Application Note

Clinical Research

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Analytical determination of testosterone in human serum using an Agilent Ultivo Triple Quadrupole LC/MS

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Introduction

This research study develops a robust, sensitive, and relatively fast analytical method for the quantitation of free testosterone in serum using a miniature Agilent Ultivo Triple Quadrupole LC/MS. Ultivo is designed to address many challenges faced by routine analytical laboratories. This research study was conducted to assess how this novel triple quadrupole mass spectrometer (MS) could perform with a typical endogenous analyte of research interest[1]. Innovative technologies within Ultivo allowed Agilent to reduce its physical footprint, while generating a comparable analytical performance level to similar, but physically larger MS systems. Instrumentation innovations such as VacShield, Cyclone Ion Guide, Dodecapole Vortex Collision Cell, and small Hyperbolic Quads were designed to maximize quantitative performance within a miniature package, and to enhance instrument reliability and robustness.

Ultivo reduces the need for user intervention for system maintenance, making the system operation and maintenance manageable for nonexpert MS users. Agilent MassHunter Software simplifies data acquisition, method setup, data analysis, and reporting, resulting in the fastest possible acquisition-to-reporting time, increasing lab productivity. This research study outlines typical confirmation performance of free testosterone in human serum using the Ultivo Triple Quadrupole LC/MS. Lower limits of quantitation, chromatographic precision, calibration linearity, range, and accuracy are outlined.

Experimental

LC configuration and parameters

Table 1. UHPLC configuration and settings.

Parameter	Value
Instruments	Agilent 1290 Infinity II high speed pump (G7120A) Agilent 1290 Infinity autosampler (G4226A) Agilent 1290 Infinity autosampler thermostat (G1330B) Agilent 1290 Infinity II multicolumn thermostat (G7116B)
Needle wash	100 % Methanol
Autosampler temperature	4 °C
Injection volume	19 µL plus 1 µL of internal standard
Analytical column	Agilent Poroshell120 EC-C18, 2.1 × 50 mm, 2.7 µm, LC column (p/n 699775-902)
Column temperature	55 °C
Mobile phase A	0.1 % Formic acid and 5 mM ammonium acetate in water
Mobile phase B	Methanol
Flow rate	0.5 mL/min
Gradient	Time (min) %B 0.0 60 4.0 95 5.0 95 5.1 60
Stop time	6 minutes
Post time	1 minute

Triple quadrupole mass spectrometer configuration and parameters

Table 2. Mass spectrometer instrument configuration and source settings.

Parameter	Value
Instrument	Agilent Ultivo Triple Quadrupole Mass Spectrometer
MS/MS mode	MRM
Ion mode	Positive
Drying gas temperature	300 °C
Drying gas flow	8 L/min
Nebulizer pressure	50 psi
Sheath gas temperature	380 °C
Sheath gas flow	12 L/min
Nozzle voltage	0 V
Capillary voltage, positive	3,000 V
Cell accelerator voltage	9 V
MS1/MS2 resolution	0.7/0.7 Unit
Dwell time	75 msec

MS/MS compound information for analytes and internal standards

Table 3. Detailed MRM Mode settings.

Compound	ISTD	Precursor ion (*m/z*)	Product ion (*m/z*)	Retention time (min)	Fragmentor (V)	Collision energy (V)
Testosterone-d3	✓	292.2	97.0	1.647 min	130 V	17 V
Testosterone		289.2	109.1	1.661 min	130 V	18 V
Testosterone		289.2	97.0	1.661 min	130 V	17 V

Chemicals and reagents

Human serum, used for matrix-matched calibrators, was sourced from Golden West Biologicals (Temecula, CA). Standards and internal standards were obtained from Cerilliant Corporation (Round Rock, TX). Sample preparation and LC solvents were from Sigma-Aldrich (St. Louis, MO) and Honeywell Riedel-de Haën (Seelze, Germany).

Sample preparation

To achieve the top concentration, negative human serum was spiked with testosterone, while lower concentrations were created by serial dilutions into clean serum. Each 250 µL sample was extracted by protein precipitation using 500 µL of acetonitrile. Samples were vortexed for 1 minute, then centrifuged for 4 minutes at 10,000 rpm. Before to introduction into the LC system, a 500 µL aliquot of supernatant was diluted with 500 µL of water. The 11-point calibration curve was prepared in triplicate, ranging from 0.001 to 100 ng/mL. Sufficient internal standard stock solution was added directly to each matrix standard and calibrator to create a consistent concentration of 25 ng/mL across all sample types injected.

