Automated Method for High-Throughput LC-MS/MS Quantitation of Testosterone from Serum: An Improved Validated Method

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Introduction

Analysis of testosterone is helpful when investigating endocrine disorders such as hypogonadism, polycystic ovarian syndrome in women, and early or late onset of puberty in boys. The naturally occurring low levels of endogenous testosterone, found in females and children, has resulted in quantitation by LC-MS/MS being the preferred analytical method for achieving relevant sensitivity and specificity[1]. Automated sample preparation of testosterone is desirable to accommodate the high sample throughput, robustness, and efficiency demands of the analysis.

An improved automated method for the extraction of testosterone in serum was developed using $ZnSO_4$ and Low Porosity Filtration Tips-Ultra Pure (LPFT-UP) on a Microlab® NIMBUS96 system (Hamilton Company, Reno, NV). The fully automated method incorporates a $ZnSO_4$ solution with acetonitrile on a Hamilton Heater Shaker (HHS) for improved recovery and precision during protein precipitation. LPFT-UP containing high purity filtration media are used to remove matrix interferences and achieve very low limits of detection. Using this quick (< 2 minutes) and effective, DPX patent-pending, Tip-on-Tip (ToT) technology, the LPFT-UP provide an automated filtration alternative to traditional centrifugation and filtration. This method allows for up to 96 samples to be protein precipitated, filtered, and ready for injection in under 5 minutes.

Benefits-Based Highlights

- 99.5% recovery from pooled patient samples
- 96 samples protein precipitated, filtered, and ready for injection in under 5 minutes

Materials and Methods

Reagents and Standards

Reference standards, testosterone-d_3 (100 µg/mL T-046) and testosterone (1.0 mg/mL T-037), were purchased commercially from Cerilliant Corporation (Round Rock, TX). Stripped human serum used for calibration preparation was purchased from Golden West Biologicals, Inc. (Temecula, CA). The quality control sample at 300 ng/dL was obtained from Utak (P/N DHEA Plus Low, C2330). $ZnSO_4$ (Fisher Scientific, Fair Lawn, NJ) was made to be 0.2 g/mL in DI water. The internal standard solution (testosterone-d_3) was made to be 20 ng/mL in 50% acetonitrile (ACN).

Sample Preparation

A 75 µL aliquot of each serum specimen, calibrator, quality control sample, and blank is transferred into a 96-well 1.2 mL plate. Each sample is spiked with 10 µL of Internal Standard (IS). The plated samples were then loaded onto the HHS on a NIMBUS96 system where the solutions are equilibrated for 20 minutes at 500 RPM. 300 µL CO-RE® tips add 25 µL $ZnSO_4$ solution. The HHS shakes for 15 seconds at a setting of 1,400 RPM. The same CO-RE tips then add 250 µL ACN to the sample solution. The HHS shakes for another 60 seconds at a setting of 1,500 RPM. After a delay of 20 seconds, 200 µL of the protein precipitated samples are aspirated using 300 µL wide bore CO-RE tips, and then dispensed through LPFT-UP. The schematic for the novel ToT method is depicted in Figure 1 (see page 2). The solutions are collected in a well plate and subsequently covered with no further sample preparation required. The deck layout for this fully automated method is shown on page 4.

Instrumentation

Analyses were performed on an Agilent 1260 HPLC system coupled with a SCIEX Triple Quad 6500+ Tandem Mass Spectrometer. A C18 column (3 x 50 mm, 2.7 µm, Poroshell) from Agilent heated to 45 °C with 0.1% formic acid in water (A) and methanol (B) was used for separation. The injection volume was 12 µL. The LC conditions were 55% A at 0.5 mL/min for 0.2 min, then ramped to 7% A at 0.8 mL/min at 2.3 min, then ramped to 0% A at 3.3 min and held for 1 min, then ramped back to 55% A for 1 min to re-equilibrate the column. Monitored testosterone transitions included 289.1/109.2 and 289.1/97.1 while testosterone-d_3 was monitored at 292.2/97.

