Quantitative Analysis of Low Testosterone Concentrations in Plasma Using the TSQ Quantiva Triple-Stage Quadrupole MS

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Key Words
Testosterone, liquid chromatography, tandem mass spectrometry

Goal
To develop an LC/MS method meeting requirements for testosterone research analysis in female and juvenile plasma.

Introduction
Analysis of testosterone in female and juvenile plasma samples for research requires an analytically sensitive method with a limit of quantitation of at least 10 pg/mL. Liquid chromatography coupled with mass spectrometry (LC/MS), an analytically sensitive and selective technique, is widely accepted for testosterone analysis in complex matrices such as human serum or plasma.

Experimental
Sample Preparation
Plasma and serum samples were spiked with an internal standard (testosterone-D_3) and subjected to liquid-liquid extraction method using methyl tert-butyl ether. The resulting organic layer was evaporated, and the residue was reconstituted in Fisher Chemical™ methanol/water (1:1). A 10 µL aliquot of processed sample was analyzed with the following LC/MS method:

HPLC

Pump:	Thermo Scientific™ Accela™ 1250 pump
Autosampler:	Accela AS
HPLC column:	Thermo Scientific™ Accucore™ aQ 100 x 2.1 mm, 2.6 µm, ambient temperature
Mobile phase A:	5 mM ammonium acetate in water/methanol (95:5 v/v) (Fisher Chemical)
Mobile phase B:	5 mM ammonium acetate in methanol (Fisher Chemical)
Mobile phase C:	Acetonitrile/isopropyl alcohol/acetone (45:45:10 v/v/v) (Fisher Chemical)
LC gradient:	Refer to Table 1

Figure 1. TSQ Quantiva triple-stage quadrupole mass spectrometer

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Table 1. LC gradient

Time (min)	A (%)	B (%)	C (%)	Flow Rate (µL/min)
0.00	95	5	0	400
0.10	60	40	0	400
3.60	20	80	0	400
3.61	0	100	0	400
4.60	0	100	0	400
4.61	0	0	100	800
5.00	0	0	100	800
5.01	95	5	0	600
6.50	95	5	0	600

Mass Spectrometry

MS analysis was performed on a Thermo Scientific™ TSQ Quantiva™ triple-stage quadrupole mass spectrometer (Figure 1). The MS conditions were as follows:

Ionization:	Heated electrospray ionization (HESI)
Vaporizer temp (°C):	500
Capillary temp (°C):	375
Spray Voltage (V):	800
Sheath gas (AU):	55
Auxiliary gas (AU):	25
Data acquisition mode:	Selected reaction monitoring (SRM)
Chrom filter peak width (s):	3
Collision gas pressure (mTorr):	2
Cycle time (s):	0.2
Q1 (FWMH):	0.7
Q3 (FWMH):	0.7
SRM parameters:	Refer to Table 2

Table 2. Optimized SRM parameters

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	CE (V)
Testosterone	289.1	97.1	30
		109.1	30
Testosterone-D_3	292.1	97.1	30
		109.1	30

Method Evaluation

Calibration standards were prepared in charcoal stripped serum (CSS) (Bioreclamation, LLC) at concentrations of 5, 10, 20, 40, 100, 200, and 500 pg/mL. QC samples were prepared in CSS at 10 and 50 pg/mL. Intra-assay precision was obtained by processing and analyzing a standard curve along with three replicates of each QC sample. Inter-assay precision was obtained by processing and analyzing a standard curve along with three replicates of each QC samples on three different days. Matrix effects were evaluated by comparing peak areas of a 25 pg/mL sample prepared in CSS to a sample prepared in reconstitution solution. Matrix effects in different lots of plasma were evaluated by comparing the internal standard signal in donor plasma samples to the internal standard signal in solvent matrix.

Data Processing

Data was processed with Thermo Scientific™ TraceFinder™ software version 3.1. The target ion ratio was calculated by averaging the values obtained for calibrators and applying a tolerance of 20% for QC and donor samples.

