

RESEARCH ARTICLE

A robust LC–MS/MS assay with online cleanup for measurement of serum testosterone

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Accurate measurement of low levels of testosterone is critical for diagnosis and treatment of androgen disorders. The very low concentrations of testosterone in children, females, and males with androgen suppression therapies necessitate the use of mass spectrometry-based methods. We aimed to develop a liquid chromatography with tandem mass spectrometry method with simplified sample preparation and online solid-phase extraction cleanup to achieve enhanced precision, accuracy, robustness, and cost-effectiveness. The assay was linear from 10 to 20 000 pg/mL with an analytical recovery of 93–104%. The total coefficient of variation was 2.5, 1.9, and 1.7% at concentration levels of 348, 5432, and 10 848 pg/mL, respectively. No significant carryover was observed from samples with concentrations up to 20 000 pg/mL. No significant interference was observed from androstenedione, dehydroepiandrosterone, epi-testosterone, and estriol. Comparison with CDC Hormone Standardization program (HoSt) reference samples with defined values ($n = 40$) showed a Deming regression slope of 0.963, intercept of 28.06 pg/mL, standard error of estimate was 66.9, a correlation coefficient of 0.9996, and a mean bias of -0.6% . The method met the accuracy criteria by the CDC HoSt program. In addition, we achieved >12 000 injections on a single analytical column without significant performance deterioration due to the specific online solid-phase extraction settings.

KEYWORDS

liquid chromatography, mass spectrometry, online extraction, testosterone

1 | INTRODUCTION

Testosterone (T) is the primary androgen hormone in men, important for the development and maintenance of male characteristics [1–3]. In females, T concentrations are associated with sexual functions [4,5]. Disorders associated with abnormal androgen metabolism include hypogonadism [3], disorders of puberty [6], amenorrhea, polycystic ovary

syndrome [7,8], and tumors of ovary, testis, breast, and prostate [9]. Recent research findings have also associated T concentrations with other conditions such as cardiovascular diseases [10], metabolic syndrome [11,12], diabetes [13,14], osteoporosis [15], Alzheimer [16], depression [17], and mortality in men [18,19]. Accurate measurement of T in circulation is important for clinical diagnosis and treatment of androgen disorders in men, women, and children [2].

There are many assays available for measuring T in circulation. Immunoassays are widely available on automated immunochemistry platforms. However, these immunoassays present analytical challenges: 1) there are significant interferences with positive bias at low concentrations, which are commonly observed in T-deficient men, women, and children;

Article Related Abbreviations: HoSt, hormone standardization program; IS, internal standard; PBS, phosphate-buffered saline; RMP, reference measurement procedure; SV, single vortex method; T, testosterone; VFV, vortex-freeze-vortex method.

2) there is poor agreement among different immunoassays [20]. Mass spectrometry based assays offer high sensitivity and specificity [21–24]. In order to improve consistency of different methods, CDC established the Hormone Standardization program (HoSt) for T assays [25]. The key elements of this program include: 1) the use of a certified pure reference material to calibrate the reference method and establish the metrological traceability [26]; 2) the reference measurement procedure (RMP) to provide highest available reference measurement [27]; 3) reference samples with target values assigned by RMP to provide assay calibration and calibration verification for participants with a mean bias of 6.4% or less required for sufficient accuracy [28]. Currently 12 assays (11 LC–MS/MS, and 1 immunoassay) are certified by the CDC HoSt program [27]. Endocrine Society and Urology Society guidelines recommend using CDC-certified assays that have similar sensitivity, specificity, and accuracy with LC–MS/MS assays.

Sample preparation for LC–MS/MS assay is very important to assure appropriate performance. Liquid-liquid extraction (LLE) and SPE are the most commonly used methods by the CDC HoSt certified methods. LLE [29] usually offers very clean samples with the caveat of being labor-intensive and time-consuming. Online SPE [30,31] or turbulence flow extraction is attractive for routine clinical use because of the simplicity, cost-effectiveness, and ease of automation. However, the online extraction methods by the CDC HoSt certified methods showed lower sensitivity than the offline SPE or LLE methods [32].