Data analysis

Data were acquired and analyzed using Agilent MassHunter software suite C.01.00 for data collection from the Ultivo. MS/MS transitions were obtained using Agilent MassHunter Acquisition Optimizer software to determine optimal precursor and product ions, fragmentor voltages, and collision energies. This procedure was done upon injection of a neat solution of each individual compound or internal standard at a concentration level of 1,000 ng/mL, 2 µL injection volume in flow injection mode.

Results and Discussion

Linearity

The calibration concentrations ranged from 1 pg/mL to 100 ng/mL for the testosterone analyte. R^2 values were greater than 0.999, n = 3, with the testosterone compound displaying linear responses throughout the concentration range with a 1/x weighting factor applied. Precision data observed over the three batches resulted in a %RSD variation of <5 % across all calibration levels.

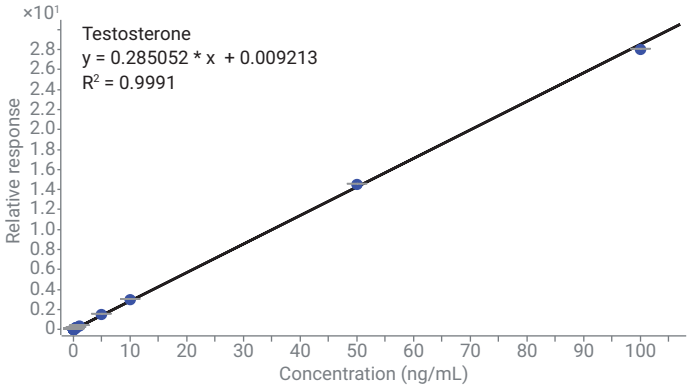

Figure 2. Example calibration curve for testosterone, using a 1/x weighting factor.

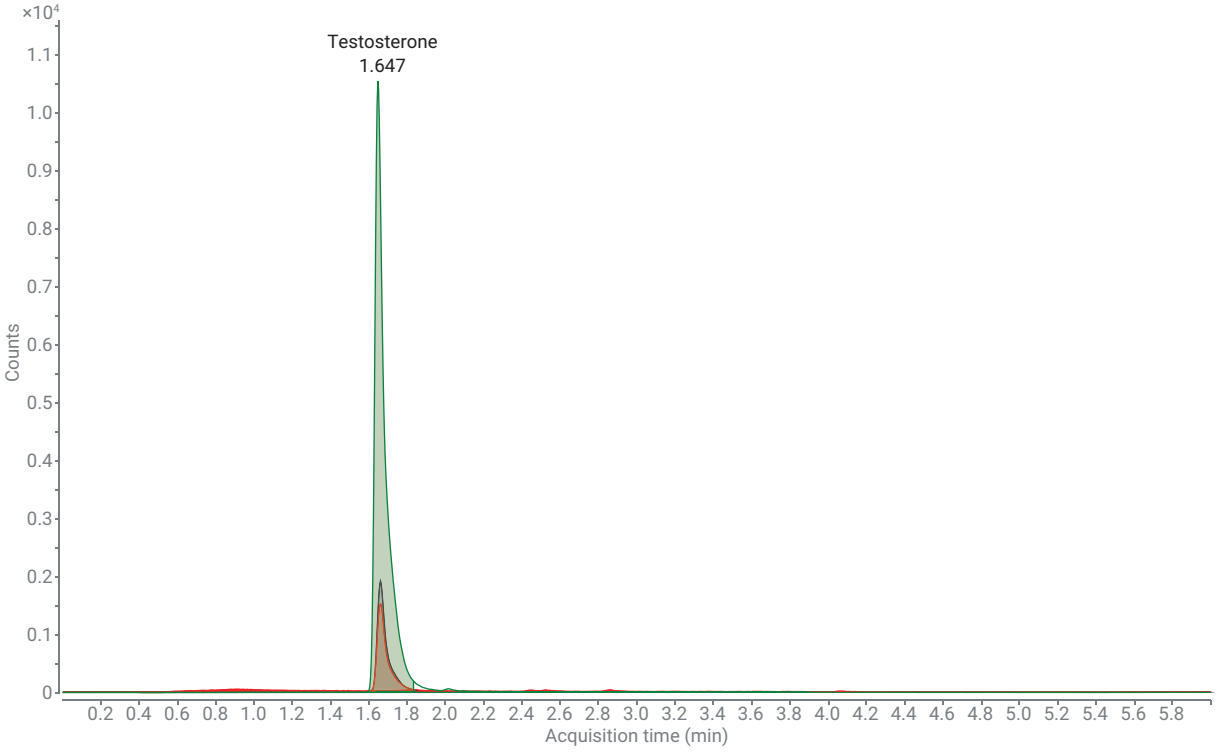

Figure 1. Overlaid MRM chromatograms, showing elution of testosterone (500 pg/mL) and the d_3 internal standard (25 ng/mL).

