

Rapid determination of serum testosterone by liquid chromatography-isotope dilution tandem mass spectrometry and a split sample comparison with three automated immunoassays

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Abstract

Objectives: To develop a rapid convenient-to-implement high performance liquid chromatography-isotope dilution tandem mass spectrometry (LC-IDMS/MS) method for determination of serum testosterone concentration in routine clinical laboratories.

Methods: Following extraction by organic solvents, an Agilent 1200 Series HPLC system coupled to an API 5000 mass spectrometer equipped with an atmospheric pressure chemical ionization ion source was used to separate, detect and quantify serum testosterone. Ion-transitions of m/z 289.2 → 109.1 and 294.2 → 113.2 were used to monitor testosterone and testosterone-2,2,4,6,6-d₅, respectively.

Results: Functional sensitivity was 0.056 nmol/L (CV 20%). Within-run and total imprecision were 4.6% and 5.2% at 1.3 nmol/L, 2.4% and 4.3% at 11.0 nmol/L, and 1.9% and 1.9% at 23.4 nmol/L respectively. The LC-MS/MS method agreed closely with three automated immunoassays when the concentration of testosterone exceeded 3 nmol/L. However, the immunoassays showed a positive bias at concentrations below 3 nmol/L.

Conclusion: This method provides a rapid, simple, highly selective and sensitive procedure that can be easily used for determination of serum testosterone in routine clinical laboratories. It measures serum testosterone precisely and accurately at concentrations found in children and adults of both genders.

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Introduction

Testosterone is a potent androgen that is primarily secreted by Leydig cells in men and Thecal cells in women. Secretion is regulated by luteinizing hormone derived from the pituitary gland. Lesser amounts of testosterone are secreted by the adrenal cortex. Testosterone plays critical roles in spermatogenesis, the development and maintenance of the internal and

external genitalia and secondary sex characteristics in men, and the control of libido in both genders [1–4]. Clinically, the measurement of serum testosterone is useful in the investigation of suspected disorders of excessive and insufficient androgen production in the adult, precocious and delayed puberty in children and ambiguous genitalia in the neonate.

In most clinical laboratories the analysis of serum or plasma testosterone is performed using automated immunoassays. However, existing testosterone measuring immunoassays suffer from a number of serious problems including insufficient sensitivity, cross-reactivity, inaccuracy, limited linear range, poor inter-method agreement and imprecision. Immunoassays perform particularly poorly when quantifying the relatively low

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circulating levels of testosterone in children and women due to their limited sensitivity and unacceptable cross-reactivity, the latter leading to a substantial positive bias [5]. In one study of serum testosterone analysis in women [6], the mean immunoassay result exceeded that determined by gas chromatography mass spectrometry (GC-MS) by 46%, with levels in some individuals overestimated five-fold. The same study showed that immunoassay underestimated testosterone in men by 12% compared to GC-MS [6]. In another study highlighting the inaccuracy of immunoassays, this time compared to liquid chromatography-tandem MS (LC-MS/MS), Wang et al. [7] showed that the former still significantly overestimated testosterone at concentrations less than 3 nmol/L, even after specimens had been pretreated with extraction. In addition, immunoassay precision is often less than optimal. Between assay imprecision of 15–32% has been reported [5]. The above problems with immunoassay have drawn significant attention recently, and prompted a number of clinical societies, such as the Endocrine Society, the Androgen Excess Society, and the American Society of Andrology [5,8,9], to issue statements recommending the use of more accurate, precise and consistent methods to measure testosterone for clinical care.

There are several published GC-MS [6] and LC-MS/MS methods for the analysis of testosterone [7,10–12] that qualify as reference assays. Four of the published LC-MS/MS methods have been compared and showed good agreement [13]. In general, these methods display good sensitivity and specificity and can be used to measure serum testosterone in women and children as well as men. However, current GC-MS and LC-MS/MS methods have demonstrated technical challenges that complicate and hinder their implementation in laboratories providing a routine clinical service. For example, some methods require large quantity of serum up to 2 mL [6,7,14]. The sample run time of some published methods are lengthy from 12 min to even 40 min leading to low throughput [6,14–16]. Many of published methods demonstrate sensitivity not superb to routine immunoassays in that limits of quantification (LOQ) are between 0.17 and 2 nmol/L [15,17–19]. Some methods use laborious and extensive sample preparation including solid phase extraction (SPE) and/or derivatization in addition to liquid–liquid (L–L) extraction [6,10,16,20]. Recognizing these limitations, we set out to develop a LC-MS/MS method that required minimal sample preparation, less sample volume than other published LC-MS/MS methods, high throughput and yet still achieved the sensitivity, specificity and precision commensurate with the reliable analysis of circulating levels of testosterone in children and adults of both genders. Here we describe the method, its validation and a split sample comparison against three automated immunoassays currently widely used in the clinical service laboratories.

