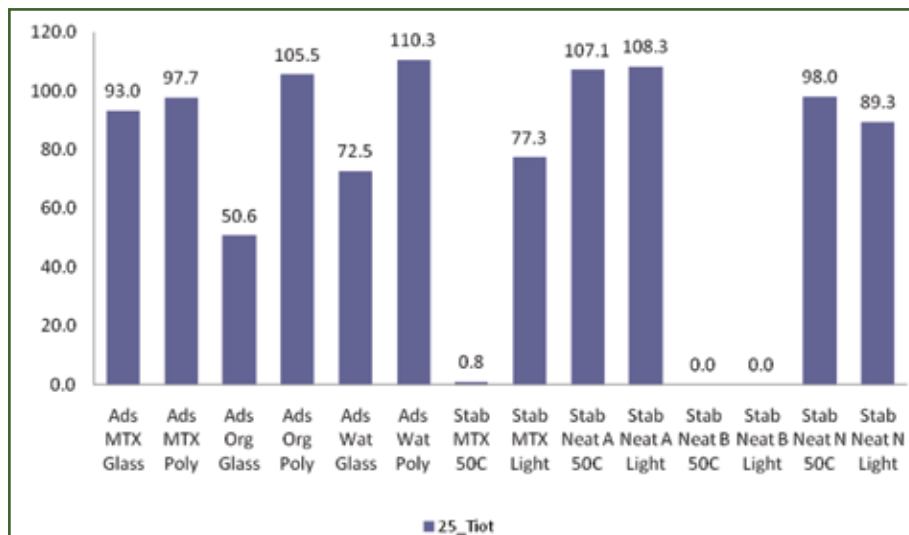


Results and Discussion

METHOD DEVELOPMENT

- **Chromatography development:** Retention proved to be a problem with C18 and silica columns with both low and high pH due to the polarity of the quaternary amine. We then focused on using the aromatic functionality and after testing a biphenyl and a C6 phenyl column, found that Tiotropium still eluted in the void volume. We then switched to a PFP column and had great retention with acidic conditions and isocratic conditions.
- **Stickiness evaluation:** There are no stickiness issues in plasma, for neat solution, or with polypropylene, though a glass container is problematic.
- **Stability evaluation:** The stability profile showed that Tiotropium was unstable in plasma at high temperature. It was also not stable in neat basic solution conditions and formed the N-demethyl product. (Figure 2).
- **Sample extraction:** The standard extraction preparation screen experiment yielded unexpected results with poor recovery for cation exchange. Since the compound contains a quaternary amine we had expected the cation exchange sorbent to show good recovery and selectivity for the compound. In order to get retention of the sorbent we switched the conditioning step to basic conditions to remove the proton on the sorbent's carboxylic acid and then load the extract with neutral conditions to achieve the ionic interaction. Elution was then accomplished with acidic conditions to protonate the sorbent and selectively releases the Tiotropium.

FIGURE 2: Stickiness and Stability Test





Results and Discussion (continued)

