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Validation of a total testosterone assay using high-turbulence liquid chromatography tandem mass spectrometry: Total and free testosterone reference ranges

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ABSTRACT

Accurate measurement of testosterone concentration is of critical importance when diagnosing and treating male hypogonadism, congenital adrenal hyperplasia, premature or delayed puberty, and androgen excess in polycystic ovary syndrome or other virilizing conditions. However, some assays have inherent limitations and biases that affect measurement of low-testosterone values. Therefore, we developed a highly specific online mass spectrometry method. Sera were extracted online using high-turbulence flow liquid chromatography coupled to analytical HPLC and atmospheric pressure chemical ionization tandem mass spectrometry (HTLC–APCI-MS/MS). Analyte ions were monitored by multiple reaction monitoring (MRM). Total analysis time was 1.15 min per sample when using the multiplexing system. Testosterone concentrations were measured directly from 150 μL of serum or plasma without derivatization or liquid–liquid extraction. The lower limit of quantification was 0.3 ng/dL, and the assay was linear up to 2000 ng/dL. The method compared very well with an established RIA: $y = 1.02x + 1.5$, $r^2 = 0.994$. Comparison with a platform immunoassay confirmed the previously reported ICMA positive bias at low concentrations. Male and female adult and pediatric reference ranges were developed for this very sensitive and accurate high-throughput LC–MS/MS method. This method is suitable for measuring the expected low-testosterone concentrations seen in women, children, and hypogonadal males and for monitoring testosterone suppressive therapy in prostate cancer patients.

1. Introduction

The importance of accurate total testosterone (TT) measurements, especially of low concentrations, has been the subject of much discussion in the literature [1–15]. Accurate measurements are particularly critical in five clinical scenarios: (1) detection of the initial rise in testosterone at the onset of puberty [16,17]; (2) diagnosis of polycystic ovary syndrome (PCOS), the most common endocrinopathy in women of reproductive age [18–20]; (3) diagnosis of hypoandrogenism in surgically induced menopause [5,8,21,22]; (4) diagnosis of mild hypogonadism or waning testosterone concentrations in aging males [23,24]; and (5) dose titration of GnRH analogues used to achieve medical castration in men with prostate cancer [25–27].

Prior to mass spectrometry and over the past 30 years, the accurate measurement of testosterone and other steroids for clinical diagnostic purposes has required use of methods involving organic extraction, column chromatography, and radioimmunoassay (RIA). The specificity of these reference methods relies on completeness of the separation of closely related steroids in the chromatography step and the specificity of the primary antibody employed. Their availability is now limited to a few reference laboratories owing to development of platform immunoassay methods. Although seen as easy and rapid means of measuring TT concentrations, a growing body of literature unequivocally demonstrates that platform immunoassays lack the accuracy essential for the above clinical scenarios [1,2,4–9].

Recently, the Endocrine Society has issued a position statement [15] detailing the limitation of TT platform immunoassys and recommending use of either extraction chromatography RIA or tandem mass spectrometry methods when TT concentration is expected to be low as in the scenarios described above. LC–MS/MS has several advantages relative to extraction chromatography RIA. The liquid chromatography step lends itself to automation, and MS/MS has an obvious advantage in accuracy and specificity over immunoassays. Accuracy is gained through use of internal standards that are stable isotopes of the same compound being measured, giving accurate corrections for procedural losses. MS/MS provides increased specificity owing to its ability to select for the mass of the compound of interest (parent ion) and to fragment the

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parent ion into specific, smaller ions (daughter ions). Selecting one or more specific daughter ion(s) permits positive identification of the compound being measured. On the other hand, quality of available RIA methods is highly dependent on the affinity and specificity of the antibody used. Because of these limitations, the LC–MS/MS is rapidly becoming the preferred reference method for measurement of steroids and other small molecules.

Following improvements in the sensitivity and robustness of MS/MS that have occurred in recent years, we undertook development of a highly sensitive and accurate LC–MS/MS method for measuring TT over a wide dynamic range. Our intent was to address the inaccurate low concentrations that result from the platform assays. We report herein the total testosterone HTLC–MS/MS method validation, comparisons with RIA, an immunoassay platform assay, and determination of new normal TT reference ranges for both children and adults. Furthermore, we report development of new normal reference ranges for free testosterone (equilibrium dialysis method), since the accuracy of free testosterone (FT) concentrations derived from equilibrium dialysis is entirely dependent on accuracy of the TT measurement.

2. Materials and methods

2.1. Subjects

Serum samples were collected from 499 apparently normal volunteers age 8–90 years at Quest Diagnostics Nichols Institute for reference range determinations. Tanner staging was performed by self-reporting. Informed consent and IRB approval was obtained. Anonymized remnant sera, submitted for routine laboratory testing, was also used in the study.