Results and Discussion

The limit of quantitation (LOQ) was 5 pg/mL, equivalent to 100 fg on column, with excellent signal-to-noise. The LOQ was limited by the presence of endogenous testosterone in CSS (about 1 pg/mL). Figure 2 shows chromatograms for testosterone quantifier and qualifier ions at a concentration of 5 pg/mL in CSS. The calibration range is 5–500 pg/mL. Figure 3 shows a representative calibration curve. Intra-assay precision was better than 3.4% RSD for the 10 pg/mL QC and 2.0% RSD for the 50 pg/mL QC (Table 3). Inter-assay precision was 2.4% and 4.6% RSD for the 10 and 50 pg/mL QCs, respectively. Matrix effects in CSS were not observed. The average percentage recovery calculated against the spiked solvent was 94.8%. Limited matrix effects were observed in donor plasma. Internal standard signal in donor plasma was about 30% lower when compared to signal in solvent samples. Ion ratios passed for all calibration standards, QCs, and donor samples. Figures 4 and 5 present a TraceFinder chromatogram and calculated ion ratio for selected donor samples obtained in separate analytical runs.

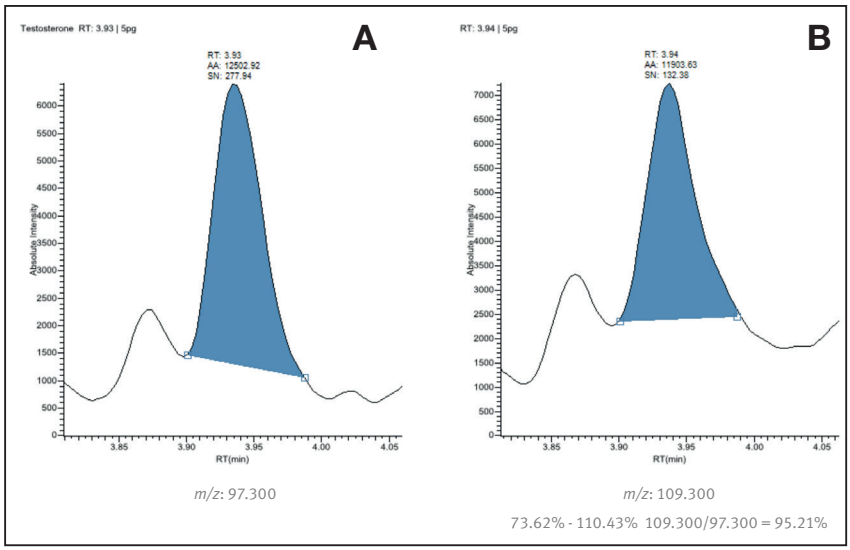
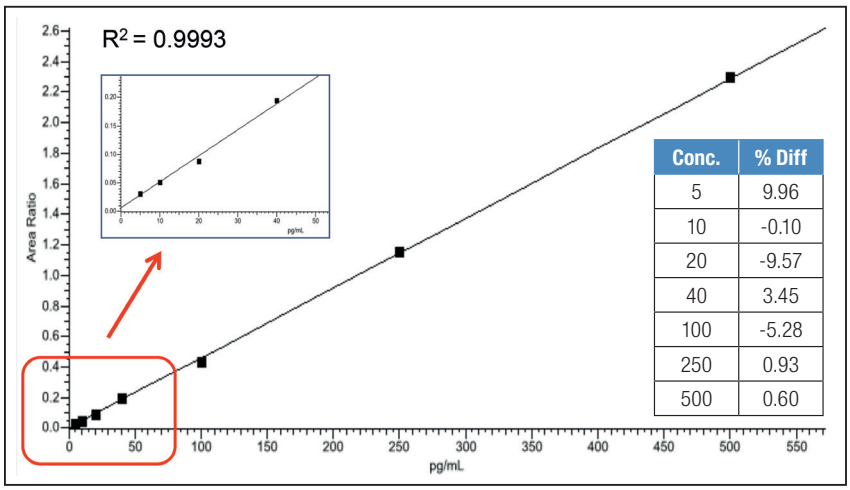


Figure 2. Chromatogram of the lowest calibration standard 5 pg/mL in CSS: (A) quantifier and (B) qualifier peaks



Conc.	% Diff
5	9.96
10	-0.10
20	-9.57
40	3.45
100	-5.28
250	0.93
500	0.60

Figure 3. Representative calibration curve for testosterone

Table 3. Intra-assay precision

	Concentration (pg/mL)		
QC1- 10 pg/mL	**Batch #1**	**Batch #2**	**Batch #3**
QC1-1	10.6	11.1	11.0
QC1-2	11.4	11.4	11.1
QC1-3	11.0	11.4	10.9
% RSD	**3.4**	**1.5**	**0.58**