FIGURE 3: Sample Preparation Screening Experiment

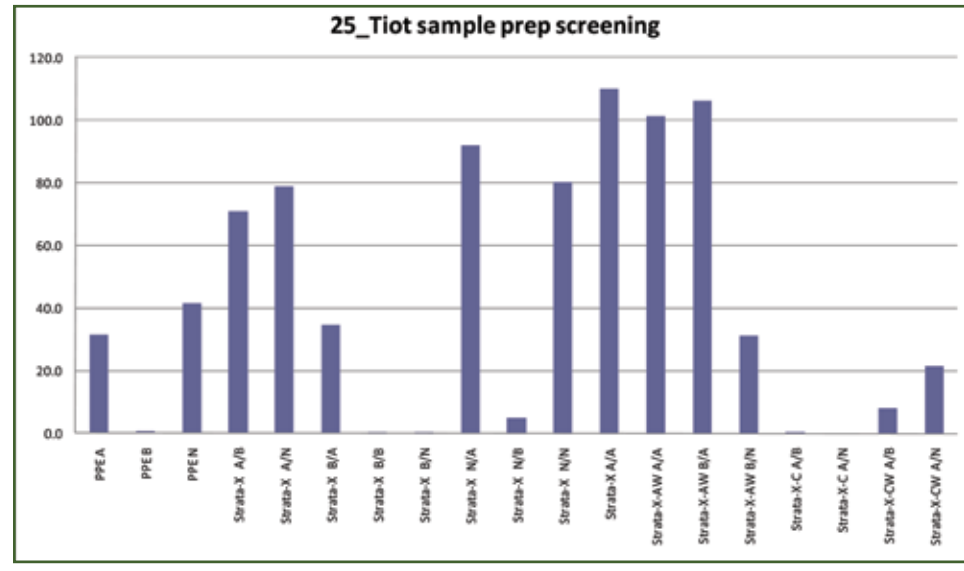
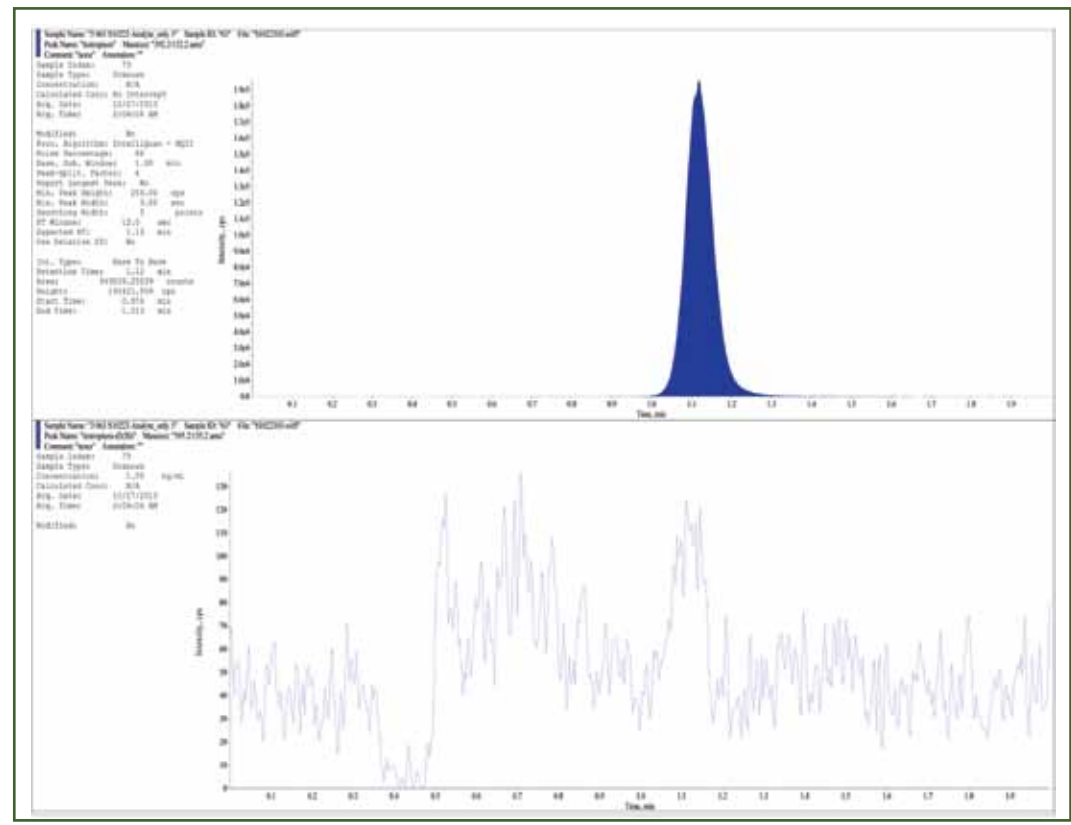


FIGURE 2: Analyte only at High Standard Without Internal Standard (400 pg/mL)





Results and Discussion (continued)

