Automated High Throughput LC-MS/MS Quantitation of Testosterone from Serum: Validation and Inter-laboratory Comparison

Nicholas R. Chestara[1], Kaylee R. Mastrianni[2], Evan DiVirgilio[2], William E. Brewer[2], William E. Kemnitzer[1], Run-Zhang Shi[3], Kevin W.P. Miller[4]

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.

We report the quantitation of testosterone using a patent pending Tip-on-Tip (ToT) technology. This technology utilizes a proprietary Low Porosity Filtration Tip (LPFT) from DPX Technologies on a Hamilton MICROLAB® NIMBUS96. Hamilton Compression O-Ring Expansion (CO-RE) technology alleviates any back-pressure concerns during the unique ToT method for protein precipitation.

The DPX ToT method was compared to Stanford Healthcare's traditional method. A blind inter-laboratory study of 61 patient samples was performed with Stanford Healthcare (Palo Alto, CA) that independently determined testosterone concentrations using liquid-liquid extraction (LLE) and LC-MS/MS.

METHODS & INSTRUMENTATION

Reagents and Standards

Reference standards, testosterone-D3 (16, 16, 17-D3), 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.

DPX Sample Preparation

A 50 µL aliquot of each serum specimen, calibrator, quality control sample and blank was transferred into a 96-well 1.2 mL plate. Each sample was spiked with 10 µL of testosterone-D3 at 10 ng/mL in methanol. The plated samples were incubated for 25 minutes using a Labnet VorTemp™ 56 Benchtop Incubator/Shaker (Woodbridge, NJ) at room temperature (RT) to ensure the internal standard was properly equilibrated in the samples. The plate was then moved to the MICROLAB® NIMBUS96 system. Using 300 µL wide bore tips, the robot transferred 200 µL of the precipitation solvent (acetonitrile 100%, Fisher) to each well. The serum was protein precipitated utilizing the method indicated in the DPX ToT Schematic (Figure 2). The samples were dispensed from the wide bore tip through LPFT into a new collection plate. A 100 µL aliquot of the filtered supernatant was transferred and diluted with 100 µL water and then injected (30 µL) on the LC-MS/MS.

Stanford's Sample Preparation

Patient samples were obtained by venous phlebotomy using serum BD vacutainer coagulation tubes and serum was obtained after centrifugation. 50 µL of internal standard solution (20 ng/mL d3-testosterone in methanol; Sigma-Aldrich) were added to 200 µL of quality control, calibrator or patient sample and incubated for 20 minutes at RT. Next, samples were extracted for 30 minutes at RT using 1 mL of methyl t-butyl ether. Then the organic phase of each sample was transferred to new vials and the solvent was evaporated. The residue of each sample was reconstituted in 150 µL water-methanol solution (1:1 v/v).

Stanford's Validated Method

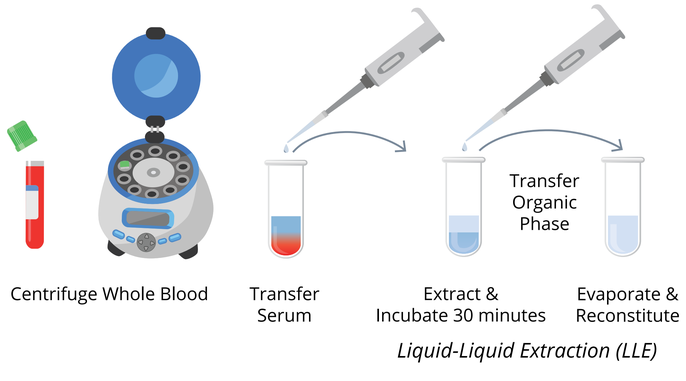

Figure 1. Stanford's validated method which takes >30 minutes to perform.