We aimed to develop a novel online SPE extraction workflow for routine measurement of T in serum, which is simple, accurate, cost-effective, and easy to automate.

2 | MATERIALS AND METHODS

2.1 | Materials

LC–MS grade acetonitrile and methanol were purchased through Thermo Fisher Scientific (Fair Lawn, NJ). Ultrapure water was prepared with a Milli-Q water purification system (Billerica, MA). T and T- $^{13}\text{C}_3$ were from Cerilliant (Round Rock, TX). Androstenedione, dehydroepiandrosterone, epi-T, estriol, and Fluka formic acid were purchased through Sigma-Aldrich (St. Louis, MI). In-house calibrators were prepared in phosphate-buffered saline (PBS) from Thermo Fisher Scientific (Vilnius, Lithuania) with 1% BSA from Sigma-Aldrich (St. Louis, MI), via serial dilution, at the following concentrations: 10, 39, 156, 625, 2500, and 10 000 pg/mL. In-house quality controls (QC) were prepared in female patient sample pools with spike-in concentrations of 150 and 10 000 pg/mL. Internal standard (IS) solution (T- $^{13}\text{C}_3$, 250 pg/mL) was prepared in ACN.

2.2 | Sample preparation

Patient samples were collected in serum tubes without gel separator. Two hundred microliters of sample (calibrators, QCs, and patient samples) was mixed with 400 μL of IS solution. To facilitate protein precipitation, the mixture was vortexed for 10 min, stored at -20°C for 7 min, then vortexed again for 10 min. After centrifugation at $4000 \times g$ for 10 min, 200 μL supernatant was mixed with 200 μL water, and the resulting solution (60 μL) was injected for LC–MS/MS analysis (Figure 1).

2.3 | Online SPE and LC–MS/MS method

The analysis was performed on an LX2 system coupled with a Thermo TSQ Altis tandem mass spectrometer (San Jose, CA). The online SPE procedure was performed on a C18 SecurityGuard cartridge from Phenomenex (Torrance, CA). The valve switching and gradient conditions are shown in Table 1 and Figure 1. First, 60 μL of sample prepared as specified in section 2.2 was directly injected onto the SPE cartridge with six-port injector valve in 6-1 position to waste. Second, the six-port valve was switched to 2-1 position to connect the SPE cartridge with the C18 analytical column (100×2.1 mm, 5 μm , Phenomenex) maintained at 30°C . Last, the SPE cartridge and the analytical column were washed and conditioned prior to the next injection.

The mass spectrometer was in positive ESI mode with the voltage set to 1000 V and turbo ion spray source temperature at 350°C . The following transitions were monitored: m/z 289 \rightarrow 97 (quantitation), m/z 289 \rightarrow 109 (identification) for T; m/z 292 \rightarrow 100 (quantitation), and m/z 292 \rightarrow 112 (identification) for the IS. Quantitation was based on peak area ratio of the T over IS using TraceFinder software (Thermo Fisher).

2.4 | Method validation

To validate the method, the following experiments were performed: ion suppression/enhancement, mixing study, interference, analytical measurement range (AMR), carry-over, stability, precision, method comparison, and reference interval verification. From the stability experiment onwards, QCs at two levels were analyzed with every batch. The use of leftover patient samples in this study was approved by the Cleveland Clinic Institutional Review Board.

2.4.1 | Matrix effects

To assess qualitative matrix effects, post-column infusion experiments were performed. The qualitative ion suppression/enhancement was evaluated by infusing a 200 ng/mL solution of the IS through a tee connection post-column at 10 $\mu\text{L}/\text{min}$, while a solvent blank and ten serum samples (five males and five females) after extraction without IS

