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Using mass spectrometry to overcome the longstanding inaccuracy of a commercially-available clinical testosterone immunoassay

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ABSTRACT

Accurate measurement of testosterone is important for the diagnosis of gonadal disorders in men, women, and children. Testosterone measurement has limited accuracy at low concentrations by most commercially available immunoassays. We aimed to develop an LC-MS/MS assay to address the inaccuracy of the in-house immunoassay observed over the past decade and to replace it with the new assay. Testosterone in serum/plasma was extracted with commercial supported liquid extraction plates. Method validation was performed following the CLSI C62-A guideline. A total of 126 samples were used for method comparison between the Beckman UniCel DxI immunoassay and LC-MS/MS. Results by immunoassay were 20% lower compared with LC-MS/MS and had minimal correlation (R^2 = 0.403) with LC-MS/MS below 100 ng/dL. When comparing specimens from the Accuracy-Based Survey from the College of American Pathologists, the newly developed assay agreed well with the CDC reference measurement procedure. In summary, immunoassay measurement of testosterone can be significantly inaccurate, especially at low concentrations. The newly developed LC-MS/MS assay provides accurate results across the entire measurable range.

1. Introduction

Testosterone is a cholesterol-derived androgen (sex hormone) with a molecular mass of 288.4 Da. Testosterone is primarily produced by the Leydig cells in the testes and by the theca cells in the ovaries, with small quantities produced by the adrenal glands in both sexes. Testosterone is responsible for primary sexual development and regulation of secondary male characteristics. In addition, it also plays systemic roles in the maintenance of bone density, muscle mass, and erythropoiesis [1]. Measurement of testosterone is central in the workup of hypogonadism in men, hyperandrogenism in women (e.g., idiopathic hirsutism, congenital adrenal hyperplasia, polycystic ovarian syndrome, and androgen-secreting ovarian or adrenal tumors), and delayed or precocious puberty in children [2–4]. It is also a useful biomarker to monitor testosterone supplementation in patients with hypogonadism and transgender men, as well as to monitor testosterone suppression in specific patients with prostate cancer [2,5].

In circulation, testosterone binds to sex hormone binding globulin (SHBG) with high affinity and binds to albumin with low affinity. Only a small fraction of testosterone is present as free testosterone. Because the concentration of total testosterone can be affected by the abundance of SHBG and albumin, the concentration of free or bioavailable testosterone (the sum of free testosterone and that loosely bound to albumin) may be more informative in cases where SHBG or albumin are abnormal [2].

Immunoassay measurements of total testosterone can be significantly inaccurate, especially at low concentrations (i.e., <100 ng/dL) [6,7]. This is partially due to the technical challenges around making the signal of low concentrations of analyte statistically different from noise. In addition, different testosterone immunoassays use different approaches to calibration, which contributes to a lack of concordance of results between platforms and laboratories. The CDC formally harmonized the reference range for total testosterone in men in 2017 [8]. However, the application of this reference range to the diagnosis of

Abbreviations: **SHBG**, sex hormone binding globulin; **SLE**, supported liquid extraction; **MTBE**, methyl tert-butyl ether; **ABS**, accuracy-based survey; **HoSt**, hormone standardization.

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hypogonadism in adult males across all laboratories requires that testosterone results be comparable to those generated by the CDC reference measurement procedure.

Our laboratory has been using the testosterone immunoassay from Beckman Coulter (UniCel DxI). Clinical demand from our endocrinologists prompted us to set up a better in-house assay for accurate measurement of testosterone. Given the cost of purchasing another immunoassay platform, we decided to develop a testosterone assay on our existing mass spectrometry platform to replace the immunoassay. The assay described in this manuscript only measures testosterone; however, it has the capacity for a multi-plex panel covering other clinically relevant steroid hormones in the future. Our method takes advantage of supported liquid extraction (SLE) to simplify sample preparation, which can be easily automated. We confirmed the inaccuracy of the Beckman UniCel DxI immunochemiluminescent total testosterone assay observed over the past decade.

2. Materials and methods

An abbreviated list of materials, methods, and procedural steps is included here. A complete standard operating procedure is provided in **Supplementary Material**.