DPX ToT Method

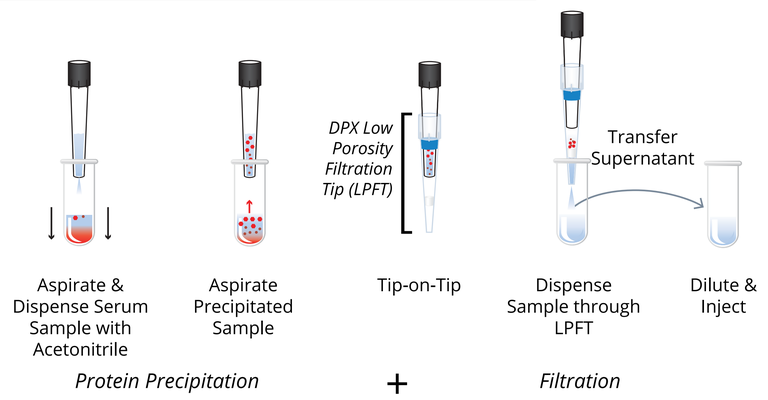

Figure 2. DPX ToT method which takes <5 minutes to perform. Sample preparation was performed on a MICROLAB® NIMBUS96 open layout liquid handler.

Instrumentation

Analyses were performed on an Agilent 1290 LC system coupled with a SCIEX QTRAP® 6500 mass spectrometer. A C18 column (2.1 x 50 mm, 5 um) from Peeke Scientific (heated at 60°C) was used for separation with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) . The LC method was less than 5.5 minutes. MS source parameters were the following: Curtain Gas (CUR): 25; Collision Gas: medium; IonSpray Voltage (IS): 4500; Temperature (TEM): 750°C; and Ion Source Gas 1 and 2: 70°C. Monitored testosterone transitions included 289.2/109 and 289.2/97 while testosterone-D3 was monitored at 292.2/97. A declustering potential of 135 and an entrance potential of 10 were used.

RESULTS

This method was evaluated for linearity, precision (inter-day, and intra-day), accuracy, extraction recovery, and limit of quantitation (LOQ). Linearity was assessed by analyzing serum samples at six concentration points (0.02, 0.10, 0.50, 1.0, 5.0, 10.0 ng/mL) with two replicates at each point. The linear regression of the calibration resulted in a correlation coefficient of 0.9997. The Stanford Healthcare method had an LOQ of 0.02 ng/mL. In order to adopt this LOQ for the DPX method, a signal to noise (S/N) of greater than 10:1 and a relative standard deviation of less than 20% for replicate calibrators (n=6) was verified. Inter-day precision and accuracy was determined by analyzing 28 QC points over a seven day period. The results included an in-house QC (0.75 ng/mL), UTAK low (Lot C2330) and UTAK high (Lot C1474) with a minimum of three intra-day QC replicates per validation batch. Inter and intra-day precision were calculated to be 3.75%, 8.47% and 9.45% by evaluating the in-house, UTAK low and UTAK high quality control samples, respectively. Accuracy was determined by comparing the same replicates to the reported target concentration of the quality controls. The results were determined to be 96%, 81%, and 106% for the in-house, UTAK low and UTAK high quality control samples, respectively. Accuracy and Precision are shown in Table 1. The recovery of the DPX Tip-on-Tip was also evaluated at low and high concentration, which was determined to be 88% and 97%, respectively.

Table 1. Accuracy, Inter- and Intra-day Precision for three quality controls levels over four days.

	IN-House QC	UTAK QC_LOW	UTAK QC_HIGH
Reported Conc. (ng/mL)	0.75	3.77	5.87
Accuracy	96%	81%	106%
Intra-day Precision (%CV)	-	-	-
Day 1	2.4	3.4	2.0
Day 2	3.4	7.9	4.8
Day 3	0.6	4.9	3.9
Day 4	N/A	2.7	8.5
Average	2.1	4.7	4.8
Inter-day Precision (%CV)	3.8	8.5	9.5

Testosterone case samples that had been previously analyzed were provided by Stanford Healthcare. These patient samples (61 in total) were analyzed using Stanford's existing validated testosterone method involving protein precipitation, LLE and an API 5000 LC-MS/MS system (SCIEX). These patient samples were analyzed in our laboratory in duplicate by our method delineated above. The standard deviation of the duplicate patient sample concentrations was below 20% for all samples, with the majority falling at or below 15%.

The correlation coefficient for measured testosterone concentrations determined by our method versus the corresponding lab's results was 0.9695. (Shown in Figure 6.) Further, the average percent bias between the average concentration from the DPX method and the concentration reported from Stanford was -3.2%. The overall success of this inter-laboratory study further confirms the validity of this automated method for the quantitative analysis of testosterone.