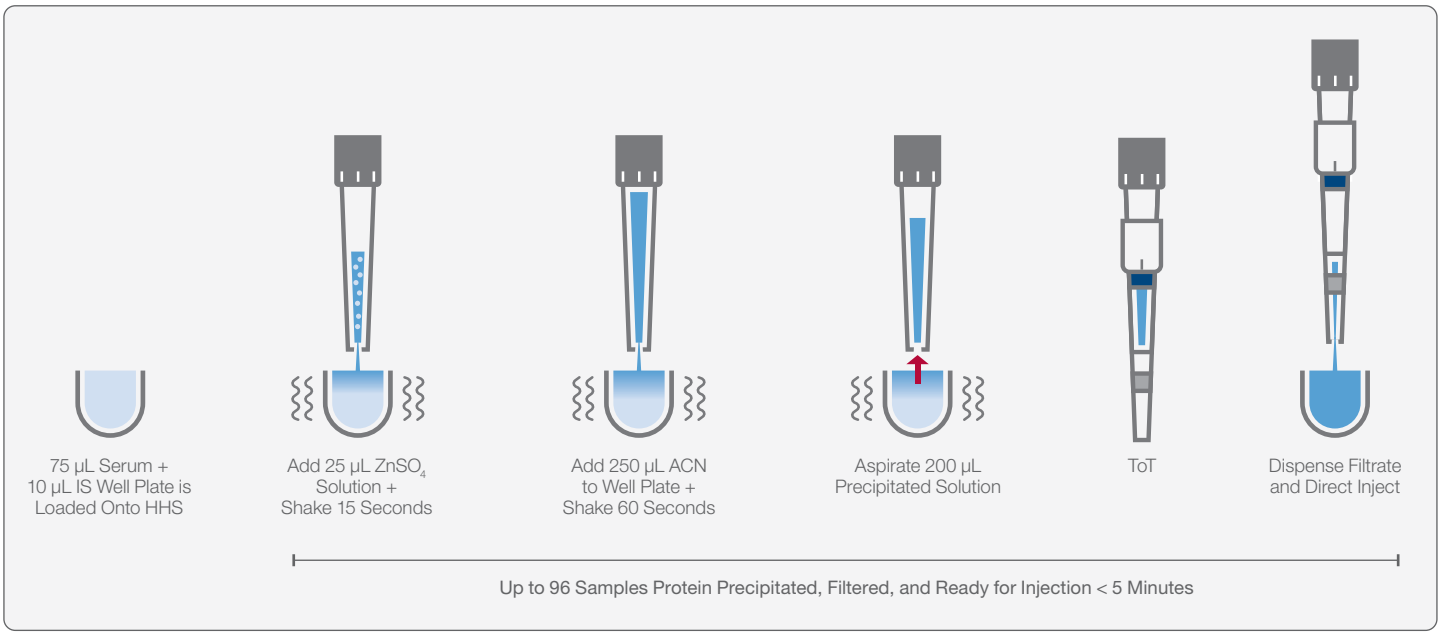


Figure 1: Schematic for Tip-on-Tip (ToT) Filtration method. Blue gasket within filtration tips creates an air tight seal for efficient dispense of solution.

Results and Discussions

This method was evaluated for linearity, precision (interday and intraday), accuracy, extraction recovery, and limits of detection and quantitation for three separate days of analyses. Linearity was assessed by analyzing serum samples at eight concentration points (2, 5, 10, 20, 50, 100, 500, and 1,000 ng/dL) with three replicates at each point and duplicate at 1,000 ng/dL (Figure 2). The linear regression of the calibration resulted in correlation coefficients of greater than 0.999 each day (0.99955, 0.99976, and 0.99975 using weighting of 1/x).

The %RSDs for the quality control sample were 3.5%, 4.5%, and 2.9% for each day. The interday was calculated to be 4.6% with an average accuracy of 96%. The limit of detection was calculated according to SWGTOX[2] guidelines to be 0.97 ng/dL. The LOQ was calculated to be 2.9 ng/dL, and 2.6 ng/dL based on a signal to noise of ten. If an LOQ of less than 2.0 ng/dL is desired, solvent evaporation could be readily incorporated. It is also possible to improve the sensitivity of the method by monitoring just one transition for testosterone (at very low concentrations).

The percent recovery was calculated using pooled patient samples (that had less than detectable levels of testosterone) in order to have representative results. We estimated the total volume of ACN that could be collected (using centrifugation) from the patient sample, and calculated a recovery of 99.5% with no detected matrix effects (see Table 1 for results). This demonstrates that the use of $ZnSO_4$ and ACN is very efficient in extracting testosterone, and the use of ultra pure grade tips (LPFT-UP) resulted in negligible losses.

We also analyzed patient samples from Stanford Healthcare and compared quantitative results. A chromatogram from a representative patient sample at a low concentration is shown in Figure 3. The correlation coefficient for measured testosterone concentrations determined by our method versus the corresponding lab's results was 0.973 for 88 patient samples. This exemplifies the accuracy of the automated protein precipitation with ToT Filtration.