Materials and methods

Materials

Testosterone (1 mg/mL) was purchased from Grace Davison Discovery Sciences (Deerfield, IL, USA). Testosterone-

2,2,4,6,6- d_5 internal standard (isotopic enrichment >98%) was from CDN Isotopes (Pointe Claire, QC, Canada). The Eclipse C8 HPLC column (50 × 3.0 mm, 1.8 μ m) was purchased from Agilent Technologies (Santa Clara, CA, USA). HPLC grade ethanol, methanol, methyl tert-butyl ether (MTBE) and heptane were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). All other chemicals were analytical reagent grade.

Sample preparation

0.25 mL of serum was spiked with testosterone-2,2,4,6,6- d_5 (400 fmol) and vortex-mixed for 5 s and incubated for 5 min at room temperature. Testosterone was extracted with 1 mL of MTBE. MTBE was evaporated under a stream of nitrogen gas at 40 °C and the residue was redissolved in 80% methanol and extracted with 1 mL of heptane. The top heptane layer was discarded and the bottom methanol layer was transferred to clean tubes and evaporated to dryness. The residue was dissolved in 100 μ L of 50% methanol and a 40 μ L aliquot was analyzed by LC-MS/MS.

LC-MS/MS

HPLC was carried out using an Agilent Technologies 1200 series system in linear gradient mode at a flow rate of 0.85 mL/min on an Eclipse C8 column employing a mobile phase consisting of methanol–water (20:80) increasing to 100% methanol over 4 min and maintained at 100% methanol for one min. The column was then re-equilibrated with methanol–water (20:80) for 1 min. The column temperature was maintained at 50 °C.

An API 5000 (Applied Biosystems/Sciex, Concord, ON, Canada) mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode. The ion source temperature was maintained at 400 °C, the corona current adjusted to 3.0 μ A, collision gas, nebulizer gas and curtain gas pressures set to 5.0 U, 30.0 U, and 30.0 U, respectively, the collision energy set to 35 V and the declustering potential set to 90 V. The ion-transitions of m/z 289.2 → 109.1 and 294.2 → 113.2 were monitored to detect and quantify testosterone and d_5 -testosterone, respectively. The dwell time per transition was set to 50 ms.

Analyst software (version 1.4.2) controlled the system and mediated data acquisition, peak-area integration and comparison against the standard curve to calculate the concentration of unknowns. The standard curve was derived from calibrants analyzed within the same analytical run.

Method validation

Interference from hemolysis, lipemia and icterus in the LC-MS/MS assay of testosterone was assessed by analyzing patient serum samples with levels of 2.5 g/L haemoglobin, 40 mmol/L triglyceride and 500 μ mol/L total bilirubin, respectively. Serum containing 1.3 nmol/L of testosterone was spiked with supra-physiological levels of estradiol and cortisol at final concentrations of 50 nmol/L and 50 μ mol/L,