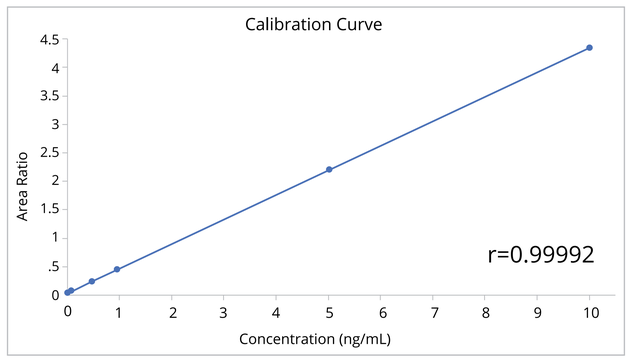

Figure 3. Representative example of a six-point calibration plot calculated from this Tip-on-Tip Method (r = 0.99992)

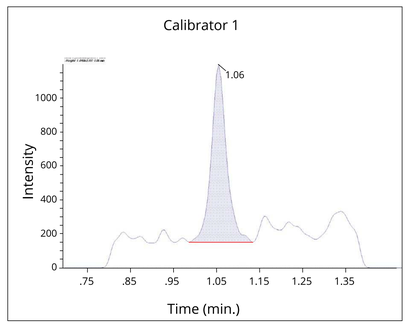

Figure 4. Chromatogram for a calibrator at the Limit Of Quantitation (LOQ), 0.02 ng/mL.

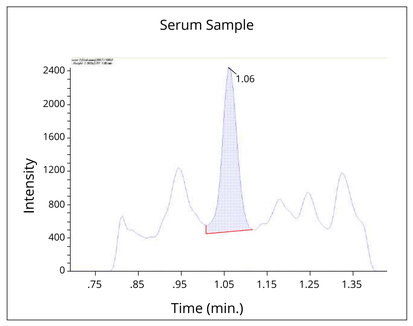

Figure 5. Patient serum sample near the LOQ concentration, 0.025 ng/mL.

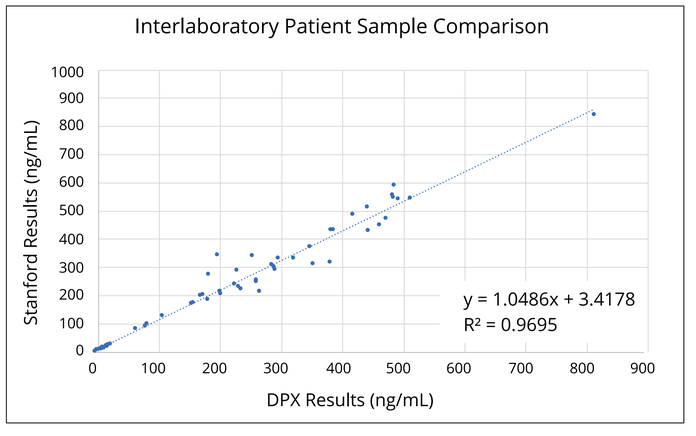

Figure 6. Inter-laboratory patient sample correlation data with a correlation coefficient of 0.9695 for the results between Stanford and DPX inter-laboratory analysis of 61 serum samples.

CONCLUSIONS

Tip-on-Tip is a robust methodology for automated protein precipitation that can provide a fast solution for a variety of complex applications. Here the technology has been validated as an effective sample preparation approach for the analysis of testosterone in serum. In addition to performing method validation, the DPX ToT method was compared to a manual sample preparation technique involving protein precipitation and liquid-liquid extraction via patient sample comparison (61 samples). The results of the comparison showed a correlation coefficient of 0.9695 and an average bias of -3.2%.

REFERENCES

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, 1706-1709.

[1]DPX Technologies, West Coast Research Center, 9924 Mesa Rim Rd, San Diego, CA 92121
[2]DPX Technologies, 19 Technology Circle, Columbia, SC 29203
[3]Stanford Healthcare, Clinical Chemistry and Immunology of Clinical Laboratories, 3375 Hillview Ave., Palo Alto, CA 94304
[4]Hamilton Company, 4970 Energy Way, Reno, Nevada