Analytical sensitivity

Negative serum sample blanks show measurable amounts of endogenous testosterone present. Figure 3A shows a small testosterone response in blank serum, n = 3. Figure 3B illustrates negative serum spiked with 1 pg/mL testosterone. A significant difference in area count and signal-to-noise ratio can be seen from these calibrators over the respective matrix blanks; therefore, the calibration curves were created, and calculations undertaken using a blank-offset feature.

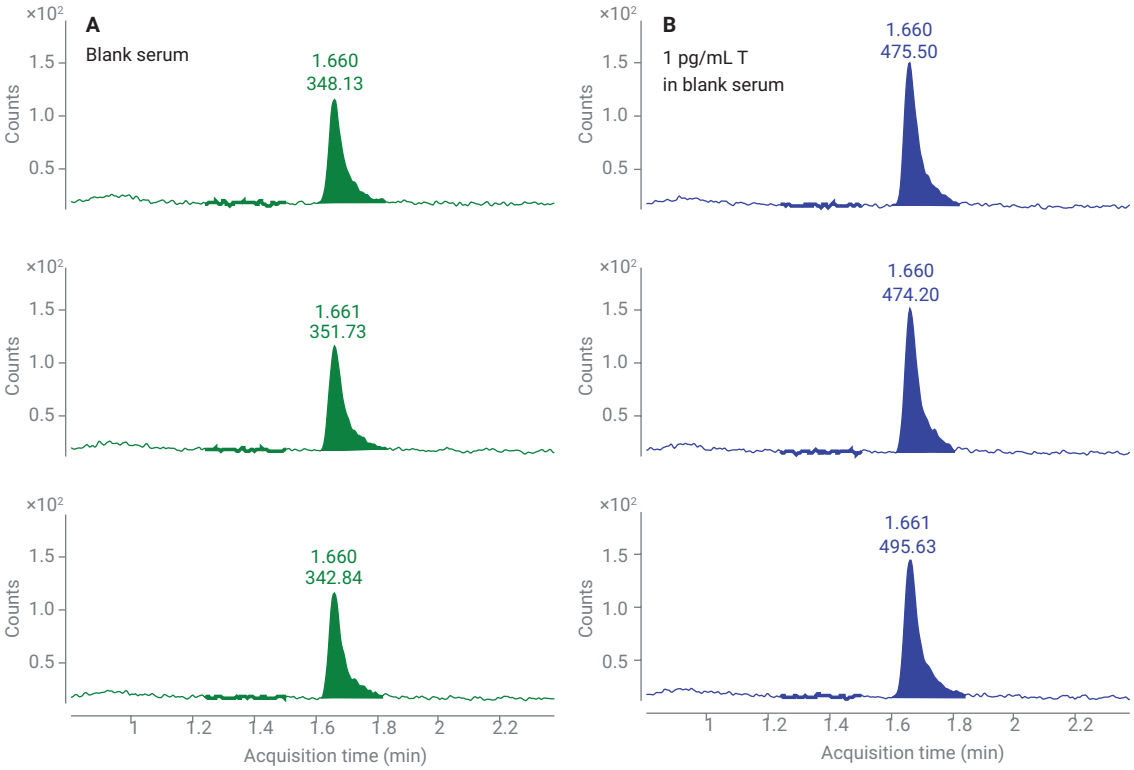

Figure 3. Triplicate injection of blank serum (A) and blank serum spiked with 1 pg/mL testosterone (T) (B).

Conclusion

This research project demonstrates that the Agilent Ultivo Triple Quadrupole LC/MS produces excellent linearity, precision, and analytical sensitivity across a range of 1 pg/mL through 100 ng/mL for free testosterone in human serum in a 6-minute analysis cycle time.

Future work is required to assess potential interferences for this analytical method over serum and blood matrices sourced from different suppliers and prepared for LC/MS analysis through further sample preparation techniques.

Reference

1. Analysis of testosterone and dihydrotestosterone in mouse tissues by liquid chromatography-electrospray tandem MS. *Anal Biochem*. **2010**, *15*, *402(2)*, 121-128.

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Printed in the USA, January 17, 2018
5991-8847EN

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