2.1. Sample collection and storage

The analysis of de-identified clinical samples for aggregation and publication was approved by the Human Subjects Division at the University of Washington (STUDY00013082). Samples were centrifuged and serum or plasma was removed from cells within 6 h and stored at 2–8 °C.

2.2. Materials and instruments

The following reagents, materials, and equipment were commercially available: testosterone (T-037, Cerilliant), testosterone-2,3,4-^{13}C (730610-1ML, Sigma-Aldrich), DC Mass Spect Gold human serum (MSG4000, Golden West Biologicals), HPLC grade heptane (H350-1, Fisher), HPLC grade methyl *tert*-butyl ether (MTBE; E127-4, Fisher), ammonium acetate (100651, ICN Biomedicals, Inc.), Optima LC/MS methanol (A454-4, Fisher), Opitima LC/MS acetonitrile (A9554-4, Fisher), formic acid (88%; JT0128-1, VWR), Optima LC/MS water (W6-4, Fisher), sodium chloride 0.9% solution (2F7122, Baxter Healthcare), ISOLUTE SLE + 200 µL SLE plate (820–0200-P01, Biotage), 96-Well 2 mL SQ well collection plate (186002482, Waters), Biotage Turbovap 96-well plate evaporator, Biotage PRESSURE + positive pressure manifold, and Beckman Allegra X-22 Centrifuge. For HPLC-MS/MS analysis, samples were analyzed using an Acquity HPLC T3 C18 1.8 µm, 50 × 2.1 mm analytical column (186003538, Waters) on a Waters Acquity UPLC system coupled to a Waters/Xevo TQ MS API tandem mass spectrometer.

2.3. Calibrators, internal standard, quality control materials, and samples from the College of American Pathologists survey

An intermediate testosterone standard solution of 1,000 ng/mL was made by diluting the stock (100 µg/mL) testosterone solution in methanol. A seven-point calibration curve was prepared by diluting the intermediate standard with MSG4000, resulting in target concentrations of 10, 50, 200, 400, 600, 800, and 1,000 ng/dL. The internal standard of testosterone was prepared by diluting the 0.1 mg/mL ^{13}C-testosterone in methanol solution to 10 µg/mL of stock solution and subsequently to 1 ng/mL of working solution in saline. Testosterone with incorporated ^{13}C was chosen instead of deuterated internal standard, because it had the same chromatographic retention time as native testosterone (**Figure S3**).

Two levels of quality control materials were from BioRad (Liquicheck Immunoassay plus). A negative control was made from DC Mass Spect Gold Human Serum. Calibrators and internal standards were stored in aliquots at −80 °C. Twelve residual samples from the 2019–2020 Accuracy Based Steroid Survey from the College of American Pathologists (CAP) were also used to evaluate accuracy.

Table 1
MRM transitions.

Compound	Transition	Dwell time (ms)	Cone (V)	Collision (V)
Testosterone	289.3/97.0*	0.078	30	24
Testosterone	289.3/109.1	0.078	30	24
Testosterone-2,3,4-^{13}C	292.3/100.1*	0.078	34	21
Testosterone-2,3,4-^{13}C	292.3/112.1	0.078	34	21

* Indicates the quantifier.

2.4. Sample preparation

The workflow is depicted in **Figure S1**. Samples and reagents were brought to room temperature, as necessary. A volume of 120 µL of internal standard was added to each well of a Waters 96-well 2 mL deep-well plate, followed by 120 µL of calibrator, control, or patient sample. Samples were sealed with a WebSeal mat and mixed on a multi-tube vortex mixer at speed of 5–6. A volume of 200 µL of each sample was transferred to a SLE plate above a Waters 96-well 2 mL deep-well plate . The stacked plates were put onto the positive pressure manifold and the solution was pushed into the sorbent at 3 psi until all fluid was absorbed. After 5 min of incubation, samples were twice extracted with 375 µL of heptane/MTBE (50:50, v:v). The fluid was drained completely from the extraction plate into the collection plate by applying pressure of 3 psi with the positive pressure manifold. Eluent (~750 µL) in the collection plate was dried in a Turbovap concentrator for 20–30 min at 30 °C at a nitrogen flow rate of 20F/hr. Samples were reconstituted with 100 µL of methanol/water (55:45, v:v).