FIGURE 3: Internal Standard Only

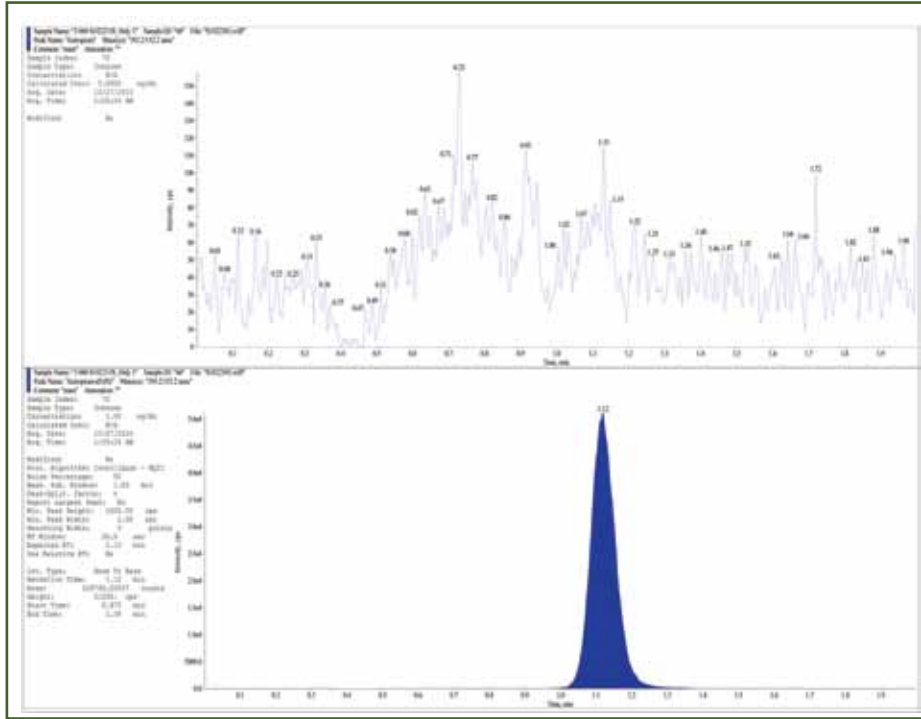
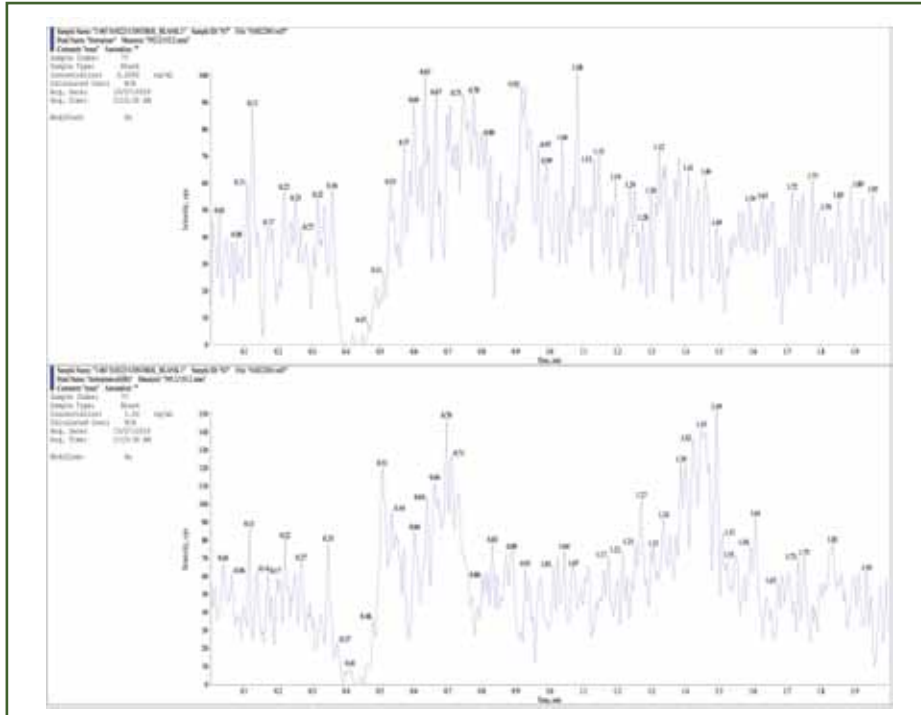


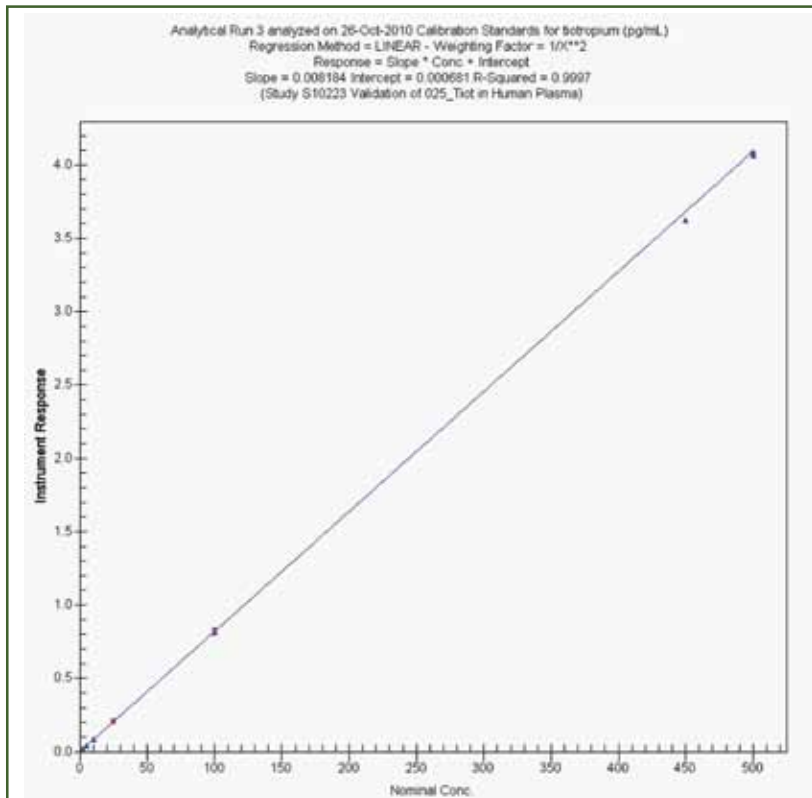
FIGURE 4: Representative Plasma Blank Control