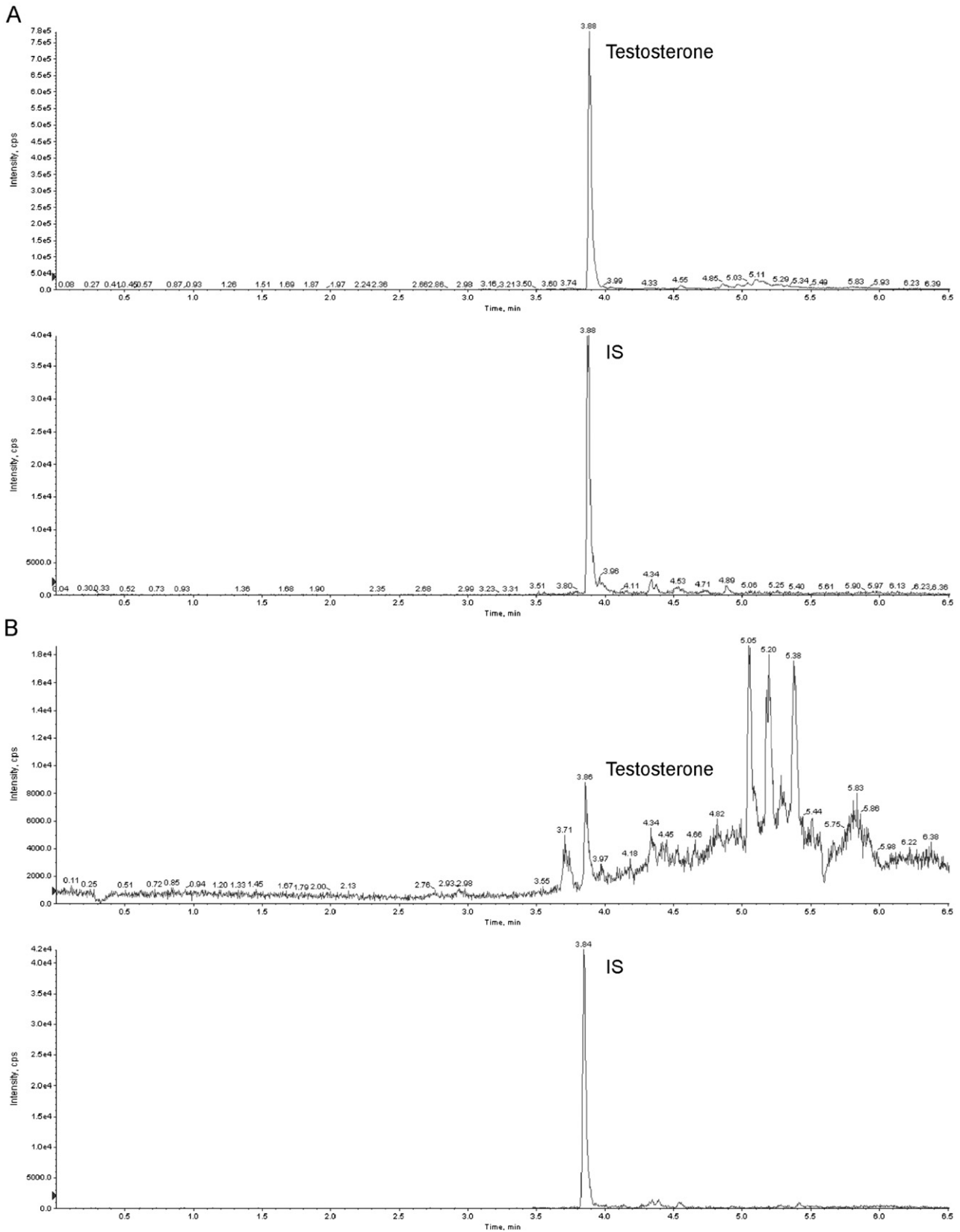


Fig. 1. Representative LC-MS/MS chromatograms of testosterone. (A) Serum pool containing testosterone at a concentration of 23.4 nmol/L with its internal standard (IS) testosterone-2,2,4,6,6-d₅ (400 fmol). (B) Serum containing testosterone at a concentration of 0.06 nmol/L along with the internal standard.

Table 1
Method imprecision (n=20)

CV (%) levels	Within-run	Overall
1.3 nmol/L	4.5	5.2
11.0 nmol/L	2.4	4.3
23.4 nmol/L	1.9	1.9

respectively, to determine whether interference from these two steroids could be ruled out under conditions of normal clinical practice.

Within-run (n=20 replicates) and overall imprecision (20 runs for 10 days) was determined using patient serum pools with testosterone concentrations of 1.3, 11.0, and 23.4 nmol/L.

Carryover was assessed by measuring 3 successive aliquots (a1, a2, a3) of serum containing a high level of testosterone followed by 3 successive aliquots (b1, b2, b3) of serum containing a low level. Carryover, k, was calculated by the following equation: $k = (b1 - b3) / (a3 - b3)$.

