Sample Multiplexing: Increased Throughput for Quantification of Total Testosterone in Serum by Liquid Chromatography-Tandem Mass Spectrometry

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BACKGROUND: For high-volume assays, optimizing throughput reduces test cost and turn-around time. One approach for liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays is sample multiplexing, wherein the analyte of interest is derivatized in different specimens with reagents of different molecular weight (differential mass tagging). Specimens can then be combined and simultaneously analyzed within a single injection to improve throughput. Here we developed and validated a quantitative, sample-multiplexed LC-MS/MS assay for serum total testosterone (TT) based on this approach.

METHODS: For the sample-multiplexed assay, calibrators, controls, and patient specimens were first extracted separately. After mass tagging with either methoxyamine or hydroxylamine, they were combined and injected into the LC-MS/MS system. To evaluate assay performance, we determined limit of quantification (LOQ), linearity, recovery, and imprecision. A method-comparison study was also performed, comparing the new assay with the standard LC-MS/MS assay in 1574 patient specimens.

RESULTS: The method was linear from 2.5 to 2000 ng/dL, with accuracies from 93% to 104% for both derivatives. An LOQ of 1.0 ng/dL was achieved. Intra-assay and total CVs across 4 quality control concentrations were less than 10%. The assay demonstrated good agreement (Deming regression, 1.03x + 6.07) with the standard LC-MS/MS assay for the patient specimens tested (TT, 3 to 4862 ng/dL).

CONCLUSION: Sample multiplexing by differential mass tagging of TT increases LC-MS/MS throughput 2-fold without compromising analytical accuracy and sensitivity.

Introduction

A goal of large clinical laboratories is to optimize throughput, especially for high-volume liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays such as serum total testosterone (TT). Reducing the turn-around time for patient testing is key but must be done in a manner that maintains the quality of the assay. Increasing the speed of analysis on LC-MS/MS platforms can be accomplished by shortening and optimizing liquid chromatography (LC) methods through the appropriate selection of solvents, LC columns, and gradient conditions. Multiplexing LC systems that operate simultaneously in tandem with a single mass spectrometer can also increase throughput.

Once LC parameters have been optimized to maximize the speed of analysis, the options to further increase LC-MS/MS throughput become limited. One methodology, sample multiplexing, involves introducing more than one sample per injection into the LC-MS/MS instrument *(1, 17-19)*. This approach uses reagents of differing mass (differential mass tagging) to derivatize separate specimens, which are then combined prior to injection. The individual specimens are identified based on their corresponding modified molecular weights *(1, 17-19)*.

Methoxyamine (MOA) and hydroxylamine (HOA), popular Girard reagents used in both gas chromatography-tandem mass spectrometry and LC-MS/MS applications, enhance the ionization of ketosteroids *(2–6, 16, 20)*. We chose MOA and HOA as derivatization reagents for TT because of the similarity in reaction conditions and unique, stable derivatives (Fig. 1) *(2–6, 20)*. Both reagents react with the carbonyl group of testosterone to form an oxime derivative *(2–6, 11, 16, 20)*. Our goal was to develop and validate a sample multiplexing assay using MOA and HOA to quantitate TT in serum via LC-MS/MS.

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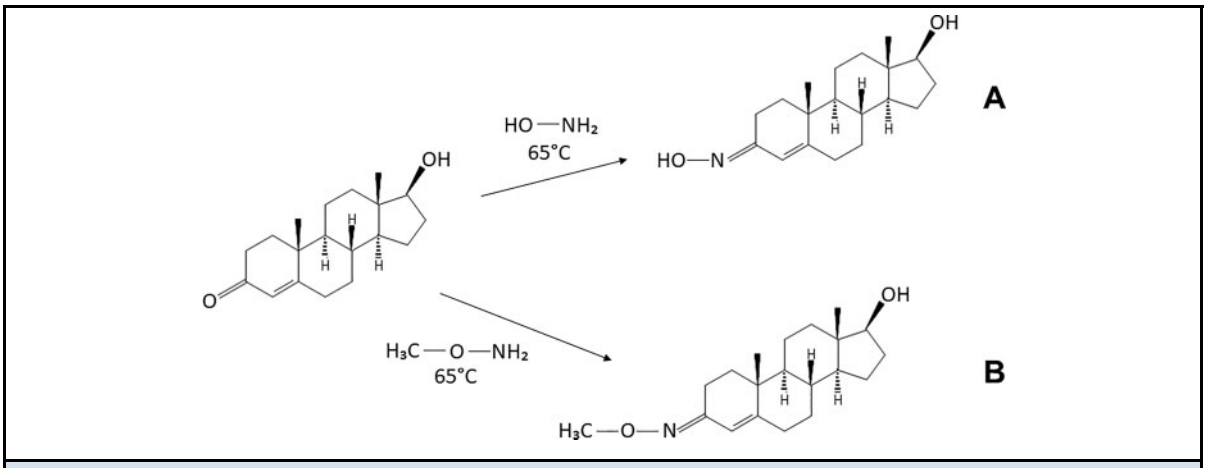

Fig. 1. Derivatization of testosterone with HOA (a) and MOA (b). HOA-T, hydroxylamine derivative of testosterone; MOA-T, methoxyamine derivative of testosterone.

