Achieving Lower Limits of Quantitation for Testosterone by LC-MS/MS

Chris Vanselow, Xiaolei Xie, Kristine Van Natta & Joe Di Bussolo, Thermo Fisher Scientific, San Jose, CA

ABSTRACT

Purpose: To demonstrate quantitation of total testosterone in serum/plasma at concentrations below picogram/milliliter (pg/mL) levels without derivatization steps.

Methods: Commercial and donor serum specimens spiked with testosterone-D_3 internal standard (IS) were subjected to liquid-liquid extraction and reconstitution to the same volume of the specimen extracted (no concentration step). The extracts were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with electro-spray ionization. The sensitivities of two MS/MS systems were compared. Testosterone peak areas from neat solutions and serum specimens, which had known amounts of testosterone, were compared to assess the lowest limits of quantitation (LLOQ) and the effects of serum matrix on quantitation limits. On-line solid-phase extraction/concentration methodology was also evaluated to improve LLOQ.

Results: LLOQ for testosterone in neat solutions when making 50 µL injections was 1 pg/mL using the Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer and 0.5 pg/mL using the Thermo Scientific™ TSQ Altis triple quadrupole mass spectrometer. TurboFlow on-line extraction/concentration of 80 µL injections showed LLOQs of 0.5 pg/mL and 0.25 pg/mL for the TSQ Quantis MS and TSQ Altis MS, respectively. However, TurboFlow results exhibited more chemical noise and carryover. Chemical noise, presumably from persistent environmental traces of testosterone and isobaric compounds, prevented reliable quantitation below 0.25 pg/mL using the TSQ Altis MS. Extracts of serum samples exhibited IS peak area that were 30% of those from neat solutions.

INTRODUCTION

Many researchers want to quantitate various steroids in biological fluids at concentrations below pg/mL levels without derivatization steps. Current designs of tandem mass spectrometers (MS/MS) coupled to liquid chromatography (LC) systems can achieve lower limits of quantitation (LLOQ) of 2 pg/mL for testosterone from human blood-plasma/serum specimens subjected to liquid-liquid extraction and concentration. We recently attempted to approach LLOQs down to 0.25 pg/mL by making large injections and by using TurboFlow on-line extraction and concentration coupled to LC-MS/MS.

MATERIALS AND METHODS

Sample Preparation

Fisher Scientific™ Optima™ solvents were used for LC mobile phases, wash solutions as well as calibrator and sample preparations. Testosterone Certified Reference Materials (CRMs) of acetonitrile and stripped serum for clinical research applications and testosterone-D_3 in acetonitrile were purchased from Cerilliant (Round Rock, TX). Neat solutions and calibrators were made from the acetonitrile products using a diluent of 30% methanol in water. Other reagents and consumables were purchased from Fisher Scientific™.

Test Method(s)

200 µL aliquots of commercial and donor serum specimens spiked with testosterone-D3 internal standard (IS) were extracted with methyl tert-butyl ether. Extracts were evaporated and reconstituted with 200 µL of diluent. 50 µL injections were made with Figure 1 parameters into Thermo Scientific™ Accucore™ aQ columns (2.6 µm, 2.1 x 50 mm) on either a Thermo Scientific™ Transcend™ TLX-2 or Prelude™ SPLC dual-channel LC system with TurboFlow™ technology. Mobile phase gradients from 30% methanol in water containing 0.25 mM ammonium fluoride to 100% methanol, using Figure 2 parameters, isolated and eluted the analytes into the heated electro-spray ionization probe on either a TSQ Quantis MS or TSQ Altis MS triple-quadrupole mass spectrometer. Analytes were measured by selected-reaction monitoring of positive-ion transitions for testosterone and IS using Figure 3 parameters. We added on-line extraction/concentration steps utilizing a Thermo Scientific™ Cyclone™ TurboFlow™ column (50 x 0.5 mm) to accommodate 80 µL injections using Figure 4 parameters. Thermo Scientific™ Arija™ MX software, Thermo Scientific™ Xcalibur software and Thermo Scientific™ TraceFinder™ software were used for instrument control and data acquisition.

Data Analysis

TraceFinder was used for data analysis and reporting.

Figure 1. Autosampler method.

