A streamlined SPE-LC-HRMS workflow for sub-pg/mL quantitation of tiotropium in human plasma

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This technical note demonstrates a sensitive quantitation workflow for tiotropium, a long-acting bronchodilator, in human plasma on the ZenoTOF 8600 system. A lower limit of quantitation (LLOQ) was reached at 0.05 pg/mL using a solid phase extraction (SPE) method in a 96-well format (Figure 1).

Tiotropium is an inhaled long-acting muscarinic antagonist used for maintenance treatment of obstructive airway diseases. Despite its favorable positive-ion MS response as a permanently charged quaternary ammonium compound, bioanalysis remains challenging due to its low systemic exposure[2] and poor chromatographic behavior.[3] Reliable characterization of the pharmacokinetic profile requires the quantitation of tiotropium at sub-pg/mL concentrations, particularly at later time points when circulating drug levels rapidly approach the quantitation limit.[2]

Here, a streamlined LC-MS workflow for tiotropium quantitation in human plasma, combining weak cation-exchange (WCX) extraction and conventional LC separation with the sensitivity and selectivity of the ZenoTOF 8600 system is demonstrated.[1]

Key benefits for tiotropium quantitation using the ZenoTOF 8600 system

- **Low level of quantitation:** Reach an LLOQ as low as 0.05 pg/mL in human plasma using the ZenoTOF 8600 system
- **Robust analytical performance:** Achieve accurate and highly reproducible (%CV <6) quantitative performance at all concentration levels
- **Streamlined data management:** Easily acquire, manage, and process data using SCIEX OS software, a 21 CFR Part 11-compliant platform