Materials and Methods

STANDARDS AND REAGENTS

Testosterone and testosterone-2,3,4-$^{13}C_3$ (T-$^{13}C_3$) were purchased from Cerilliant. Methoxyamine hydrochloride and hydroxylamine hydrochloride were purchased from Sigma-Aldrich. Stripped, delipidized human serum was purchased from Golden West Biologicals. HPLC grade acetonitrile and water were obtained from Honeywell, HPLC grade methanol from Fisher Scientific, and formic acid from EMD Millipore. For interference studies, 32 steroidal compounds (Supplemental Table 1) were obtained from Steraloids and Cerilliant. Standard Reference Material® (SRM) 971 was obtained from the National Institute of Standards & Technology (NIST).

PREPARATION OF CALIBRATORS, CONTROLS AND INTERNAL STANDARD

For the preparation of calibrators, a working solution of testosterone was prepared in acetonitrile at 10 μg/mL, then diluted in stripped serum to create a working standard at a concentration of 100 ng/mL. This standard was aliquoted into microcentrifuge tubes (0.5 mL/tube) and stored frozen (−20.0 to −30.0 °C), then thawed as needed and further diluted in stripped serum to create 10 calibrators at concentrations of 2.5, 5, 10, 25, 50, 100, 500, 1000, 1500, and 2000 ng/dL. Two additional working solutions were prepared in acetonitrile at 10 μg/mL and 100 ng/mL to be used for the preparation of the quality control material. Quality controls at ultra-low (10 ng/dL), low (50 ng/dL), medium (250 ng/dL), and high (1200 ng/dL) concentrations were prepared in stripped serum by dilution of these working solutions to achieve concentrations across the linear range of the assay. An internal standard solution (400 ng/dL of T-$^{13}C_3$) was prepared in acetonitrile and acted as the precipitating reagent in the sample preparation process. Derivatization reagents were prepared fresh daily at a concentration of 0.3 M; the solution of MOA was prepared in methanol, and the HOA solution was prepared in 70:30 methanol: water.

SAMPLE PREPARATION

The LC-MS/MS assay for quantifying serum TT performed at Quest Diagnostics at the time of this study was identified as the reference method. This method was certified through the CDC Laboratory/Manufacturer Hormone Standardization (HoSt) Program for testosterone and utilized a comparable LC-MS/MS platform to the new, sample-multiplexed method. The sample preparation for the standard assay involved acidification of serum specimens and the addition of internal standard by a liquid handling robot, followed by incubation at room temperature for 30 min. Prepared samples were then extracted online using high-turbulence flow liquid chromatography coupled to analytical HPLC and atmospheric pressure chemical ionization tandem mass spectrometry (HTLC-APCI-MS/MS) *(7)*.

For the sample-multiplexed method, serum samples were initially divided into 2 sets of equivalent number of samples. Each set included blank matrix, 10 calibrators, patient specimens, and two sets of quality control samples bracketing the patient specimens. A Microlab STAR liquid handling robot (Hamilton) aliquoted 200 μL of all samples within each set, including standards and quality controls, into a 2-mL/well, 96-well protein precipitation plate. The liquid handler then

dispensed 400 μL of the internal standard solution into all wells to precipitate proteins. After vortex-mixing each plate (10 min, 1200 rpm), samples were filtered by centrifugation through the protein precipitation plate (10 min, 1847 × g) and collected into a 1-mL/well 96-well collection plate.

For differential mass tagging, the liquid handler was used to add 100 μL of MOA solution to all wells of one plate and 100 μL of HOA solution to all wells of the second plate. After vortex-mixing each plate (2 min, 1200 rpm), samples were incubated at 65 °C for 60 min, then cooled for 10 min at 2 °C to 8 °C to stop the derivatization reaction. Differentially derivatized calibrators, controls, and serum specimens were combined at the solid phase extraction (SPE) step, which was performed on a Cerex® IP8 system (SPEware). The IP8 system used optimized pressure profiles to push solvents and samples through the 25-mg/well C18 96-well plate. Solvents were prepared as needed and stored at room temperature until expiration. Each well was conditioned with 1 mL of methanol, then equilibrated with 1 mL water. After dilution with 1 mL of water, 600 μL of all calibrators, controls, and serum specimens derivatized with MOA were loaded onto the SPE plate. Next, 600 uL of all calibrators, controls, and serum specimens derivatized with HOA were diluted with 1 mL of water, then loaded onto the same SPE plate into the corresponding wells. Samples were then washed with 1 mL water, followed by 1 mL of 80:20 water: methanol. Analytes of interest were eluted with 800 μL methanol into a 1-mL/well 96-well plate and dried down under nitrogen heated at 60 °C. Finally, the dry extracts were reconstituted on the Hamilton STAR system with 150 μL 90:10 methanol: water and vortex-mixed (10 min, 1200 rpm) prior to injection.