	Concentration (pg/mL)		
QC2- 50 pg/mL	**Batch #1**	**Batch #2**	**Batch #3**
QC2-1	46.7	49.3	44.3
QC2-2	47.4	50.1	43.8
QC2-3	46.6	48.1	44.9
% RSD	**0.89**	**2.0**	**1.2**

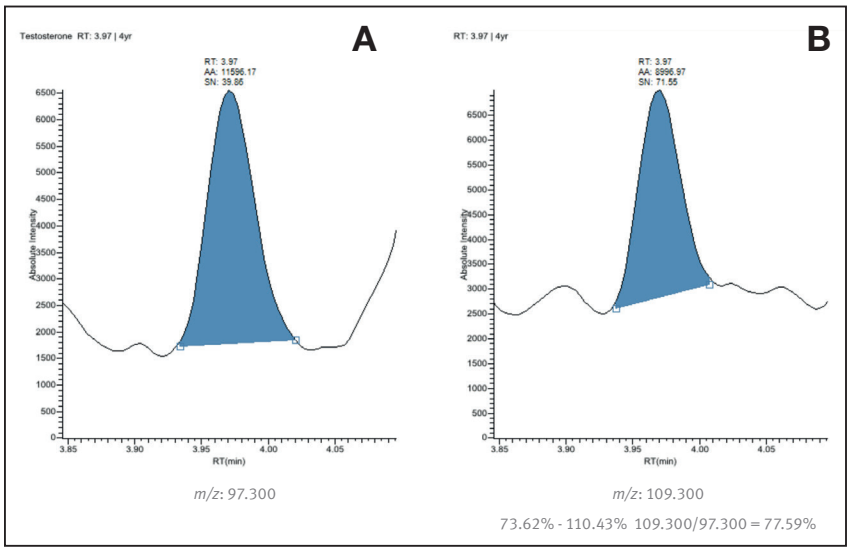


Figure 4. Chromatogram of testosterone (A) quantifier and (B) qualifier peaks in sample from a 4-year-old donor at concentration of 6.29 pg/mL

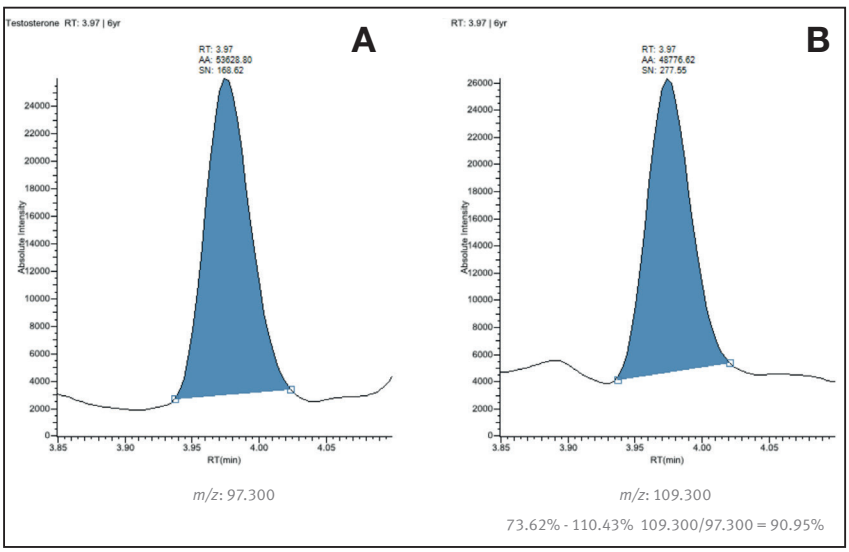


Figure 5. Chromatogram of testosterone (A) quantifier and (B) qualifier peaks in sample from a 6-year-old donor at concentration of 33.2 pg/mL

Conclusion

Using the TSQ Quantiva mass spectrometer, an analytically sensitive and robust method was evaluated for the research analysis of low testosterone concentrations in human plasma. The LOQ of 5 pg/mL is lower than that reported with other research methods. Precision was better than 5% RSD, and the ion ratios easily met industry standard criteria. The data show the TSQ Quantiva mass spectrometer has excellent analytical sensitivity and robustness to facilitate research.

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