Linearity was tested over the range 0–80 nmol/L using eleven different dilutions of testosterone from a stock solution. The 1 mmol/L stock solution was prepared by dissolving 2.9 mg of testosterone in 10 mL of methanol. Further dilutions were carried out in methanol.

The limit of quantification of the LC-MS/MS assay corresponding to a functional sensitivity of 20% precision was determined from 5 replicate measures of serum pools containing one of six concentrations of testosterone ranging from 0.06 to 11.0 nmol/L. 5 of the 6 concentrations were below 2 nmol/L.

Method comparison

The LC-MS/MS method was compared to immunoassays available from the manufacturers of three automated platforms, namely the Abbott Architect i2000, Roche Modular E170, and DPC Immulite 2500. The automated immunoassays were carried out according to the manufacturer’s instructions. Serum specimens from 32 male and 30 female adult subjects with testosterone concentrations ranging from 0.3 to 39 nmol/L were included in the comparison.

Statistical analysis

Statistical analysis was carried out by Microsoft Excel. The dilution curve was compared to the best fitted line determined by linear regression analysis to assess linearity. Functional sensitivity was calculated from the formula generated from the LOQ curve by Microsoft Excel. Regression analysis expressed

Table 2
Carryover

High value, a (nM)	Low value, b (nM)	$k = (b1 - b3) / (a3 - b3)$
22.29, 22.40, 21.07	0.44, 0.45, 0.42	0.001045
29.20, 30.08, 29.26	0.54, 0.59, 0.57	-0.00113

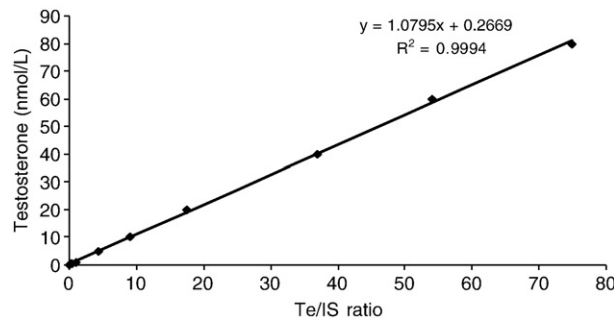


Fig. 2. Standard curve. The method shows a dynamic linear response up to 80 nmol/L.

as Bland and Altman plots was used to assess systematic bias between methods [6,7].

Results

LC-MS/MS characteristics of testosterone and interference study

Testosterone specific transition 289.2→109.1 and d₅-testosterone transition 294.2→113.2 eluted at approximately 3.9 min from the LC column (Fig. 1A). Column elution and reconditioning took a total of 6.5 min. Hemolysis, lipemia, and icterus did not interfere in the assay. Testosterone was also well separated from estradiol and cortisol, even when the latter two were present at higher concentrations than those normally encountered clinically. Fig. 1B shows the LC-MS/MS chromatogram obtained with testosterone and the internal standard, respectively, when testosterone is present at concentrations of 0.06 nmol/L near the LOQ of the method.

Imprecision

Within-run and total precision were 4.6% and 5.2% at 1.3 nmol/L, 2.4% and 4.3% at 11.0 nmol/L, and 1.9% and 1.9% at 23.4 nmol/L patient serum pools respectively (Table 1).

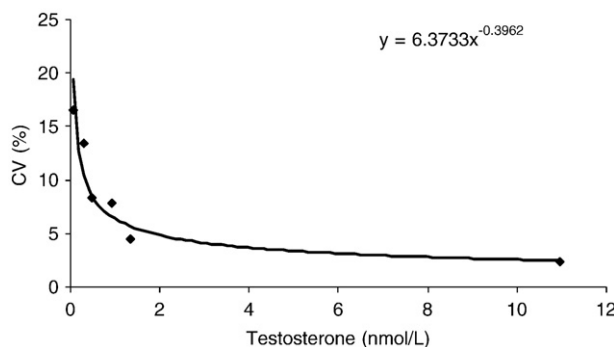


Fig. 3. Functional sensitivity curve demonstrates a limit of quantitation (LOQ) of 0.056 nmol/L.