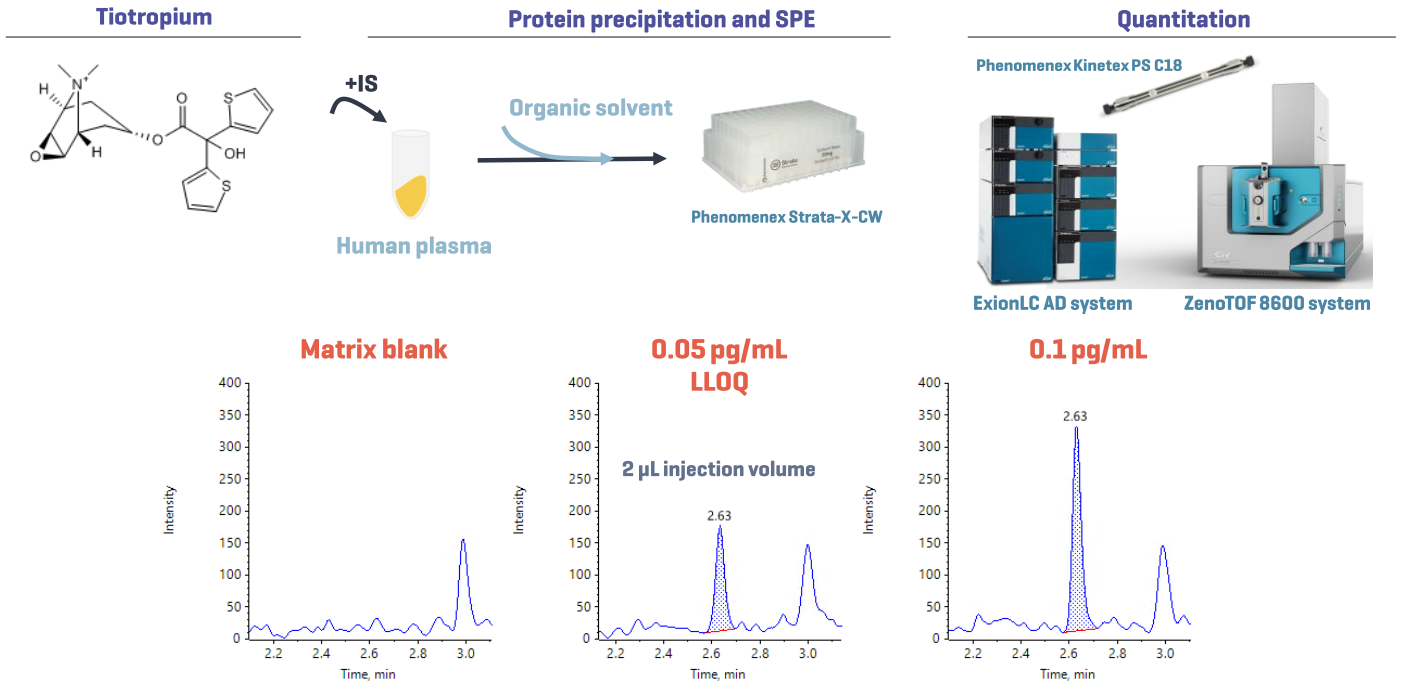

Figure 1. Overview of the quantitation workflow of tiotropium in human plasma on the ZenoTOF 8600 system. Tiotropium bromide was spiked in human plasma. WCX-based SPE was used to separate the analyte from the plasma matrix. Representative extracted ion chromatograms (XICs) from blank, LLOQ, and a low concentration are shown. An LLOQ of 0.05 pg/mL was achieved for the quantitation of tiotropium. No interference was detected in the matrix blank. Ipratropium was used as an internal standard (IS).

Introduction

Tiotropium is administered as an inhaled therapy, resulting in low pg/mL systemic plasma exposure. As plasma concentrations can decline rapidly after dosing, insufficient low-end sensitivity can lead to missing or poorly defined PK time points, limiting accurate characterization of exposure, elimination, and bioequivalence.[2,4] Despite favorable ionization behavior associated with its permanently charged quaternary ammonium structure, tiotropium remains challenging to quantify at sub-pg/mL levels due to chromatographic and matrix-related challenges. At these concentrations, chromatographic performance becomes critical because poor retention, peak broadening, matrix interference, nonspecific adsorption or carryover can directly compromise sensitivity, reproducibility, and quantitative accuracy. Therefore, sensitive and robust LC-MS methods are required to achieve sub-pg/mL level LLOQs for reliable pharmacokinetic characterization in complex biological matrices.

Efficient sample cleanup is critical for bioanalysis requiring sub-pg/mL sensitivity. In this workflow, WCX SPE was used to isolate tiotropium from human plasma through cation-exchange interaction between its permanently charged quaternary ammonium group and the carboxylate sites of the WCX sorbent. This selective retention enables strong organic washes for matrix cleanup, while acidic elution disrupts the ion-exchange interaction, thereby supporting efficient recovery.

A Phenomenex Kinetex PS C18 column was selected to improve chromatographic performance for tiotropium. The C18 phase provides reversed-phase retention, while the positively modified surface helps minimize secondary interactions between cationic tiotropium and negatively charged sites on the stationary phase. Reducing these interactions supports improved peak shape, reproducibility and carryover performance, which are critical for reliable sub-pg/mL quantitation.

The HRMS-based workflow plays a critical role in addressing matrix-related selectivity challenges that can remain even after SPE cleanup and chromatographic optimization. High mass resolution and accurate-mass detection enhance selectivity by enabling better differentiation of the analyte from co-eluting matrix components. This capability is especially valuable for reliable low-level quantitation in complex biological matrices, making HRMS an increasingly attractive option for quantitative bioanalysis in challenging matrices. The ZenoTOF 8600 system provides the selectivity needed to distinguish tiotropium from endogenous interferences in human plasma. Improved front-end design enhances sensitivity through efficient ion generation, capture, and transmission, while advanced TOF optics support robust detection across a wide concentration range.[1] Together, these workflow elements enable reliable quantitation of tiotropium in human plasma across 3.6 orders of magnitude, with an LLOQ of 0.05 pg/mL.