2.5. HPLC-MS/MS parameters

A volume of 15 µL of reconstituted extract was injected onto the HPLC column for chromatographic separation and MS/MS analysis. Mobile phase A was composed of 2 nM ammonium acetate/0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The flow rate was 0.3 mL/min over a 4-min gradient: 0–1 min, 45 % B; 1–3.5 min, 45–65 % B; 3.5–3.51 min, 65–98 % B; 3.51–4 min, 98–45 % B. Two transitions were monitored per molecule (Table 1). The MS parameters are summarized in **Table S1**.

2.6. Method comparison for total testosterone by LC-MS/MS

The accuracy of the method was evaluated by two approaches. First, interlaboratory comparison with LC-MS/MS assay at Mayo Clinic Laboratories, Rochester, MN was performed. For the comparison, both laboratories analyzed 40 de-identified individual serum samples ranging 7–1600 ng/dL and the total testosterone concentrations were compared. Second, residual Accuracy-Based Steroid Survey (ABS) proficiency testing samples from calendar years 2019 and 2020 were tested using the newly developed method. Total testosterone concentration was compared to the target concentration determined by the CDC testosterone reference measurement procedure. We also compared our current Beckman UniCel DxI 800 total testosterone assay to the LC-MS/MS assay by analyzing in parallel 126 clinical samples covering the full range of the AMR.

2.7. Automated liquid handling

To reduce the manual steps involved in this relatively simple assay,

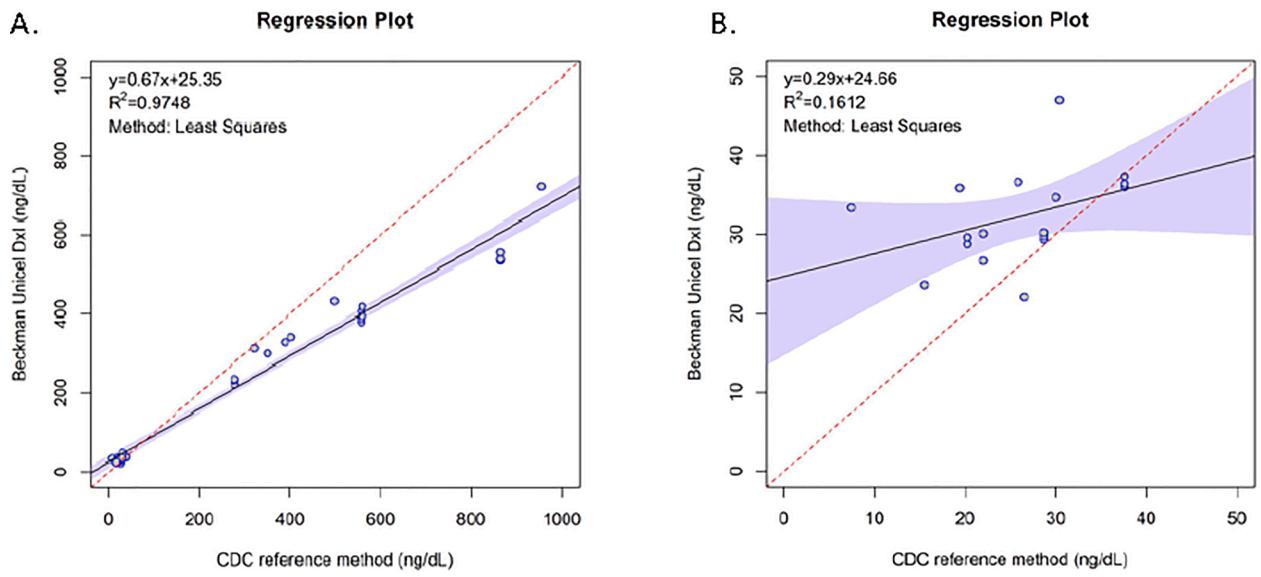

Fig. 1. Historical performance of Beckman UniCel DxI testosterone immunoassay. Data from ABS participant summary reports were compiled for 2010–2018. Peer group means for the Beckman UniCel DxI platform are plotted vs. the values provided by the CDC reference measurement procedure (N = 34, two peer group means were unavailable due to insufficient number of laboratories). The full range (***A***) and low concentrations<100 ng/dL (***B***) are shown.

we tested the performance of a Hamilton automated liquid handling system (Starlet 8; Reno, NV). Method comparison was made using clinical samples prepared using manual or automated pipetting.