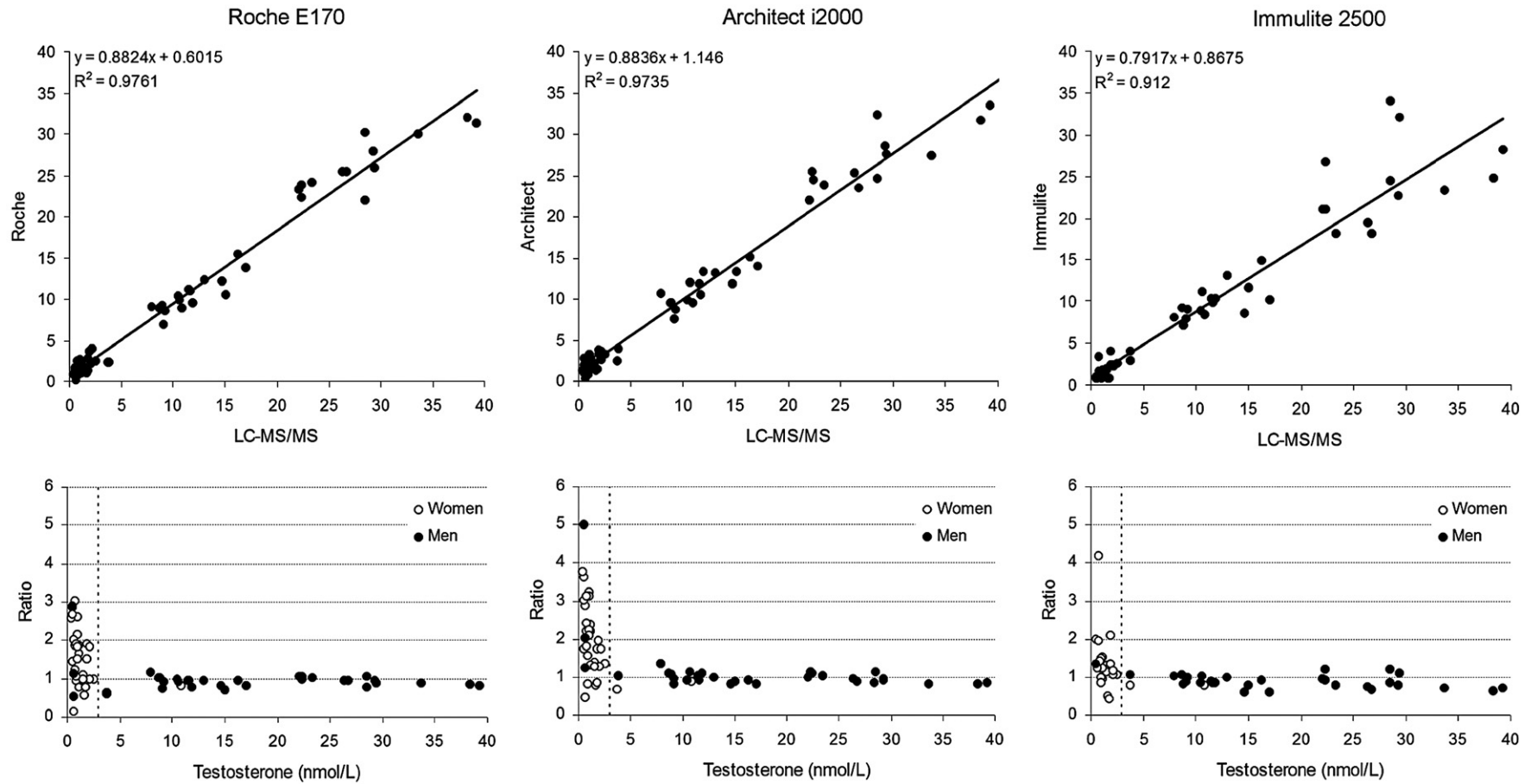


Fig. 4. To investigate how the LC-MS/MS method compared against existing immunoassays, 30 female and 32 male samples were analyzed both by LC-MS/MS and by immunoassays on the Roche Modular E170, Architect i2000 and DPC Immulite 2500. The upper panel shows the correlation of immunoassays with LC-MS/MS for all 62 samples. The lower panel shows a plot of the ratio of immunoassay to LC-MS/MS measured serum testosterone (Y axis) against the LC-MS/MS measured testosterone concentration. The vertical dotted line denotes a testosterone concentration of 3 nmol/L. Seven samples (5 female, and 2 male) were excluded from analysis on the Immulite because the testosterone concentration was below the detection limit defined by the manufacturer.