Methods

Commercially available tiotropium bromide and ipratropium bromide monohydrate (IS) were reconstituted in DMSO and diluted in 70:30 (v/v) methanol:water containing 2% formic acid. Tiotropium was spiked into 100 µL of human plasma at concentrations ranging from 0.05 pg/mL to 200 pg/mL. Ipratropium was used as an internal standard and was spiked at 10 pg. Protein precipitation was performed using 200 µL of methanol. Supernatant was diluted with water (pH 6.5) to reach a final 15% organic content before loading on the Phenomenex WCX microelution SPE plate. The plate was activated and equilibrated with methanol and water (pH 6.5), respectively. Wells were washed with water (pH 6.5), then with 100% methanol. Elution was performed using methanol containing 5% formic acid. Eluents were dried under a nitrogen flow at 40°C and reconstituted in 50 µL of 1% formic acid in methanol and diluted in 50 µL of water.

Chromatography: Sample separation was performed using an ExionLC AD system (SCIEX) at a flow rate of 0.4 mL/min on a Phenomenex Kinetex PS C18 column (2.1 x 50 mm, 2.6 µm, 100 Å). The column temperature was maintained at 55°C. An 8-minute gradient was run using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B (Table 1). An injection volume of 2 µL was used for analysis. A 1:1:1 (v/v/v) acetonitrile/methanol/water containing 0.02% difluoroacetic acid (DFA) was used as the needle wash solvent.

Table 1. LC gradient conditions.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	98	2
0.5	98	2
3.5	45	55
4.5	5	95
6	5	95
6.1	98	2
8	98	2

Mass spectrometry: Analysis was performed on the ZenoTOF 8600 system. The optimized parameters are listed in Table 2 for source and gas and in Table 3 for MRM^{HR}.

Table 2. Source and gas parameters.

Parameter	Value
Polarity / Workflow	Positive / Small molecule
Spray voltage	3500 V
Ion source gas 1	50 psi
Ion source gas 2	60 psi
Curtain gas	45 psi
Source temperature	500 °C
CAD gas	9

Table 3. MRM^{HR} parameters on the ZenoTOF 8600 system.

Parameter	Value
Method duration	8 min
TOF MS start-stop mass	250 - 1000 Da
TOF MS accumulation time	0.05 s
QJet DP	30 V
TOF MS/MS start-stop mass	80 – 500 Da
TOF MS/MS Accumulation time	0.06 s
Zeno threshold	200000 cps
Collision energy (CID)	50 V
Tiotropium Q1/Q3 mass	392.1 >> 170.118* Da 392.1 >> 152.107 Da
Ipratropium Q1/Q3 mass	332.20 >> 166.159 Da

**Transition used for quantitation*

Data processing: Data collection and analysis were performed using SCIEX OS software, version 4.0. Peaks were integrated using the MQ4 algorithm. Peak width was set to 0.05 Da for analyte and IS. Peak area ratio was used, and a weighting of $1/x^2$ was applied for tiotropium quantitation.

Quantitation of tiotropium on the ZenoTOF 8600 system

This technical note demonstrates streamlined sample preparation and sensitive quantitation of tiotropium in human plasma using the ZenoTOF 8600 system.An LLOQ was reached at 0.05 pg/mL for tiotropium. No interferences were observed in the human plasma matrix blank (Figure 1).

The calibration range for tiotropium was between 0.05 and 200 pg/mL (Figure 2). Linearity was achieved with a coefficient of determination (r^2) of ≥0.994, across a linear dynamic range (LDR) of 3.6 orders of magnitude.