2.8. Data analysis and illustrations

MS data were processed using the TargetLynx software. Excel and cp-R [9] were used for linear regressions. The workflow in Figure S1 was created with BioRender.com.

3. Results

3.1. Validation of the LC-MS/MS method

The LC-MS/MS method to quantify total testosterone in human serum and plasma was validated according to CLSI C62-A [10]. Validation experiments are described in **Supplemental Material**. The lower limit of the measuring interval was 5 ng/dL. The assay was linear up to 3,000 ng/dL. We chose 10–1,000 ng/dL as the analytical measurement range. The intra-assay CV was 1.02 and 1.01% for the low and high levels of QC material, respectively. Since going online clinically, the inter-assay CV has been < 3% for both levels of QC materials in 67 runs within four months (data not shown). The recovery was 100–106%. No obvious carryover or interferences were observed. Serum and EDTA-anticoagulated plasma from phlebotomy tubes without gel separation barriers were determined to be acceptable. Samples were stable after separation at 4 °C for at least 18 days.

3.2. Beckman UniCel DxI immunoassay and accuracy- based proficiency testing

To evaluate the performance of Beckman testosterone immunoassay, historical CAP survey results were analyzed. Linear regression demonstrated a significant bias in our current Beckman UniCel DxI 800 immunoassay compared with the reference measurement procedure at the CDC [11]: $\text{Testosterone}_{(\text{Beckman})} = 0.67\ \text{Testosterone}_{(\text{CDC})} + 25.4$ ng/dL, with a Pearson correlation coefficient (R^2) of 0.975 across low, medium, and high levels of ABS samples (specimens that are similar to

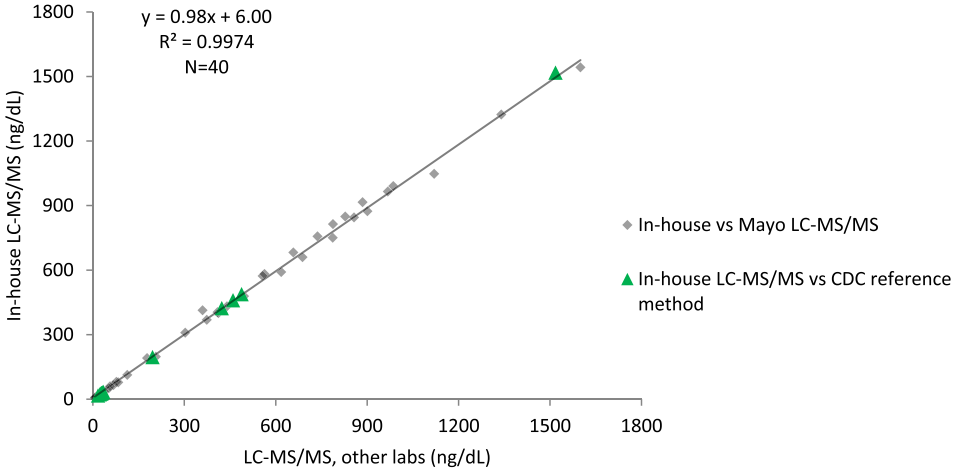

Fig. 2. Accuracy assessment. Two sets of specimens were used. One set was from Mayo Clinic Laboratories (N = 40), and the other was from residual ABS samples from 2019 to 2020 (N = 12). Total testosterone concentrations measured by the newly developed LC-MS/MS assay are plotted vs. Mayo Clinic Laboratories (grey diamonds) or against the values by the CDC reference measurement procedure (green triangles).