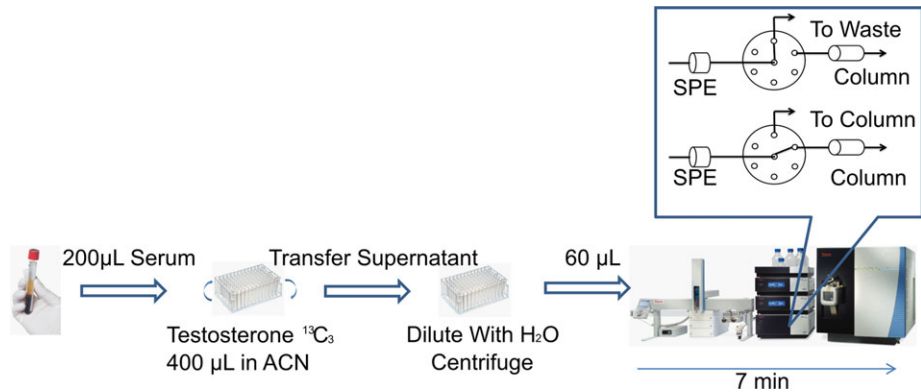


FIGURE 1 Streamlined workflow for serum testosterone measurement by LC-MS/MS with online SPE extraction, requiring minimal sample processing and enabling >12 000 injections on single analytical column

TABLE 1 Online SPE and LC condition

Time (min)	Flow rate (mL/min)	A% ^a	B% ^b	Valve switching	
				Time (min)	Position
0	0.6	80	20	0	1-6 (to waste)
1.5	0.6	80	20	1.6	1-2 (to column)
2.5	0.6	40	60	5.8	1-6 (to waste)
4.5	0.6	25	75		
5.0	0.6	0	100		
5.5	0.6	0	100		
5.8	0.6	5	95		
5.9	0.6	5	95		
6.0	0.6	80	20		
7	0.6	80	20		

^aMobile phase A: 5.0 mM ammonium formate with 0.1% formic acid in H₂O.

^bMobile phase B: 5.0 mM ammonium formate with 0.1% formic acid in methanol.

were injected individually. The signal intensity for the IS was monitored throughout the chromatogram. A significant reduction or enhancement in signal intensity at the expected retention time in the patient samples compared to the solvent blank indicated matrix effects.

2.4.2 | Mixing study

The acceptability of a candidate matrix for calibrator preparation and patient sample dilution was determined via a mixing study. The candidate matrix PBS with 1% BSA spiked with 10 000 pg/mL of T was mixed 1:1 with patient samples (five males and five females) individually. The candidate matrix, the patient serum sample, and the mixture were then extracted and analyzed. The response ratio (T/IS) of each mixture was expected to be within 20% of the calculated value based on the corresponding results from the candidate matrix and patient sample.

2.4.3 | Interferences

Four potential non-specific endogenous interfering conditions were evaluated: lipemia (L index: 689), hemolysis (H

index 288), icterus (I index: 12), and uremia (urea nitrogen: 10 mg/dL). The serum indices were measured by Roche Cobas c502 or c702. This experiment was performed at two concentrations (spiked 70 µg/mL and 10 000 pg/mL in PBS with 1% BSA solution), by mixing the spiked samples 1:1 with the interferent samples individually. Significant interference was observed if the response ratio of the mixture was >20% different from the mean response of the corresponding spiked solution and the interferent sample.

Androstenedione, dehydroepiandrosterone, epi-T, and estriol are structural analogs of T with close molecular weights. These potential interferents were spiked in PBS with 1% BSA at the concentration of androstenedione 5000 pg/mL, dehydroepiandrosterone 80 ng/mL, epi-T 10 000 pg/mL, and estriol 28 ng/mL, respectively. Each spiked sample and a blank PBS with 1% BSA solution were processed as patient samples. The absence of a peak at the retention time for T and IS indicated no obvious interference from these analogues.

Stable isotope-labeled ¹³C₃-T might include a small amount of unlabeled T, which could interfere with the quantitation. The potential impurity of internal standard was

evaluated by injecting a 1000 pg/mL $^{13}\text{C}_3$ -T solution and monitoring the T transitions.

Phospholipids can be a major cause of matrix effects. Potential interference from phospholipids was monitored at transition m/z 184→184 [33].