Analytical performance was evaluated for accuracy and precision. The accuracy of the calculated mean was expected to be between 80% and 120% at the LLOQ and between 85% and 115% at higher concentrations. The %CV of the calculated mean for each concentration was expected to be <20% at the LLOQ.[5]

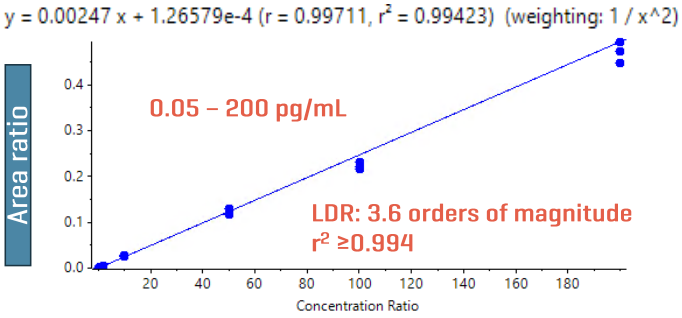

Figure 2. Calibration curve of tiotropium. The area ratio between tiotropium and ipratropium (IS) was used to generate a calibration curve. Good linearity was observed with r^2 ≥0.994 for tiotropium.

The assay accuracy was within ±8% of the actual concentration, and the %CV was less than 6% at all concentrations. The calculated percentage accuracy and %CV values were within the acceptance criteria at each concentration level (Figure 3).

Actual Co...	Num. Values	Mean	Standard...	Percent CV	Average Accuracy across...
0.050	3 of 3	0.050	0.003	5.76	101.
0.100	3 of 3	0.097	0.003	3.33	96.8
0.500	3 of 3	0.522	0.017	3.30	104.
2.000	3 of 3	2.098	0.081	3.85	105.
10.000	3 of 3	10.888	0.161	1.47	109.
50.000	3 of 3	49.085	2.778	5.66	98.2
100.000	3 of 3	90.243	2.919	3.23	90.2
200.000	3 of 3	191.135	9.079	4.75	95.6

Figure 3. Quantitative performance of tiotropium in human plasma. Reproducibility and accuracy results were determined using the calibration curve standards across 3 replicates at each concentration. Statistical results were summarized using the Analytics module in SCIEX OS software.

Compliance-ready SCIEX OS software

Equivalent SCIEX OS software capabilities for small molecule analysis can be executed on the ZenoTOF 8600 system, ensuring high fidelity when performing method transfers while retaining critical compliance features.

SCIEX OS software is a closed system and requires records and signatures to be stored electronically, meeting the regulations outlined by 21 CFR Part 11. SCIEX OS software can open raw data files from any visible storage location within a closed network by using designated processing workstations.

Figure 4 illustrates the features of SCIEX OS software used to monitor the audit trail, acquire and process data, and configure user access. The audit trail feature enables users to audit critical user actions and locks in data integrity.

The Central Administrator Console (CAC) feature allows users to centralize acquisition and processing using a single platform to maximize efficiency for multi-instrument laboratories, independent of compliance standards. The configuration module allows users to assign roles and access as the administrator, method developer, analyst, and reviewer.