Unless otherwise stated, all samples in the following studies have been multiplexed.

LC-MS/MS PARAMETERS

The LC-MS/MS conditions for the standard TT assay were as previously described *(7)*. For the new method, pooled, extracted specimens were analyzed on a TSQ Quantum Ultra Triple Quadrupole mass spectrometer (Thermo Fisher Scientific). A CTC-PAL autosampler (Cohesive Technologies) injected 40 μL of each well onto the TLX-4 HPLC system (Thermo), where additional SPE was performed using a C18-E SP 50x1mm Turboflow column (Thermo). Chromatographic resolution was accomplished using a Synergi Max-RP 2.5 μ 50x2 mm column (Phenomenex) with a 6.5-min stepwise binary gradient of acidified water (mobile phase A) and 50:50 methanol: acetonitrile (mobile phase B). Introduction of samples and gradient conditions were controlled by Aria operating software (Thermo). A 1.25-min data acquisition window enabled column multiplexing, wherein 4 HPLC channels were run in staggered parallel in order to increase LC-MS/MS throughput.

Table 1. Q1 and Q3 ions for testosterone derivatives and corresponding internal standards.

Analyte Name	Q1 Mass (m/z)	Q3 Mass (m/z)
HOA-T	304.2	112.0, 124.0
HOA-T-$^{13}C_3$	307.2	127.1
MOA-T	318.2	126.1, 138.1, 152.1
MOA-T-$^{13}C_3$	321.2	129.1

HOA-T, hydroxylamine derivative of testosterone; HOA-T-$^{13}C_3$, hydroxylamine derivative of testosterone-$^{13}C_3$; MOA-T, methoxyamine derivative of testosterone; MOA-T-$^{13}C_3$, methoxyamine derivative of testosterone-$^{13}C_3$.

The methoxyamine derivative (MOA-T), hydroxylamine derivative (HOA-T), and the respective internal standard ion pairs were analyzed using multiple reaction monitoring in positive electrospray ionization mode (Table 1, Supplemental Fig. 1). Source parameters were initially optimized by direct infusion and further optimized under HPLC conditions. These include a spray voltage of 3500, vaporizer temperature of 380 °C, capillary temperature of 350 °C, sheath gas of 50, and aux gas of 10. All acquired data were processed using LC Quan Software (Thermo). The responses of the derivatives were normalized against the corresponding internal standard, and the concentration was calculated based on the calibration curve.

Ion suppression due to matrix effects was evaluated through post column infusion. A constant flow of an organic solution of each derivative of testosterone (100 ng/dL in acetonitrile) was infused into the mass spectrometer while simultaneously injecting extracted stripped serum and extracted serum specimens with low concentrations of TT. Additionally, mobile phase A, mobile phase B, and reconstitution buffer were injected for comparison. The total ion chromatograms were then visually inspected for ion suppression, characterized by a depression in signal intensity for the sum of the respective product ions for MOA-T and HOA-T.

LIMIT OF QUANTIFICATION

The limit of quantification (LOQ) was considered the lowest value of meaningful data for this assay. It was determined by diluting the 5 ng/dL standard with stripped serum to achieve 3 samples at 3.0, 2.0, and 1.0 ng/dL. These dilutions were then assayed 30 times across 5 runs. The LOQ was set at the lowest concentration that gave an inter-assay CV of ≤20% while maintaining accuracy within ±20% of the nominal concentration. A supplemental LOQ study was performed in patient serum to verify precision

and signal-to-noise ratios (S/N) at a concentration comparable to the first calibrator (2.5 ng/dL). For this study, patient specimens with low concentrations of endogenous testosterone were pooled and extracted across 3 runs (10 replicates per run). The low-concentration pool was additionally spiked with 5 ng/dL testosterone and extracted across 3 runs (5 replicates per run), and percent recoveries based on the expected value as calculated from the neat specimen pool were calculated.

LINEARITY, RECOVERY, ACCURACY AND IMPRECISION

The linearity of the assay was determined by diluting the highest calibrator with stripped serum to make 10 samples with concentrations ranging from 2.5 ng/dL to 2000 ng/dL, then each sample was assayed 4 times within one run. The mean of observed concentrations was then plotted against the expected concentration, and the respective deviation of the mean observed versus expected concentration was calculated.