	Step Type	Comment
1	Rinse Needle (@Waste) with Wash1 for 2 s	Aqueous rinse
2	Rinse Injector (SEQ.Injector) with Wash1 for 2 s	Aqueous rinse
3	Airgap (10 ul)	Separate sample from washes
4	Get Sample (SEQ.Tray:SEQ.Index): SEQ.Volume	
5	Inject Sample (Syringe Content) to SEQ.Injector	
6	Rinse Needle (@Waste) with Wash1 for 2 s	Aqueous rinse
7	Rinse Injector (SEQ.Injector) with Wash1 for 2 s	Aqueous rinse
8	Rinse Needle (@Waste) with Wash1 for 2 s	Organic rinse
9	Rinse Injector (SEQ.Injector) with Wash2 for 2 s	Organic rinse

Wash 1: Water with 0.1% formic acid
Wash 2: 45% acetonitrile 45% isopropanol 10% acetone

Figure 2. Reversed-phase LC method for testosterone.

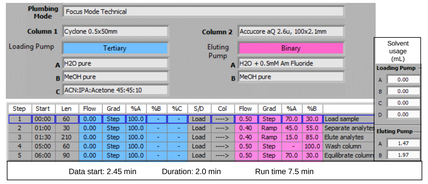


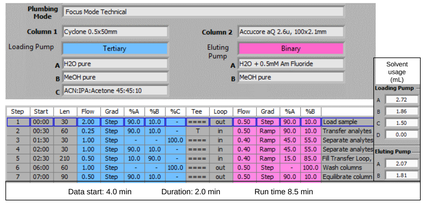
Figure 3. TSQ Altis or TSQ Quantis ESI-MS/MS method for testosterone.

Ion Source Type = H-ESI
Spray Voltage: Positive Ion (V) = 1250
Spray Voltage: Negative Ion (V) = 1500
Sheath Gas (Arb) = 40
Aux Gas (Arb) = 15
Sweep Gas (Arb) = 2
Ion Transfer Tube Temp (°C) = 350
Vaporizer Temp (°C) = 375

Experiment Type: SRM
Cycle Time (sec): 0.400
Chromatographic Peak Width (sec): 6.0
Data Mode: Centroid
Collision Gas Pressure (mTorr): 1.5
Q1 Resolution (FWHM): 0.7
Q3 Resolution (FWHM): 0.7
Source Fragmentation (V): 10.0

Compound Name	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens
Testosterone	0	1.9	Positive	289.2	97.1	23	50
Testosterone	0	1.9	Positive	289.2	109.1	26	50
Testo IS	0	1.9	Positive	292.2	100.1	23	50
Testo IS	0	1.9	Positive	292.2	109.1	26	50
Electro-clean	1.9	2	Negative	292.2	109.1	26	50

Figure 4. TurboFlow – LC method for testosterone.



RESULTS

LLOQs for neat calibrators using LC -, TurboFlow-LC – TSQ Quantis MS

The Transcend TLX-2 – TSQ Quantis system achieved linear quantitation of neat calibrators between 1 and 500 pg/mL (Figure 5) using the LC-MS/MS method without TurboFlow. An interfering peak in Cal 0 required its exclusion from the calibration curve. Cal 1 (0.25 pg/mL) barely passed acceptance criteria and results from multiple injections varied by more than 20% CV. Although Cal 2 (0.5 pg/mL) had a low-enough %difference between calculated and theoretical amounts and passed ion ratio, results from multiple injections showed a positive bias of more than 150%. Cal 3 (1 pg/mL) passed all acceptance criteria including bias and %CV less than 10% (Figure 6). These data give us confidence in achieving a testosterone LLOQ of 1.0 pg/mL in neat solutions using this system.

The TurboFlow method exhibited similar performance with this system. An interfering peak in Cal 0 required its exclusion from the calibration curve. The interference was persistent among multiple blank injections prior to injecting Cal 0 as well as in Cal 1 (0.25 pg/mL), which also had to be excluded from the calibration curve. Apparently, the TurboFlow column enriched a contaminant during equilibration at high flow rate. This contamination limited the LLOQ of the method. Cal 1 (0.5 pg/mL) passed all acceptance criteria including bias and %CV less than 10%, as shown in Figure 7. Therefore, adding TurboFlow on-line extraction/concentration did not improve testosterone LLOQ.

Figure 5. Transcend – TSQ Quantis LC-MS/MS method linearity for neat calibrators.

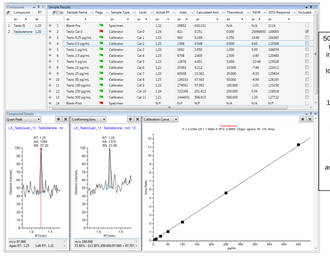


Figure 6. Transcend – TSQ Quantis LC-MS/MS method LLOQ for neat calibrators.