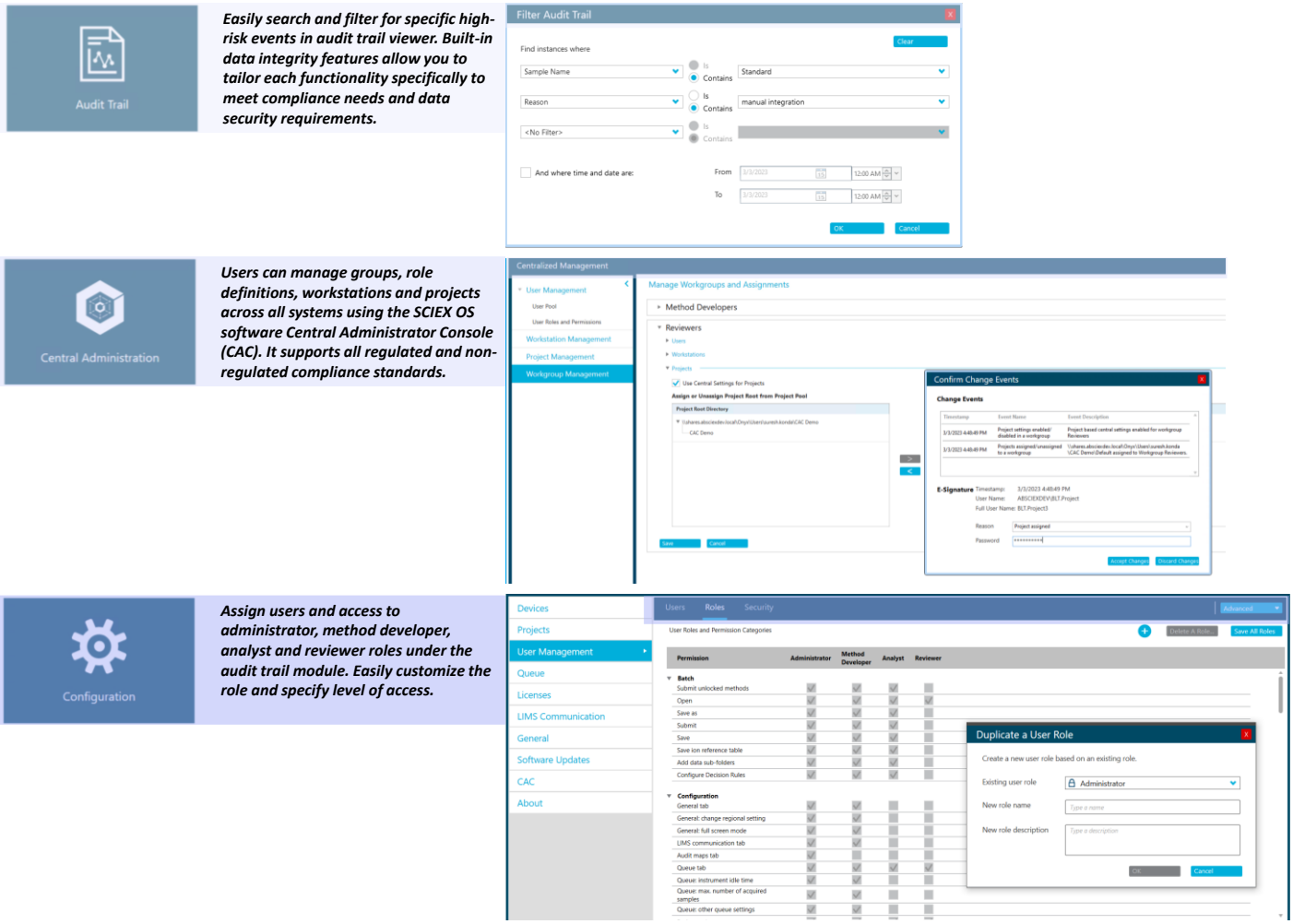

Figure 4. Features of SCIEX OS software for monitoring user access and evaluating the audit trail. The audit trail view allows users to easily filter high-risk events and enables data integrity features to meet compliance requirements. The software features a Central Administrator Console (CAC) to manage users and groups, role definitions, workstations, and projects across all systems. The CAC feature supports both regulated and non-regulated compliance standards. The configuration module enables users to quickly set up roles and levels of access for the administrator, method developer, analyst, and reviewer levels.

Conclusions

- Sensitive tiotropium quantitation was achieved with an LLOQ of 0.05 pg/mL using only a 2 µL injection volume on the ZenoTOF 8600 system
- Linearity was demonstrated across 0.05 to 200 pg/mL, resulting in a wide LDR of 3.6 orders of magnitude (r^2 ≥0.994)
- Robust sample preparation and conventional chromatography, paired with the inherent selectivity of the ZenoTOF 8600 system, supported reliable tiotropium quantitation in human plasma at sub-pg/mL levels
- Accurate and highly reproducible tiotropium quantitation in human plasma was demonstrated on the ZenoTOF 8600 system, with %CV <6 across all concentration levels
- Data management and compliance-readiness (21 CFR Part 11) features were shown using the SCIEX OS software to support small molecule quantitation on the ZenoTOF 8600 system

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