Accuracy of the sample-multiplexed method was evaluated through the CDC HoSt Program and the CDC Accuracy-based Monitoring Program (AMP). Four sets of 10 serum specimens (22 males, 18 females) provided by the CDC HoSt Program, were measured over the course of 12 months. The laboratory received 1 set per quarter and the samples in each set were analyzed in duplicate on 2 different days. The mean of observed measurements ($n = 4$) for each specimen was then compared to the reference value assigned by the CDC isotope dilution LC-MS/MS Reference Method for Total Testosterone. The acceptable performance criterion for this program is $\pm 6.4\%$ overall mean bias. Evaluation of accuracy through the CDC AMP involved measuring 6 serum specimens (3 males, 3 females) provided by the program over a 12-week period. Each sample was assayed in duplicate 4 times within this time frame, with one male and one female sample chosen at random each week. The mean of observed measurements for each specimen was then compared to the reference value assigned by the CDC isotope dilution LC-MS/MS Reference Method for Total Testosterone and deemed acceptable if within the maximum allowable error of $\pm 12\%$. Per the criteria outlined by the CDC, only the first reported result from each run was used in the evaluation of accuracy (samples in patient care are measured only once).

Accuracy was additionally verified by measuring NIST SRM. The SRM consisted of 2 serum specimens, 1 from a pool of healthy, premenopausal adult females, and 1 from a pool of healthy adult males.

To determine imprecision, 5 replicates of each quality control concentration were assayed using each derivatizing reagent across 5 runs. Percent CV was calculated for each derivative. Recovery was also calculated by comparing the mean calculated concentration to the target concentration of each control.

INTERFERENCE, SAMPLE TYPE, STABILITY AND CARRYOVER

Interference from hemolysis, icterus, and lipemia was assessed by spiking patient specimens with known amounts of hemolysate, bilirubin (conjugated and unconjugated), and triglycerides to achieve samples with slight, moderate, and gross levels of each potential interference. The samples were then assayed using the sample-multiplexed method.

To determine possible interference with testosterone, a selectivity study involving 32 endogenous and exogenous steroidal compounds was performed (Supplemental Table 1). Compounds similar to testosterone in structure and molecular weight were chosen, as well as compounds known to potentially cause interference in the standard LC-MS/MS assay. A serum specimen with a known testosterone concentration (14 ng/dL) was spiked with 1000 ng/dL of each steroidal compound and assayed using the sample-multiplexed method. The calculated concentration of each spiked sample was then compared to that of the neat sample.

A sample-type study was performed by analyzing blood samples from 10 individuals (4 males, 6 females) collected in 4 different tube types, including plain red-top with no additive, red-top with gel serum separator tubes (SST), and tubes containing sodium ethylenediaminetetraacetic acid (EDTA) or sodium heparin. These samples were then extracted using the sample-multiplexed method, and results from the SST, sodium EDTA, and sodium heparin tubes were compared against those of the corresponding plain red-top tube, using both linear and Deming regression analysis.

Specimen stability over time was evaluated by analyzing 5 patient specimens of varying concentrations of TT stored frozen, refrigerated, and at room temperature. Storage conditions were monitored and maintained in the following temperature ranges: frozen ($-20.0\,^{\circ}C$ to $-30.0\,^{\circ}C$), refrigerated ($2.0\,^{\circ}C$ to $8.0\,^{\circ}C$), and ambient ($18.0\,^{\circ}C$ to $26.0\,^{\circ}C$). Extracted sample stability was also evaluated by extracting 20 patient specimens, then storing them at 2.0 to 8.0 °C for 3 days in the autosampler of the LC-MS/MS system. Samples were deemed stable if the difference in calculated concentration at a specified time interval relative to the baseline (Day 0) was $\leq 10\%$.

Carryover was assessed by injecting 2 replicates of a low concentration sample ($\sim$10 ng/dL), followed by 2 replicates of a high concentration sample ($\sim$2000 ng/dL), followed by 3 replicates of the same low-concentration sample. This injection sequence was run 3 times per HPLC channel, with all 4 HPLC channels tested.

METHOD COMPARISON

Method comparisons were performed on 1574 residual, de-identified patient serum specimens submitted to Quest Diagnostics for TT testing and previously analyzed using the standard LC-MS/MS method. Specimens were selected at random without consideration of demographics or medical history. Sets of specimens already batched for standard method analysis within the time frame of this validation study were chosen. Specimens were excluded if the minimum required residual volume (200 μL) did not remain, or if possible interference was observed when analyzed using the standard method. Repeat analysis was performed on these selected specimens using the sample-multiplexed method, and the results were compared against those reported by the standard method using both linear and Deming regression analysis.