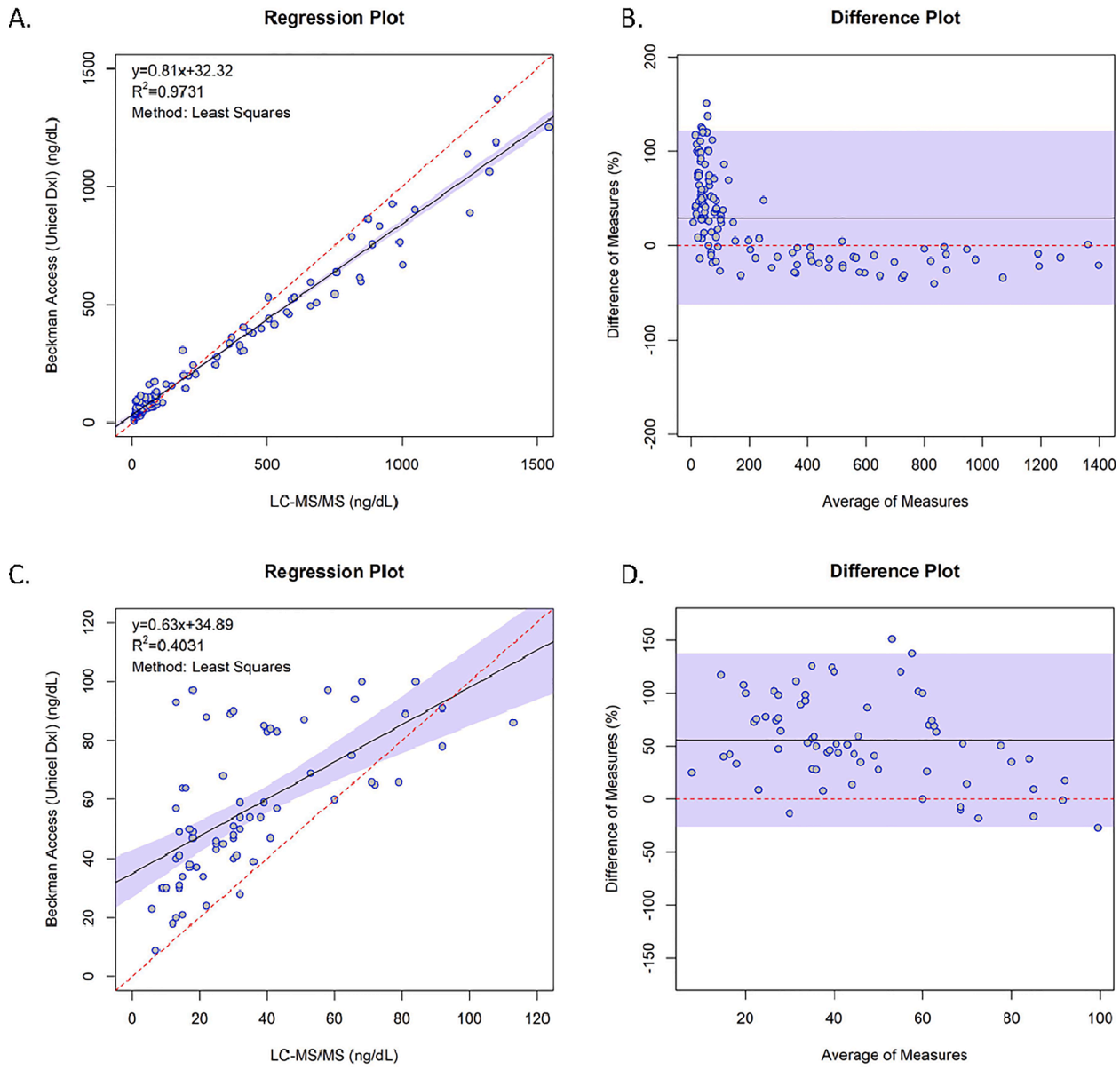

Fig. 3. Method comparison with Beckman immunoassay. A total of 126 clinical specimens spanning the full analytical measuring range were measured by Beckman UniCel DxI immunoassay and the LC-MS/MS assay in parallel. The regression plots (***A & C***) and difference plots (***B & D***) of all concentrations (***A & B***) and low concentrations<100 ng/dL (***C & D***) are displayed.

patient samples) from 2010 to 2018. When focusing on low concentrations below 50 ng/dL, the R^2 was significantly lower: 0.162 (Fig. 1).