Results and Discussion (continued)

FIGURE 7: Representative Calibration Curve



ASSAY VALIDATION

- **Accuracy/precision:** Demonstrated at n=6 at LLOQ, Low, Medium, High concentrations over 3 validation runs. (Tables 1-2)
- **Selectivity:** Demonstrated with blank and low QC concentrations in six sources of human plasma. (Tables 3)
- **Ability to dilute:** Demonstrated above ULOQ at DF=10 and DF=100. (Table 4)
- **Extraction recovery:** Evaluated for analyte at Low, Medium, High concentrations and IS at working concentration (n=6). (Table 5)
- **Matrix factor:** Evaluated for analyte at medium QC concentration and IS at working concentration (n=6). (Table 6)
- **Carryover:** Evaluated in each run. No carryover present.



Results and Discussion (continued)

- **Stock solution stability:** Room temperature for analytes and the I.S. for 6 hrs and 104 days at 1-8°C in DMF
- **Stability in matrix:** Freezing/thawing, 4 cycles
Room temperature, 6hrs
21 days at -70°C and -20°C
- **Reinjection Reproducibility:** Batch reinjection stability for 146 hours at RT

TABLE 7. Back-Calculated Concentrations of Calibration Standards for Tiotropium
(All concentrations are expressed as pg/mL)

Nominal Conc.	1.00	200	5.0	10.0	25.0	100	450	500
Mean	1.01	1.97	4.95	9.95	25.4	103	442	496
S.D.	0.0371	0.110	0.167	0.189	0.463	2.76	7.80	7.31
%CV	3.7	5.6	3.4	1.9	1.8	2.7	1.8	1.5
%Bias	1.0	-1.5	-1.0	-0.5	1.6	3.0	-1.8	-0.8
n	6	6	6	6	6	6	6	6

TABLE 2. Intra- and Inter-Assay Accuracy and Precision of Quality Control Samples for Tiotropium (ANOVA)

	LLOQ	Low	Medium	High
Mean Observed Conc.	0.942	3.02	203	400
%Bias	-5.8	0.7	1.5	0.0
Between Run Precision (%CV)	9.1	0.0	1.1	0.6
Within Run Precision (%CV)	7.4	5.5	1.1	1.2
Total Variation (%CV)	11.7	5.3	1.6	1.3
n	18	17	18	18
Number of Runs	3	3	3	3



Results and Discussion (continued)

TABLE 3. Selectivity at the Low QC Concentration for Tiotropium

Run Date	Run Number	Low QC 3.00 pg/mL
26-Oct-2010	3	2.81
		3.05
		2.96
		3.06
		3.00
		2.98
Mean		2.98
S.D.		0.0905
%CV		3.0
%Theoretical		99.3
%Bias		-0.7
n		6

TABLE 4. Dilution Quality Control Samples for Tiotropium

	Dilution QC 2000 pg/mL DF=10	Dilution QC 20000 pg/mL DF=100
	1840	18600
	1920	18900
	1940	19700
	1910	19200
	1920	19100
	1930	19800
Mean	1910	19200
S.D.	35.8	462
%CV	1.9	2.4
%Theoretical	93.5	98.8
%Bias	-6.5	-1.3
n	6	6

TABLE 5. Relative Extraction Recovery for Tiotropium and Tiotropium-d3 (IS)

	Low (3.00 pg/mL)	Medium (200 pg/mL)	High (400 pg/mL)
Mean Recovery for Tiotropium	98.2%	93.2%	95.3%
Recovery for Tiotropium-d3	95.5%		

TABLE 6. Matrix Factor for Tiotropium and Tiotropium-d3 (IS)
(Results are expressed as area counts)

	MF Extracted Tiotropium	MF Neat Tiotropium	MF Extracted Tiotropium-d3	MF Neat
Mean	424136	405557	248892	243225
S.D.	12101	15367	8303	9365
%C.V.	2.9	3.8	3.3	3.9
n	6	6	6	6
Matrix Factor	1.0		1.0	



Conclusion

A robust, sensitive and high throughput method was developed and validated to quantitatively analyze tiotropium in human plasma.