Results

LC-MS/MS

Baseline separation of the chromatographic peaks representing HOA-T and MOA-T was achieved when injecting 2 mixed samples (Fig. 2). Ion suppression due to matrix effects was observed in patient serum for both MOA-T and HOA-T (Supplemental Fig. 2). Despite this, the isotopically labelled internal standard sufficiently corrected for the suppression to achieve the performance characteristics described below.

With an equivalent data collection window of 1.25 min for both the standard and sample-multiplexed methods, the ability to analyze 2 extracted samples within a single injection doubled the number of samples analyzed from 48 to 96 specimens per hour. Solvent usage was reduced by decreasing both the loading flow rate (4.5 mL/min to 2.5 mL/min) and eluting flow rate (0.9 mL/min to 0.45 mL/min).

LIMIT OF QUANTIFICATION

The limit of quantification was determined for both derivatives in stripped serum and set at 1.0 ng/dL, matching that of the standard method *(8)*. At 1.0 ng/dL, the inter-assay CVs were 18.3% for HOA-T and 19.8% for MOA-T, with recoveries of 104% and 101%, respectively (Supplemental Table 2). The 30-

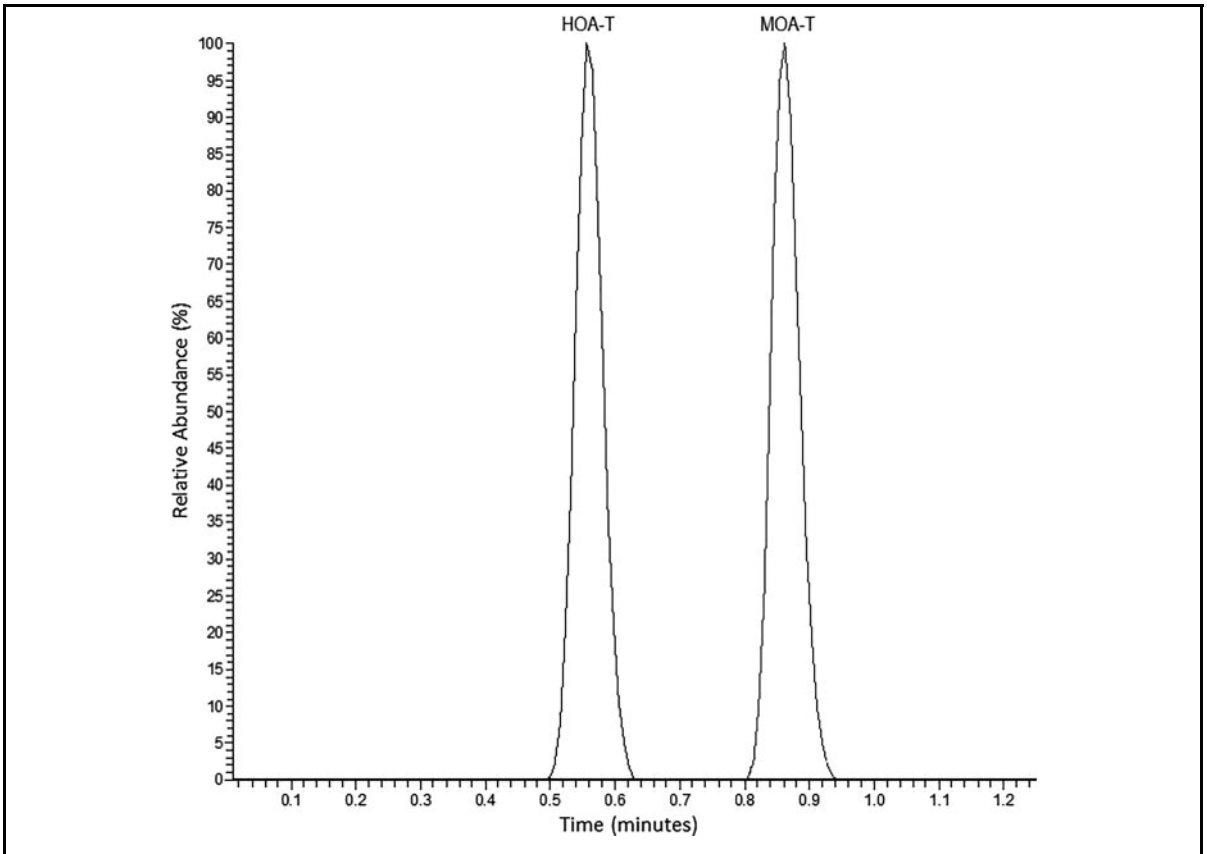

Fig. 2. Representative chromatogram of HOA-T and MOA-T. HOA-T, hydroxylamine derivative of testosterone; MOA-T, methoxyamine derivative of testosterone.

replicate inter-assay CVs of the low-concentration testosterone serum pool extracted as part of the supplemental LOQ study were 10.1% for HOA-T and 11.4% for MOA-T, with mean observed concentrations of 2.8 ng/dL (mean S/N = 20) and 2.6 ng/dL (mean S/N = 15), respectively (Supplemental Table 3). Mean recoveries of the known amount of testosterone (5 ng/dL) spiked into the serum pool were 100% for HOA-T and 95% for MOA-T (Supplemental Table 4).