3.3. Accuracy assessment of the newly developed testosterone LC-MS/MS assay

To assess the accuracy of the newly developed LC-MS/MS assay, method comparison to the Mayo Clinic Laboratory LC-MS/MS method that has been certified by the CDC Hormone Standardization Program (HoSt) was performed. The certified assay has met the performance criterion of ± 6.4% mean bias compared with the reference measurement procedure over the concentration range of 2.50–1,000 ng/dL. Interlaboratory comparison with 40 individual serum samples resulted in a regression equation of $\text{LC-MS/MS}_{(\text{in-house})} = 0.98\ \text{LC-MS/MS}_{(\text{Mayo})} + 6.0$ ng/dL, with R^2 of 0.997 (Fig. 2). To further confirm the accuracy of our LC-MS/MS assay, we subsequently analyzed 12 samples from the 2019–2020 Accuracy Based Steroid Survey from the CAP. There was a similar correlation between our method and the reference measurement procedure at the CDC.

3.4. Method comparison with Beckman immunoassay

Due to insufficient number of laboratories in the Beckman DxI peer group, peer group mean was not reported in the CAP survey starting 2019. To fairly determine if any improvement was made to the Beckman testosterone immunoassay from 2019 to 2021, method comparison between LC-MS/MS and immunoassay was carried out after we validated the LC-MS/MS assay. Method comparison of 126 individual serum samples resulted in a linear regression equation of $\text{Testosterone}_{(\text{Beckman})} = 0.81\ \text{Testosterone}_{(\text{In-house})} + 32.32$ ng/dL, with R^2 of 0.973 across the analytical measurement range (Fig. 3). The difference plot demonstrated positive and varying biases below 100 ng/dL. In contrast, negative but relatively constant percent biases were observed above 100 ng/dL. These results confirmed the observations from the ABS survey the historically poor performance of the Beckman UniCel DxI assay below 100 ng/dL.

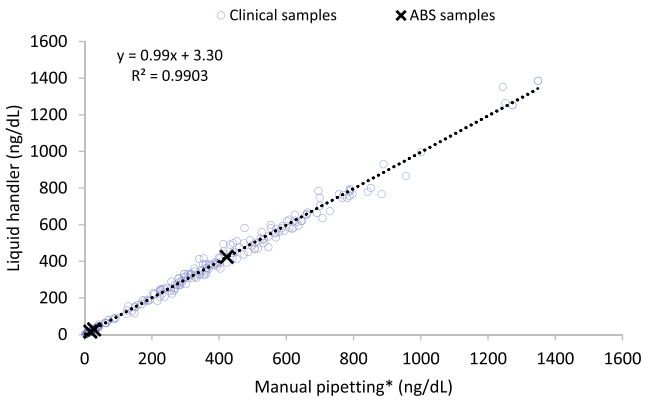

Fig. 4. Comparison of manual and automated pipetting. Clinical samples (N = 217) were prepared by either manual or automated pipetting. The testosterone concentration observed with the liquid handler (y-axis, after recalibration) is plotted against the concentration observed with manual setup (x-axis). Three ABS samples were also compared, prepared by the liquid handler, and the concentrations (y-axis) were compared to the values determined by the CDC reference measurement procedure (x-axis).

3.5. Comparison of manual and automated pipetting

Method comparison with individual serum samples was performed between manual and automated pipetting to assess if there is any bias introduced when adapting the assay to the liquid handler. It revealed that the results were highly correlated (R^2 = 0.990), but were 5.67% higher on average with the liquid handler. To resolve this bias, the set points of the calibrators were adjusted lower by 5.67%. A new comparison resulted in a linear regression equation of $\text{Testosterone}_{(\text{Liquid handler})}$ = 0.99 $\text{Testosterone}_{(\text{Manual pipetting})}$ + 3.30 ng/dL. This was confirmed with the ABS samples by comparison to the values determined by the CDC reference measurement procedure (Fig. 4).

4. Discussion

As with immunoassays in general, testosterone immunoassays are prone to interferences such as anti-reagent antibodies, structurally similar compounds, and alkaline phosphatases [12–16]. In addition to these sample-specific interferences that could cause spurious results on certain immunoassay platforms, testosterone immunoassays in general have limited accuracy, especially at low concentrations (e.g., <100 ng/dL). The results from a large survey involving 142 certified clinical laboratories using 16 immunoassays showed that the bias was high as 73.1% compared to the reference method [6].

