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# Development and validation of a serum total testosterone liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay calibrated to NIST SRM 971

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ABSTRACT

*Background:* At our institution, serum testosterone in adult males is measured by immunoassay while female and pediatric specimens are sent to a reference laboratory for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis due to low concentrations. As this is of significant cost, a testosterone LC–MS/MS assay was developed in-house.
*Methods:* A 5500 QTRAP® using electrospray ionization and a Shimadzu Prominence with a C18 column were used. Gradient elution with formic acid, water and methanol:acetonitrile at 0.5 ml/min had a 7-min run-time. A liquid–liquid extraction with hexane:ethyl acetate was carried out on 200 μl of serum. Multiple reaction monitoring was employed.
*Results:* Sample preparation took ~80 min for 21 samples. Six calibrators were used (0–1263 ng/dl; concentration assigned by NIST SRM 971) with 3 quality controls (9, 168 and 532 ng/dl). The limits of detection and quantitation were 1 and 2 ng/dl respectively. Extraction recovery was ~90% and ion suppression ~5%. Within-run and total precision studies yielded <15% CV at the limit of quantitation and <7% CV through the rest of the linear range. Isobaric interferences were baseline separated from testosterone. Method comparisons between this assay, an immunoassay, and another LC–MS/MS assay were completed.
*Conclusions:* An accurate and sensitive LC–MS/MS assay for total testosterone was developed. Bringing this assay in-house reduces turnaround time for clinicians and patients and saves our institution funds.


## 1. Introduction

Testosterone is one of the most commonly measured steroid hormones and is present in very low concentrations of around 2–45 ng/dl in the serum of females, and pediatric males of certain age groups [1–3]. Reasons for measuring total testosterone clinically include evaluation of hypogonadism in males, and in females to investigate infertility, amenorrhea, hirsutism or for diagnosis of polycystic ovarian syndrome [4–7]. In children, testosterone is measured in cases of delayed or precocious puberty and is used in the laboratory investigation of infants with ambiguous genitalia [7,8].

Serum total testosterone is most often measured clinically by immunoassay, as documented in the College of American Pathologists (CAP) proficiency testing participants (98.6% as of September 2011). However, several publications have shown that although immunoassays have sufficient accuracy and precision for measuring total testosterone in males, they are lacking in these parameters when it comes to females and pediatrics due to the low concentrations normally found in these patients [9–11]. These findings prompted The Endocrine Society to release a position statement in 2007 stating that they believe the best way of measuring total testosterone is using a method that involves extraction, chromatography and mass spectrometry [12].

A number of LC–MS/MS assays used to measure testosterone have been reported in the literature, each of which is developed in a different way, using different extraction procedures and indeed different types of ionization, although every paper reviewed herein used MS analysis in the positive mode [1,13–17]. The method of Kushnir et al. used liquid–liquid extraction with methyl t-butyl ether, hydroxylamine derivatization, 2-dimensional and then analytic chromatographic separation followed by MS analysis using electrospray ionization (ESI) [1]. The authors report a limit of quantitation (LOQ) of 1 ng/dl, an upper limit of quantitation (ULOQ) of 2500 ng/dl with no interference from 50 steroids and steroid metabolites. The assay published by Salameh et al. used online extraction by high-turbulence flow liquid chromatography followed by chromatographic separation and MS analysis using atmospheric pressure chemical ionization (APCI) with

*Abbreviations:* NIST SRM 971, National Institute of Standards and Technology Standard Reference Material 971.

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a total run time of 1.15 min per sample [13]. The limit of quantitation was determined to be 0.3 ng/dl and the ULOQ was 2000 ng/dl. Savolainen et al. also used online extraction in their assay, but in this case it was a solid phase extraction method [14]. The extraction was followed by LC–MS/MS analysis using ESI and a run time of 6.7 min. The LOQ was determined to be 7 ng/dl, and the ULOQ was 1000 ng/dl. The LC–MS/MS assay of Shiraishi et al. for testosterone used a liquid liquid extraction with ethyl acetate and hexane that was performed twice and then removal of acidic contaminants was achieved by the addition of sodium hydroxide [15]. The extracted samples were then injected onto the LC system and MS analysis using ESI was carried out, with a run time of 18.5 min per sample. The LOQ and ULOQ were 2 and 2000 ng/dl respectively. Cawood et al. used protein precipitation, followed by LC–MS/MS analysis using ESI with a run time of 4.75 min [16]. The reported LOQ was 9 ng/dl with a ULOQ of 2882 ng/dl. Finally, the LC–MS/MS assay of Guo et al. used protein precipitation, online clean-up of the injected sample followed by chromatographic separation and MS analysis using atmospheric pressure photoionization (APPI) [17]. The limit of detection was determined to be 0.15 ng/dl. These authors have also reported that using the APPI source, a soft ionization source, instead of ESI or APCI for analysis of testosterone can lead to a cleaner baseline and thereby increase the signal to noise ratio of the chromatographic peaks which is a definite advantage when measuring potentially low level analytes such as testosterone [18].