LINEARITY, RECOVERY, ACCURACY AND IMPRECISION

Linearity was verified from 2.5 ng/dL to 2000 ng/dL for both MOA-T and HOA-T, with R^2 values of linear regression >0.999 and recoveries from 93% to 104% for both derivatives (Table 2, Supplemental Fig. 3). Spiked recovery based on quality controls ranged from 100% to 107% for both derivatives (Supplemental Table 5).

Accuracy of the sample-multiplexed method in measuring CDC HoSt Program reference material met the acceptable criterion to be considered certified, with a mean assay bias of 5.1% (Supplemental Table 6). Additionally, all samples provided by the CDC AMP showed percent differences well within the ±12% maximal allowable error relative to the CDC reference values (Supplemental Table 7). Furthermore, recoveries based on the certified concentrations determined by NIST for the SRM ranged from 98% to 104% for both derivatives (Supplemental Table 8).

Mean intra-assay CVs for MOA-T and HOA-T were <10.0% for all concentrations of controls, with the ultralow concentration (10 ng/dL) showing the highest CV at 8.3% for MOA-T and 8.0% for HOA-T (Table 3). Mean inter-assay CVs for MOA-T and HOA-T were <10.0%, with the highest CV observed for the ultralow concentration at 9.6% for MOA-T and 8.7% for HOA-T (Table 3).

INTERFERENCE, SAMPLE TYPE, STABILITY AND CARRYOVER

No interference was observed from slight to gross lipemia, icterus, or hemolysis (Supplemental Table 9). Of the 32 steroidal compounds tested, one compound (5-androstene-17B-ol-3-one) caused a detectable interference in which the difference in the observed concentrations of the neat and spiked sample was >10%.

Assay results from patient specimens collected in sodium EDTA and sodium heparin tubes showed acceptable concordance with those collected in plain red-top tubes for MOA-T (Deming regression slope of 1.01 and 1.01, respectively) as well as HOA-T (Deming regression slope of 1.00 and 1.04, respectively) (Supplemental Figs. 4 and 5). However, those collected in SST tubes showed acceptable concordance for MOA-T (constant bias of −1.34 ng/dL [95% CI −5.84 to 3.16 ng/dL]), but not for HOA-T (constant bias of 20.54 ng/dL [95% CI −1.83 to 42.91 ng/dL]) (Supplemental Fig. 6).

TT in serum was stable for at least 14 days at room temperature (18.0 °C to 26.0 °C), 21 days refrigerated (2.0 °C to 8.0 °C), and 35 days frozen (−20.0 °C to −30.0 °C). MOA-T and HOA-T in extracted serum was stable for up to 3 days when stored in the cooled autosampler of the LC-MS/MS system (Supplemental Tables 10 and 11).

The maximum observed increase in the mean concentration of a low-concentration TT sample injected

Table 2. Summary of assessment of linearity for HOA-T[a] and MOA-T[b].

	HOA-T				MOA-T			
Target	Mean (ng/dL)	SD (ng/dL)	CV (%)	Recovery (%)	Mean (ng/dL)	SD (ng/dL)	CV (%)	Recovery (%)
2.5	2.4	0.2	9.1	96.0	2.4	0.2	9.8	96.0
5	4.7	0.3	7.1	94.0	4.7	0.4	9.6	94.0
10	9.8	0.4	4.4	98.0	9.3	0.5	5.7	93.0
25	25.0	1.3	5.0	100.0	25.4	1.5	5.8	101.6
50	51.2	1.0	1.9	102.4	51.2	0.6	1.2	102.4
100	101.6	1.8	1.8	101.6	104.1	1.0	1.0	104.1
500	504.3	4.1	0.8	100.9	512.8	1.3	0.3	102.6
1000	977.2	11.0	1.1	97.7	996.9	4.7	0.5	99.7
1500	1464.2	7.4	0.5	97.6	1491.5	6.0	0.4	99.4
2000	2041.8	22.0	1.1	102.1	2066.4	22.4	1.1	103.3

HOA-T, hydroxylamine derivative of testosterone; MOA-T, methoxyamine derivative of testosterone.
[a]Regression Line: y = 1.00x − 2.63 with R^2 =0.9993 (HOA-T).
[b]Regression Line: y = 1.02x − 2.09 with R^2 =0.9995 (MOA-T).