2.2. Reagents

Testosterone was purchased from Sigma–Aldrich Inc. (St. Louis, MO). Testosterone-2,2,4,6,6-d_5 was purchased from CDN Isotopes (Quebec, Canada). Both of these had a Certificate of Analysis indicating $\geq$98% purity. 1,2 ^{3}H-testosterone, specific activity 45 Ci/mmol was purchased from Perkin Elmer (Waltham, MA) and was repurified by chromatography every 2 months. Testosterone-free sera (double charcoal stripped, delipidated) was purchased form Golden West Biologicals (Temecula, CA) and was tested for the presence of detectable testosterone before use. All other chemicals were of reagent grade or better and were purchased from commercial sources.

2.3. Total testosterone by HTLC–MS/MS

The sample was prepared by acidifying 150 μL of serum or plasma with 10% formic acid to release testosterone from the binding proteins. The internal standard, prepared in deuterated methanol, was then added. After vigorous mixing, the samples were incubated at room temperature for 30 min prior to being placed in a refrigerated autosampler.

The Aria TX-4 HTLC (Cohesive Technologies Inc.; Franklin, MA; part of Thermo Fisher Scientific) injected the prepared sample onto an extraction column at high flow rate. This created turbulence inside the column, which allowed testosterone to bind to the large particles of the extraction column, while protein and other debris freely flowed through and were discarded. The flow was then reversed and slowed, and testosterone was eluted and transferred to a reverse-phase C-12 analytical column (Synergi-Max RP® from Phenomenex, Torrance, California).

TT was quantitated using a Finnigan TSQ Quantum Ultra (ThermoFisher; San Jose, CA) tandem mass spectrometer. The tandem mass spectrometer permits the isolation of the parent compound to within ±0.5 m/z within the first quadrapole (Q_1). In the second quadrapole (Q_2), the parent ion collides with an inert gas (argon) to generate daughter ions, and the appropriate daughter ion(s) are selected in the third quadrapole (Q_3). Inclusion of deuterated testosterone (d_5-2,2,4,6,6-testosterone) as an internal standard (IS) enabled absolute quantitation of testosterone by correcting for procedural losses or ion suppression caused by matrix effects in the atmospheric pressure chemical ionization (APCI) process. Detection of two daughter ions for both the endogenous testosterone and the IS increased the specificity by permitting ion-ratioing to be employed, thereby reducing the risk of quantitating isobaric interfering substances.

Testosterone was detected in the positive ionization mode. The parent ions monitored were 289.1 and 294.2 m/z; daughter ions monitored were 109.1 and 97.1 m/z for testosterone and 113.1 and 100.1 m/z for d_5-testosterone, respectively.

TT was then quantitated against a standard curve, wherein the standards were processed in the same manner as the samples. Peak area ratio between testosterone and the internal standard was used for quantification.

The whole process of extraction, separation, and detection is automated and takes 4.5 min. With the use of the Aria TX-4 system, which consists of a series of four extraction and separation systems that work in concert, the analytical time on the MS/MS can be reduced from 4.5 min per sample to 1.15 min per sample.

2.4. Total testosterone by ICMA and RIA

TT was determined in male serum samples using Bayer Advia Centaur immunoassay (Siemens Healthcare Diagnostics, Tarrytown, NY). The Advia Centaur® testosterone assay measures T concentration up to 1500 ng/dL with an analytical sensitivity of 10 ng/dL.

TT was determined in female and pediatric serum samples using an independently validated extraction/chromatography/RIA method (Quest Diagnostics Nichols Institute, San Juan Capistrano, CA). This assay employed 0.5 mL of serum to which a trace amount of tritiated testosterone was added to permit correction for procedural losses. The serum was first extracted with a cocktail of ethyl acetate and hexane, and the organic phase was collected. This was then added to a Celite column, and, after washing with different organic mixtures, the testosterone was eluted and collected in a 15% ethyl acetate/*iso*-octane solution. The eluted testosterone solution was then dried and reconstituted in RIA buffer. A portion was removed and used to determine recovery of the tracer. A second aliquot was then assayed in an RIA to obtain the TT concentration. This concentration was corrected for procedural losses. The limit of quantitation is 2 ng/dL at 20% coefficient of variation (CV), and the assay is linear to 1800 ng/dL. Linearity is extended by extraction of smaller volumes for samples with a concentration >200 ng/dL. The intra-assay CV is 11.8% at 2.5 ng/dL, while the inter-assay CV is 17.0%.

2.5. Sex hormone binding globulin (SHBG) and albumin

SHBG was determined using an automated immunoassay (IMMULITE® 2000 SHBG) on the IMMULITE instrument (Siemens; Los Angeles, CA). The IMMULITE SHBG assay was selected for use in the calculation of free and bioavailable testosterone after an internal comparison of five commercial assays and an in-house RIA.

Albumin was determined using an automated bromocresol green dye-binding assay (Albumin/BCG) on the Hitachi 917 (Boehringer Mannheim; Indianapolis, IN).