1. *Run Zhang Shi, Huub H. van Rossum, Raffick A.R. Bowen, "Serum testosterone quantitation by liquid chromatography-tandem mass spectrometry: Interference from blood collection tubes" Clinical Biochemistry. 2012, 45, 1,706–1,709.*
2. *The standard of practice adopted by Scientific Working Group for Forensic Toxicology (SWGTOX).*

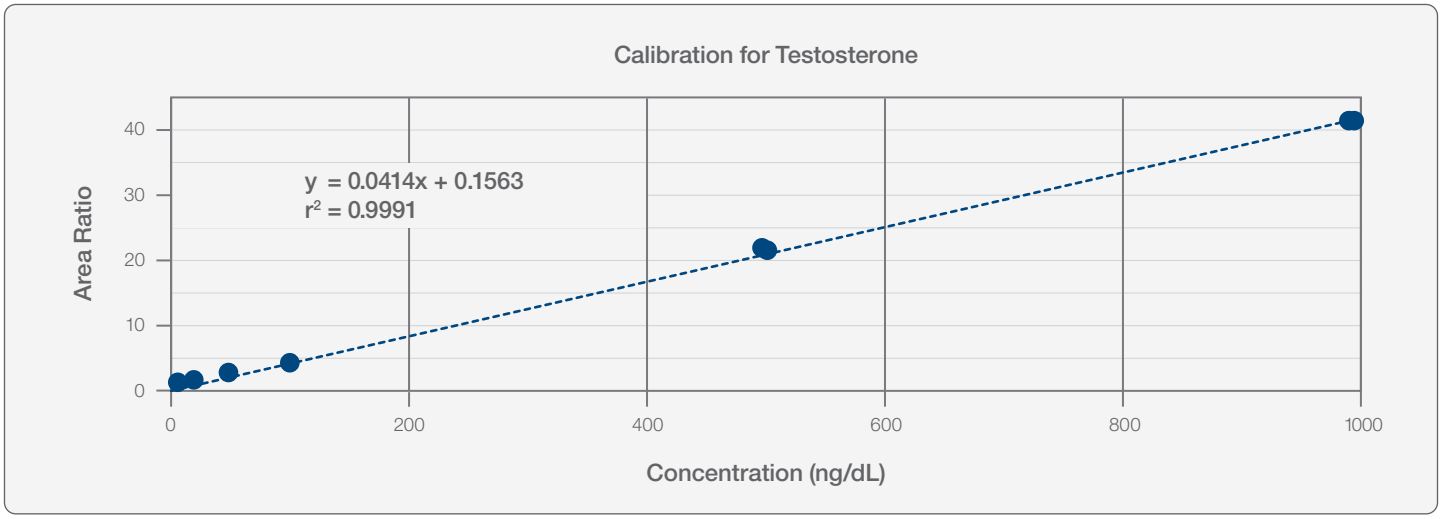


Figure 2: Calibration plot for testosterone.

Table 1: Summary of Results for Pooled Patient Samples

LOD	0.97 ng/dL
LOQ	2.9 ng/dL
Recovery	99.5%
Intraday Precision (%RSD)	UTAK QC (300 ng/dL)
Day 1	3.5
Day 2	4.5
Day 3	4.9
Interday Precision (%RSD)	4.6
Accuracy	96%