Table 3. Summary of inter- and intra-assay imprecision.

	HOA-T (ng/dL)				MOA-T (ng/dL)			
	UltraLow	Low	Mid	High	UltraLow	Low	Mid	High
Target	10.0	50.0	250.0	1200.0	10.0	50.0	250.0	1200.0
Intra-assay SD	1.0	2.6	3.5	13.8	0.9	2.8	4.3	21.0
Intra-assay CV (%)	8.0	4.5	1.3	1.1	8.3	4.9	1.6	1.6
Total SD	1.0	2.9	9.8	22.0	1.1	2.8	11.8	33.5
Total CV (%)	8.7	5.1	3.6	1.7	9.6	5.0	4.4	2.6

HOA-T, hydroxylamine derivative of testosterone; MOA-T, methoxyamine derivative of testosterone.

after a high-concentration TT sample was 6.6%, indicating no carryover for the sample-multiplexed assay.

METHOD COMPARISON

Linear regression analysis demonstrated good correlation between the standard LC-MS/MS method and the sample-multiplexed method, with an R^2 value of 0.995 and a slope of 1.03 (data not shown). Deming regression analysis confirmed these results, with a slope of 1.03 and a Pearson's *r* value of 0.997 (Fig. 3). Deming regressions of each derivative yielded similar slopes to the entire data set as well (1.03 for MOA-T and 1.04 for HOA-T, Supplemental Figs 7 and 8).

Discussion

The sample-multiplexed method for TT was validated and demonstrated acceptable analytical characteristics and good concordance with the standard method. Accuracy was verified by measurement of reference materials provided by NIST and the CDC and confirmed at serum TT concentrations below 100 ng/dL near the established reference range for adult females (5 to 50 ng/dL) *(9, 12, 13)*. Although LC-MS/MS instrumentation comparable to the standard method was used, electrospray ionization was utilized instead of atmospheric pressure chemical ionization due to an observed enhancement in analytical sensitivity of the derivatives of testosterone, which has been confirmed in literature *(2, 10, 11, 14, 15)*. Due to this enhancement in sensitivity, an LOQ matching that of the standard method (1 ng/dL) was achieved *(7)*. Although it would be ideal to perform an LOQ study in patient matrix, the difficulty of acquiring patient serum free of endogenous testosterone required it be alternatively conducted using stripped serum. Performance was verified in patient serum at a concentration close to the first calibrator (2.5 ng/dL), as serum at a lower concentration of testosterone was unable to be found in the patient population. Adequate inter-assay precision and mean S/N ratios at this concentration were demonstrated, and the difference in observed results between derivatives was <10%.

Unlike the standard method, the sample-multiplexed method has the advantage that it is not susceptible to interference in grossly lipemic and grossly hemolytic serum samples. Furthermore, of the 32 steroidal compounds tested, only 1 interfered with analytical detection of MOA-T and HOA-T, which was an exogenous compound unlikely to be used as a synthetic replacement in testosterone therapy. Similar to the standard method *(8)*, SST samples were determined to be an unacceptable sample type due to the discordance in observed TT values between HOA-derivatized SST and plain-red top samples.

Improved LC-MS/MS throughput by sample multiplexing helped offset the increase in complexity of the sample preparation process relative to the standard HTLC method. The ability to analyze 2 samples within a single injection resulted in a reduction in solvent usage (further achieved by decreasing both the loading and eluting flow rates) and a decrease in the number of LC-MS/MS instruments required to maintain the desired turn-around time. This, in conjunction with the additional sample clean-up steps incorporated to help preserve LC-MS/MS cleanliness, yielded a potential for lower instrument maintenance costs. The use of an automated liquid handler for sample preparation minimized the required manual sample manipulation, resulting in low imprecision of technical replicates, consistent batch-to-batch results and a decrease in the possibility of cross contamination between patient specimens and reagents due to human error. Automation was also critical in minimizing the impact of a lengthier sample preparation process relative to the standard method (about 4.25 h versus 1.5 h to process 384 samples). Inaccurate analysis of the mixed patient specimens was further prevented by derivatizing testosterone with reagents that allowed for a rapid and stable reaction. Additionally, each derivative was identified with unique ion pairs, and the corresponding chromatographic peaks were adequately resolved.

With confidence in the reliability and accuracy of the new method, the standard methodology was replaced with the new, multiplexed assay as the routine clinical assay to quantitate TT in serum and is now currently operational within Quest Diagnostics.

In summary, our results support sample multiplexing by differential mass tagging as an acceptable alternative to the standard LC-MS/MS assay for quantifying TT in serum. The approach achieves the higher LC-MS/MS throughput required for this high-volume assay without compromising accuracy or analytical sensitivity. Additionally, automation minimized the impact of increased complexity of sample preparation relative to the standard method.