With several years of effort from the CDC hormone standardization (HoSt) program, an evaluation of the 2016 testosterone ABS proficiency testing results showed overall improved accuracy; however, the biases at low concentrations were still undesirable for commonly used immunoassays [7]. A retrospective analysis of the ABS survey from 2010 to 2018 showed that the Beckman testosterone assay had poor correlation to the CDC reference measurement procedure at low concentrations (R^2 = 0.1612; range, 7.4–37.6 ng/dL). Evaluation of 68 clinical samples ranging from 6 to 92 ng/dL in 2020 with our newly validated LC-MS/MS assay revealed slightly improved correlation (R^2 = 0.4031).

Our assay utilized the diatomaceous earth-based SLE plate, which is different from other published assays that use liquid–liquid extraction or solid phase extraction. The workflow was simple and efficient enough for routine clinical analysis, even for laboratories lacking automated liquid handers. The newly developed LC-MS/MS assay had superior accuracy than the commercial immunoassay when compared to the CDC reference measurement procedure.

We also confirmed some technical considerations when developing LC-MS/MS testosterone assays. It has been widely accepted that the heavy isotope-labeled internal standard is essential to minimize variations and compensate for the matrix effect as the chemical properties of the isotope-labeled internal standard is theoretically the same as the native compound. Although both ^{13}C- and deuterium-labeled isotopes have been used in testosterone assays [17–19], we chose the ^{13}C-testosterone due to smaller isotopic effects during chromatographic separation than the deuterium-labeled isotope, which was also observed in the previous study [20]. Gel-containing sample collection tubes can affect steroid hormone testing in LC-MS/MS assays as shown in previous studies [21,22]. However, the direction and magnitude of how gel separators could affect the results vary. Our study showed that the BD gold-top tube caused falsely low testosterone measurement, which provides one more piece of evidence to avoid gel-containing tubes in testosterone assays.

Analyte stripped serum/plasma is often used as a base matrix for preparation of calibrators or quality controls because of its inherent characteristics of the human serum/plasma, resulting in a pool that is consistent with patient samples. DC Mass Spect Gold is processed from human plasma through a validated process that reduces hormone and steroid levels. Testosterone in this product is undetectable in our assay although the claimed testosterone concentration is no more than 250 ng/dL. However, the appropriateness of using it as a base matrix warrants thorough evaluation for other mass spectrometric hormone assays to avoid small amounts of residual steroids that could affect accuracy at low concentrations [23].

When evaluating assay accuracy, the best approach is to compare to the standard reference procedure; however, it's not always practical (e.g., cost) or necessary (e.g., whether the extent of inaccuracy could cause clinical difference). Instead, we used different approaches (i.e., inter-laboratory comparison to a HoSt certified assay during development, enrollment in the CAP survey during production) to ensure the accuracy of our assay can sufficiently meet clinical needs.

Our study also emphasized that examination of the small bias when transitioning from manual to automated pipetting should be an integral part of assay validation. Although automated liquid handlers may perform better compared with multiple technologists, bias can be introduced, for example due to different pipetting programs for calibrators vs. patient samples, as exampled in our study. This is especially important for laboratories participating in the CDC total testosterone assay HoSt program as the performance criterion ± 6.4% mean bias should be met when compared to the CDC reference measurement procedure [24]. We found that the ABS samples were instrumental to help evaluate assay accuracy during method validation.

In summary, this newly developed LC-MS/MS testosterone assay has improved accuracy and precision, and agrees well with the reference method. We believe that this assay will allow clinicians to follow the diagnostic and treatment guideline with cutoffs established based on LC-MS/MS-based assays. It will also benefit different patient populations including males, females, and pediatrics.

CRediT authorship contribution statement

Junyan Shi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Rachel Bird:** Validation, Investigation, Data curation, Writing – review & editing. **Michael W. Schmeling:** Validation, Writing – review & editing. **Andrew N. Hoofnagle:** Conceptualization, Methodology, Validation, Data curation, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122969.

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