Although mass spectrometry has high specificity, it is not without its limitations for testosterone testing. Due to other endogenous compounds that are the same (e.g., dehydroepiandrosterone; DHEA), or very similar in structure and molecular weight, testosterone must be chromatographically separated from these in order to obtain specific and accurate quantification by liquid chromatography–tandem mass spectrometry (LC–MS/MS), especially since some of these compounds are present at much higher concentrations than testosterone. Additionally, the gel in serum separator tubes in which patient blood samples are routinely collected causes interference in one of the main testosterone transitions and so clinical laboratories are required to ask clinicians to collect the sample in an alternative tube type unless they run a long chromatographic method or refrain from using these specific transitions (mass to charge ratios of 289/97 and 289/109) [19]. Matrix effects from phospholipids are another concern and they may cause a high chromatographic baseline, retention time alterations and suppression of the testosterone signal [20,21].

Another issue in total testosterone testing is the lack of standardization between different assay platforms and indeed between users of the same platform, specifically LC–MS/MS methods [12,22]. The National Institute of Standards and Technology aimed to help with this issue by releasing a Standard Reference Material 971 that contains certified concentrations of testosterone in serum [23]. This can be used by immunoassay manufacturers, or indeed mass spectrometry assay developers as a way of standardizing total testosterone assays to one measurement. The Centers for Disease Control are very aware of the lack of standardization of steroid hormone assays, and in 2010, implemented the hormone standardization program (HoST) [24]. Laboratories using self-developed methods, or assay manufacturers, can enroll in this program with an aim to improve and monitor accuracy compared to a reference method for total testosterone; however, there is a substantial cost to participate in this program which may be prohibitive for smaller laboratories.

At our institution, total testosterone in male patients is measured by chemiluminescent immunoassay, while for female and pediatric patients, samples are sent to a reference laboratory for LC–MS/MS analysis. As this practice is of significant cost, our aim was to develop a LC–MS/MS assay in-house that is suitable for the measurement of testosterone in all samples and has the calibration verified by the NIST SRM 971.

## 2. Materials

### 2.1. Experimental

Mass spectrometry-grade solvents and water were from VWR International (Brisbane, CA) or from Thermo Fisher Scientific (Pittsburgh, PA). Formic acid was from Sigma-Aldrich (St Louis, MO). Testosterone, 16, 16, 17-d3 labeled testosterone, dehydroepiandrosterone, estradiol and epitestosterone were from Cerilliant Corporation (Round Rock, TX). Hormones in frozen human serum standard reference material (NIST SRM 971) was from The National Institute of Standards and Technology (Gaithersburg, MD). Human serum calibrators containing testosterone were from UTAK Laboratories Inc. (Valencia, CA) and the concentration of these was verified by running the male and female serum samples of the NIST SRM 971 that have assigned testosterone concentrations on the LC–MS/MS assay. Lyphochek® Immunoassay Plus quality control material Levels 1 and 2 were from BioRad Laboratories (Irvine, CA). Double charcoal stripped human serum was obtained from Golden West Biologicals Inc. (Temecula, CA).

### 2.2. Patient samples

Institutional review board approval was obtained from the University of California, San Francisco Committee on Human Research for this study. Sixty-six patients who had ultra-sensitive total testosterone (LOQ = 1 ng/dl), performed by LC–MS/MS at a reference laboratory, ordered on their serum (from serum separator tubes) in 2011 and had sufficient sample volume remaining were included in this study. Four of the 66 patient specimens were from pediatric patients (two 4 years, one 7 years and one 13 years). Further, 30 patients who had total testosterone ordered on their serum (from serum separator tubes) in December 2011 performed on the Siemens Centaur® XP immunoassay by UCSF Clinical Laboratories were retrospectively included in this study. One of the 30 patient specimens was from a pediatric patient (13 years). Of these 30 patient specimens, 17 had sufficient sample volume remaining and were extracted using the liquid–liquid extraction from the LC–MS/MS method. These 17 specimens, along with the 6 calibrators from the LC–MS/MS assay were then run on the Siemens Centaur® XP immunoassay. The total testosterone concentration for each of these 96 patients using the methodologies mentioned above (and described in the method comparison study section below) was recorded and compared to the total testosterone concentration obtained from the newly developed total testosterone LC–MS/MS assay. Demographic information for the patients who the samples were obtained from is documented in Table 1.

### 2.3. Liquid–liquid extraction

Twenty-five microliters of 10 ng/ml internal standard (D3-testosterone in methanol) was added to 200 µl of serum and vortexed. One milliliter of 90:10 hexane:ethyl acetate was added to each tube, vortexed for 2 min, left at room temperature for 5 min and then centrifuged at 3000 rpm for 10 min. The tubes were then placed in dry ice until the aqueous bottom layer froze and the organic top layer was poured into a fresh tube. The solvent was evaporated to dryness under nitrogen at 45 °C and the sample reconstituted in 125 µl of methanol and water (60:40) as during method

**Table 1**
Demographic information for the patient samples utilized in the method comparison studies.

| Method | Age (years) | | | Gender | |
|---|---|---|---|---|---|
| | Mean | Median | Range | Male | Female |
| LC–MS/MS | 45.3 | 43.5 | 4–89 | 19 | 47 |
| Centaur | 55.5 | 59.5 | 13–76 | 29 | 1 |