2.4.4 | Analytical measurement range

AMR was assessed using serially diluted samples from a spiked female serum pool at these concentrations: 10, 20, 39, 78, 156, 313, 625, 1250, 2500, 5000, 10 000, and 20 000 pg/mL. These samples were extracted in triplicate and analyzed in one batch. A concentration level was considered acceptable if the analytical recovery was within $100 \pm 20\%$, coefficient of variation (CV) $\leq 20\%$, and a signal-to-noise ratio > 10 . The Deming regression with an $R > 0.9$ was considered acceptable for linearity. The AMR range was determined by the consecutive concentration levels that passed all the criteria and the lowest concentration included in the AMR was considered the lower limit of quantification (LLOQ).

2.4.5 | Carryover

Three sets of a mobile phase blank sample after a spiked serum specimen (20 000 pg/mL) were processed and injected for analysis. Significant carryover was defined as a measurable signal in the blanks $>20\%$ of the LLOQ signal.

2.4.6 | Stability

Stability in serum was evaluated for three storage conditions: ambient, 4 and -20°C at three concentration levels (160, 5660, and 11 273 pg/mL), which were prepared by spiking a serum pool. There were three aliquots for each sample at each time point of the specified storage condition (Table 2). When the designated time point was reached, the three aliquots were removed and stored at -70°C until analysis. At the end of the study, all of the samples were extracted and analyzed in a single batch. A similar study was performed for stability after extraction. Serum samples after extraction were placed in the

LC autosampler, which was maintained at 10°C . The samples were analyzed when each time point was reached (Table 2). The analyte was considered stable at the specific time point if the mean value was within 20% of the mean value of the original sample set (time 0) for each condition. Freeze-thaw stability was not performed because the information was readily available in the literature [34].

2.4.7 | Precision

Precision was evaluated based on the Clinical & Laboratory Standards Institute (CLSI) EP10-A3 guideline using spiked serum pools at levels of 348, 5432, and 10 848 pg/mL, respectively. The following sequence was run twice a day for five days: mid \rightarrow high \rightarrow low \rightarrow mid \rightarrow mid \rightarrow low \rightarrow low \rightarrow high \rightarrow high \rightarrow mid. The precision was deemed acceptable if the total CV was $\leq 20\%$ for all levels tested.

2.4.8 | Accuracy

The quantitation accuracy of the method was assessed by analyzing 40 phase I samples with values assigned by the RMP offered by CDC HoSt program. An assay with a mean bias within 6.4% was considered sufficiently accurate by the program passing criteria. Percentage of samples passing this criterion was also calculated.

2.4.9 | Data analysis and statistics

Statistical analysis was performed using EP Evaluator (10.3.0.556; Data Innovations) and Microsoft Excel 2013 (15.0.4569.1504; Microsoft).

3 | RESULTS

3.1 | Matrix effects

The post-column infusion experiment did not show significant qualitative ion suppression or enhancement. Based on the signal intensity, the estimated matrix effects (ion suppression) were about 2–15%.

3.2 | Mixing study

The percent differences of the measured ion ratios (T/IS) in the mixers and the calculated values from the corresponding patient specimens and spiked PBS with 1% BSA ranged from -6.4 to 1.4% , indicating no significant differential matrix effects. Therefore, the solution of PBS with 1% BSA was deemed acceptable to be used as the matrix for calibrators or diluent.

TABLE 2 Time points and conditions for stability evaluation

Time point	Non-extracted			Extracted
	Ambient	4°C	-20°C	10°C
Day 0	✓	✓	✓	✓
Day 2	✓			
Day 3		✓		
Day 4	✓			
Day 5		✓		
Day 7				✓
Day 14				✓
Day 30			✓	
Month 6			✓	

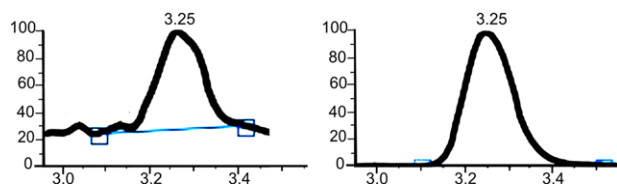


FIGURE 2 Chromatogram of a patient serum sample at 14 pg/mL for T (A) with IS (B)

3.3 | Interferences

From our preliminary experiments, we found that serum collection tube with gel separator may cause interference with testosterone measurement. Therefore, serum samples used in this study were collected in red top tubes without gel separator.