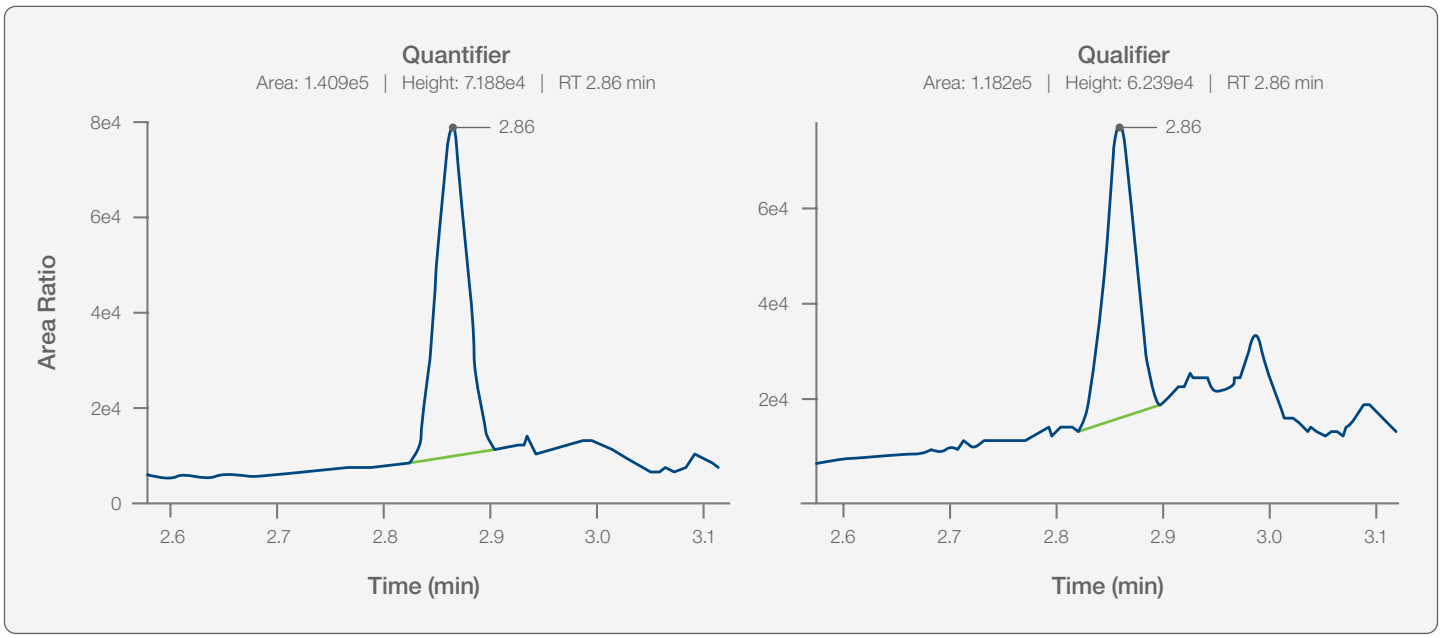
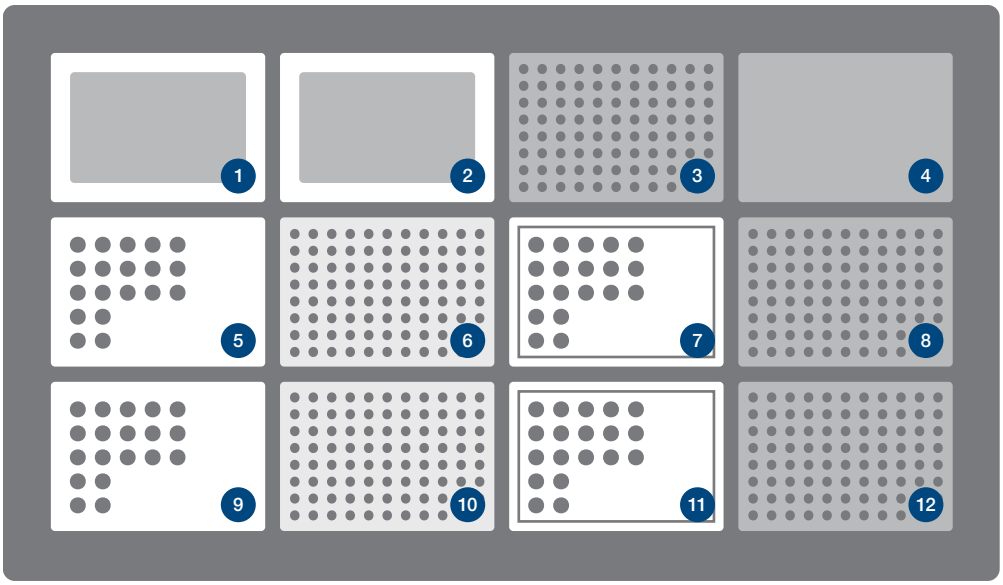


Figure 3: Quantifier (289.1/97.1) and Qualifier (289.1/109.2) extracted ion chromatograms for a patient sample calculated to be 74 ng/dL.

Conclusion

This automated $ZnSO_4$ protein precipitation and ToT Filtration method using LPFT-UP is a robust and effective sample preparation approach for the analysis of testosterone in serum. The high recoveries of testosterone from pooled patient samples highlight the importance of using $ZnSO_4$ and LPFT-UP when trying to achieve low limits of detection.

Deck Layout



1. 100% ACN
2. $ZnSO_4$ Solution
3. 300 µL CO-RE Tips
4. Empty
5. Filtrate 1
6. LPFT-UP
7. Sample 1 (75 µL Serum, 10 µL IS) Loaded on HHS
8. 300 µL Wide Bore CO-RE Tips
9. Filtrate 2
10. LPFT-UP
11. Sample 2 (75 µL Serum, 10 µL IS) Loaded on HHS
12. 300 µL Wide Bore CO-RE Tips

Deck layout for automated sample preparation using a NIMBUS96 open layout system. The layout shows sample prep for two serum well plates for processing up to 192 samples.

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