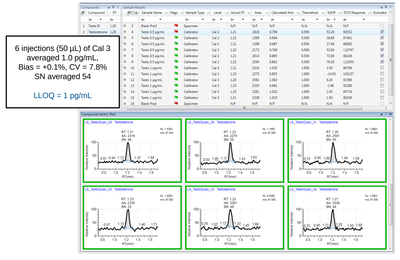
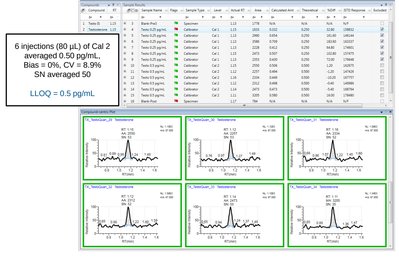


Figure 7. Transcend – TSQ Quantis TurboFlow – LC-MS/MS method LLOQ.



LLOQs for neat calibrators using LC -, TurboFlow-LC – TSQ Altis MS

The Prelude SPLC – TSQ Altis system achieved linear quantitation of neat calibrators between 0.5 and 500 pg/mL (Figure 8) using the LC-MS/MS method without TurboFlow. Cal 0 and Cal 1 (0.25 pg/mL) had interfering peaks, as in the Quantis system, and had to be excluded. Cal 2 (0.5 pg/mL) passed all acceptance criteria including bias and %CV less than 5% (Figure 9). These data give us confidence in achieving a testosterone LLOQ of 0.5 pg/mL in neat solutions using this system.

Figure 8. Prelude – TSQ Altis LC-MS/MS method linearity for neat calibrators.

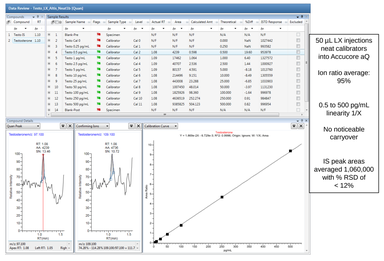
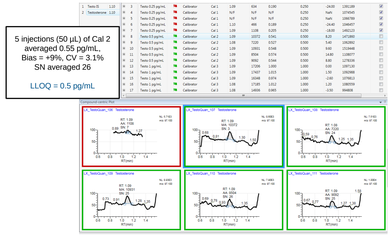


Figure 9. Prelude – TSQ Altis LC-MS/MS method LLOQ for neat calibrators.



The TurboFlow method on the Prelude SPLC – TSQ Altis system also achieved linear quantitation of neat calibrators between 0.5 and 500 pg/mL, even when making 80 µL injections, and exhibited the same interfering-peak issues with Cal 0 and Cal 1 already described.

LLOQs for serum specimens using LC -, TurboFlow-LC – TSQ Altis MS

The Cerilliant serum CRMs were extracted and quantitated as specimens using calibration curves from neat calibrators for both the non-TurboFlow and TurboFlow LC-MS/MS methods. Both methods showed agreement with expected values of the CRMs, which were typically between -4 and 12% (Figure 10). However, the "Serum Blank" consistently showed between 2 and 3 pg/mL of testosterone. The positive bias was greatest with the TurboFlow method. Testosterone measured in the donor female extract was consistently 10 pg/mL. The averages of internal standard (IS) peak areas were only 40% of the averages from non-extracted neat standards and the donor female and serum pools showed the highest and lowest values, respectively. Either extraction efficiency or ion suppression from matrix or both may have caused these recoveries which were not improved by adding TurboFlow to the LC method.

Figure 10. Prelude – TSQ Altis LC-MS/MS results for serum specimens.

DISCUSSION

Apparently, the TurboFlow column enriched a contaminant, perhaps testosterone from surrounding dust (1), in Solvent A during equilibration at high flow rate, which limited the LLOQ. The peak area of this contaminant increased proportionally as the equilibration time and/or flow rate increased and its ion ratio matched that of testosterone.

CONCLUSIONS

- We achieved LLOQs for testosterone of 0.5 pg/mL in neat solutions using conventional LC-MS/MS methodology with the TSQ Altis mass spectrometer.
- Although internal standard recoveries in extracts of serum specimens were around 40%, there was plenty of signal to permit quantitation down to sub-picogram per milliliter levels, especially if the dried extracts are reconstituted with smaller volumes. This needs to be verified using several donor specimens that contain such low levels of testosterone.
- Adding TurboFlow on-line extraction/concentration did not improve LLOQs.
- LLOQs for testosterone are chiefly limited by chemical noise, presumably traces of testosterone in the laboratory that contaminated mobile phase solvents.

REFERENCES

1. J. Di Bussolo & E. Goucher, Carryover & Contamination Causes & Cures - Assuring the Quantitative Accuracy of LC-MS/MS Methods, Poster M06, MSACL EU 2017.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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