The potential interference of common non-specific endogenous conditions including hemolysis, icterus, lipemia, and uremia was evaluated by mixing studies. The measured ion ratio (T/IS) of each mixture was within 6.6% of the calculated value, indicating that the method was free of interference from lipemia, hemolysis, icterus, and uremia at the serum indices studied: L index: 689, H index: 288, I index: 12, and urea nitrogen: 10 mg/dL. There was no obvious interference from androstenedione (5000 pg/mL), dehydroepiandrosterone (80 ng/mL), epi-T (10 000 pg/mL), and estriol (28 ng/mL). We observed no isotopic interference from potential impurity in the IS. In addition, there was no peak of m/z 184 present at the retention time T, indicating no significant interference from phospholipids.

3.4 | Analytical measurement range

AMR was assessed based on clinical needs via serial dilution of a spiked female serum pool. The CV was < 13.3% and the analytical recovery ranged from 93.0 to 104.9% for all levels studied. The lowest concentration studied and passed the pre-set criteria was 10 pg/mL and the highest was 20 000 pg/mL. The mean signal to noise ratio at the LLOQ (10 pg/mL, $n = 3$) was 33 with a CV of 13.3%. The chromatogram of a patient specimen near the LLOQ is shown in Figure 2.

3.5 | Carryover

None of the blanks after the high concentration samples (20 000 pg/mL) showed a measurable signal for T, indicating no significant carryover for specimens with concentrations up to 20 000 pg/mL.

3.6 | Stability

The unextracted serum samples were found to be stable for the entire study period: 4 days at room temperature, 14 days at 4°C, and 6 months at −20°C. After extraction the samples

TABLE 3 Precision data following CLSI EP10-A3 guidelines

Precision	Low Level	Mid Level	High Level
<i>N</i>	30	30	30
Grand Mean (pg/mL)	348.0	5431.6	10 848.8
Total CV (%)	2.5	1.9	1.7
Total SD	8.5	105.7	180.3
Within Run SD	5.8	70.4	141.6
Within Run CV (%)	1.7	1.3	1.3

were stable for five days in the auto-sampler compartment which was maintained at 10°C (Table 2). We found that the calibrators in PBS with 1% BSA were not sufficiently stable for a freeze-thaw cycle to maintain linearity. Therefore, the calibrators were aliquoted and stored in single-use tubes at −70°C.

3.7 | Precision

Precision of the method was evaluated based on CLSI EP10-A3 guidelines using spiked serum pools with 30 data points at each level. The total CV was found to be within 2.5% for all three levels studied (Table 3).

3.8 | Accuracy

Forty serum samples offered by CDC HoSt Phase 1 were analyzed to assess accuracy of this method. These samples had assigned values ranging from 72.7 to 7460 pg/mL. Deming regression of the results by this method and the assigned values showed a slope of 0.963, intercept of 28.06 pg/mL, standard error of estimate of 66.9, and a correlation coefficient of 0.9996 (Figure 3). The mean bias was −0.6% and the individual sample passing rate was 85%, indicating a high-quality method meeting the certification standard [27].

4 | DISCUSSION

There are obvious advantages of online extraction compared to the manual sample preparation. However, most of the online extraction methods certified by the CDC HoSt program are less sensitive than the offline SPE or LLE methods [32], and some online extraction methods have relatively large intra- and inter-assay variations [35], which are higher than CDC HoSt desirable CV of 5.3% [28]. The online extraction workflows need improvement for enhanced precision, sensitivity, and accuracy.

From our investigation, we found that incomplete dissociation of T from its binding proteins could result in increased variations and decreased sensitivity. We also found that workflow simplification could increase assay sensitivity, precision, and cost-effectiveness.