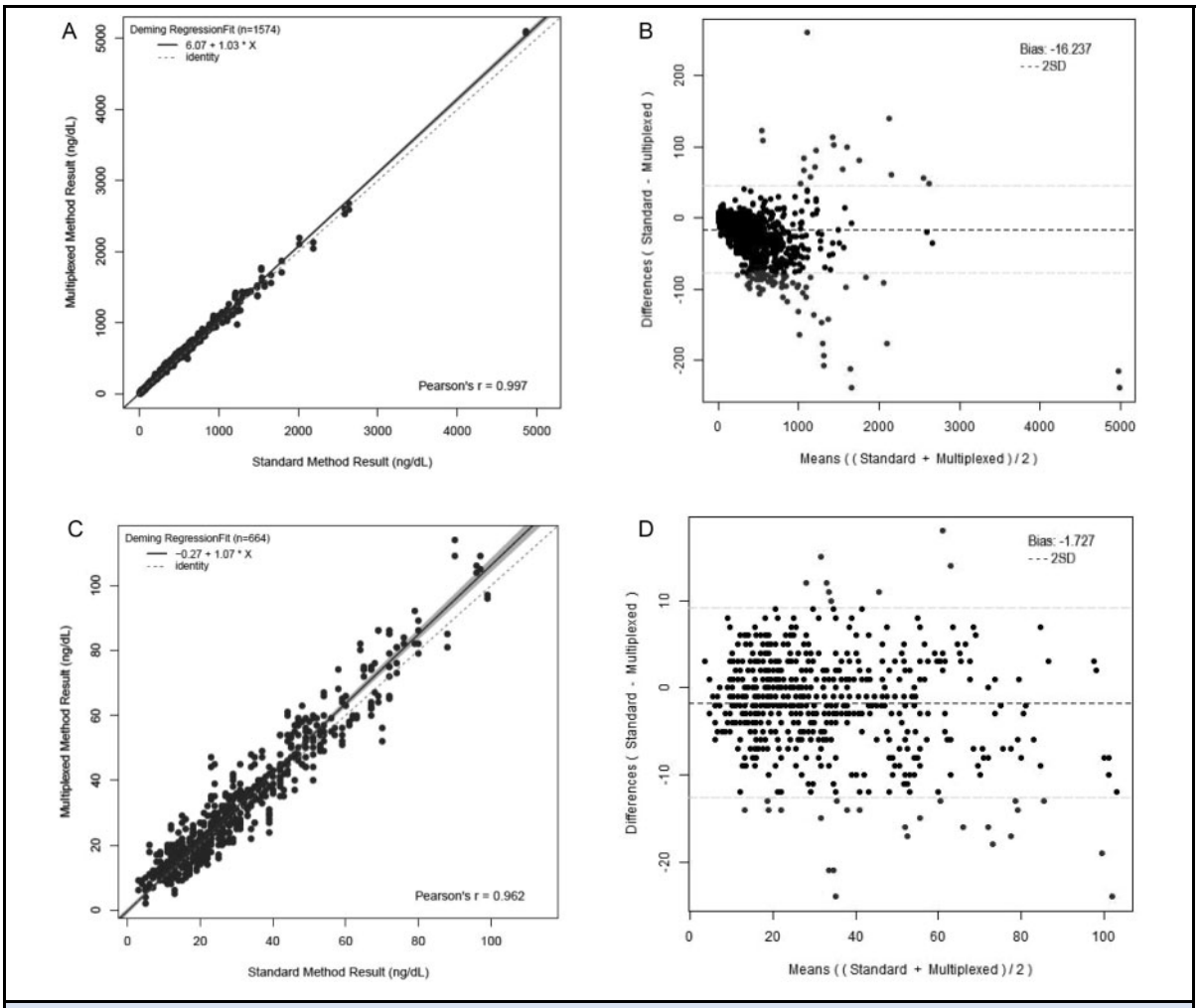

Fig. 3.. Method comparison between standard method and sample-multiplexed method. (a) Scatterplot with Deming Fit for HOA-T and MOA-T measurements for 1574 patient specimens with quantifiable concentrations of testosterone ranging from 3 to 4862 ng/dL. (b) Bland–Altman plot for (a). (c) Scatterplot with Deming Fit for HOA-T and MOA-T measurements for a subset of the 1574 patient specimens with quantifiable concentrations of testosterone ranging from 3 to 99 ng/dL (<100 ng/dL, n=664). (d) Bland–Altman plot for (c). HOA-T,hydroxylamine derivative of testosterone; MOA-T, methoxyamine derivative of testosterone.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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