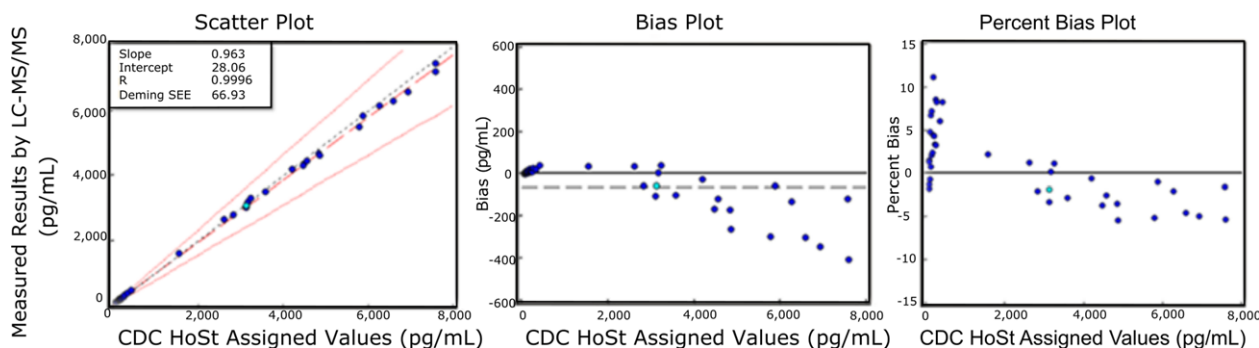


FIGURE 3 Deming regression of measured results by this LC-MS/MS versus CDC HoSt assigned values

4.1 | Optimization of protein precipitation

Extraction of the analyte with IS is a critical step that affects precision and accuracy. T exists in serum in both free and protein-bound forms, which include both sex hormone-binding globulin bound and albumin bound. To measure the impacts of the extraction methods on precision and recovery, we compared two protein precipitation methods. In one experiment we vortexed the mixture of acetonitrile and serum for 10 min (single vortex method, SV). In the second experiment, the mixture was vortexed for 10 min, kept at -20°C for 7 min, then vortexed for 10 min again (vortex-freeze-vortex method, VFV). We found that the VFV improved recovery from 82–89% to 93–104% compared to the SV in the AMR study. We also did side-by-side comparison of the two protein precipitation methods using 40 individual and 48 spiked patient samples. We found T peak areas and the ion ratios of T/IS were $\sim 7\%$ higher for the VFV than the SV. The results suggest that extraction efficiency and consistency of the VFV were better than the SV.

4.2 | Simplification of workflow

Fewer sample preparation steps may lead to better sensitivity, lower cost, higher throughput, and easier to implement and automate. We simplified and streamlined each step to avoid sample transfer and to eliminate unnecessary steps. For instance, we removed a filtration step, which was designed to remove tiny particulates before sample injection to avoid clogging of the tubing system between the injector and column. Instead, we prevented the potential clogging issues by: 1) increasing the inner diameter of the tubes; 2) centrifugation to precipitate the small particles; 3) avoiding aspirating from the bottom of sample vials. These simplified approaches improved the sensitivity by threefold compared to the method using filtration.

4.3 | System robustness

From our preliminary experiments, injection of $60\ \mu\text{L}$ without online SPE cleanup would overload the analytical column,

leading to loss in resolution. Online SPE cleanup allows large volume injection and protects the analytical column from performance deterioration.

We assessed robustness of this method by monitoring variations of back pressure, retention time, and IS areas. The back pressure did not significantly increase after $>12\,000$ injections on the analytical column. When we noticed back pressure increase (>30 bar), we changed the guard column. Usually a guard column could last >2000 injections. The variation of T retention time was $<1\%$. Variation of IS peak areas may indicate variation in instrument performance, extraction efficiency, and/or matrix effects. We found peak areas of IS in calibrators, controls, and samples were consistent within a run, showing a CV of $<10\%$. We achieved $>12\,000$ injections on an analytical column without significant performance deterioration.

5 | CONCLUDING REMARKS

In conclusion, we developed and validated a novel LC-MS/MS workflow with online SPE for total T quantification in serum. The method offers high precision, sensitivity, accuracy, robustness, and cost-effectiveness via simplification of sample preparation and online extraction.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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