Carryover

There was no detectable testosterone peak observed in methanol blanks when these followed liquid–liquid extracts of serum samples that originally contained 50 nmol/L of testosterone. Table 2 shows that carryover was of the order of ± 0.001 when serum samples with low concentrations of testosterone were run immediately after those with high concentrations of testosterone.

Linearity and functional sensitivity

The proposed method demonstrates a dynamic linear response up to at least 80 nmol/L (Fig. 2). The limit of quantification based on a functional sensitivity of 20% CV was 0.056 nmol/L (Fig. 3).

Method comparison with automatic immunoassays

At testosterone concentrations exceeding 3 nmol/L, the three immunoassays agreed reasonably well with the LC-MS/MS method giving the following comparison data (Fig. 4): Roche Modular E170 = $0.8898 \text{ LC-MS/MS} + 0.4237$, $R^2 = 0.9483$; Architect i2000 = $0.871 \text{ LC-MS/MS} + 1.445$, $R^2 = 0.9406$; and DPC Immulite 2500 = $0.7602 \text{ LC-MS/MS} + 1.6192$, $R^2 = 0.8124$. However, at concentrations below 3 nmol/L the agreement was poor with correlation coefficients less than 0.4 and all three immunoassays showing a significant positive bias as follows (Fig. 4): Roche Modular E170 = $0.9657 \text{ LC-MS/MS} + 0.551$, $R^2 = 0.3836$; Architect i2000 = $0.9434 \text{ LC-MS/MS} + 1.0093$, $R^2 = 0.3408$; and DPC Immulite 2500 = $0.8623 \text{ LC-MS/MS} + 0.548$, $R^2 = 0.2925$.

Discussion

We in this study and others before [6,7] have shown that immunoassays in common use lack sensitivity and specificity for testosterone and compare poorly to GC-MS and LC-MS/MS based methods, especially at concentrations of testosterone below the lower limit of the reference range of young adult males. Such concentrations are found in women and children

and in a proportion of aging males with androgen deficiency syndromes [4,21,22]. The use of immunoassays in these groups to measure testosterone has been of long standing concern and questionable clinical utility [5] and led to the call for more accurate, precise, sensitive and specific methods.

Our proposed LC-MS/MS method for serum testosterone measurement has a number of advantages over other published mass spectrometry methods as mentioned in the introduction of this report. We compared our method with four established mass spectrometry methods in details as shown in Table 3. Compared to the GC-MS method of Taieb et al. [6], our method requires minimal sample preparation and significantly less volume yet achieves a lower limit of functional sensitivity at half the run time. The LC-MS/MS method of Wang et al. [7] is similar to ours in limiting preliminary sample treatment to liquid liquid extraction, but suffers from poorer precision, a higher LOQ and a requirement for eight-fold more serum (2 mL vs 0.25 mL). The method of Kushnir et al. [10] actually exceeds ours in terms of functional sensitivity, shorter run time and less sample requirement, but achieves these only by employing a much more tedious sample preparation involving hydroxylamine derivatization and lengthy solid phase extraction in addition to liquid–liquid extraction. Finally, the most recent LC-MS/MS method was published by Turpeinen et al. [12] in 2008. This method demonstrated a higher LOQ and a longer sample run time of 10 min than the current method. We believe that our method with its attractive combination of simplified sample preparation, serum requirement of only 0.25 mL, short run time of 6.5 min and functional sensitivity that is adequate to measure testosterone reliably in women and children is best suited for routine use in clinical laboratories.

Our proposed LC-MS/MS method is also capable of determination of testosterone concentration in saliva and urine (data not shown) as well as serum. In addition, the functional sensitivity limit of 0.056 nmol/L is on a par with free (unbound) testosterone concentrations found in the female adult and it may be possible to extend our method, coupled with ultrafiltration, to measure free testosterone and replace the unreliable [9,23,24] analogue immunoassay methods in current clinical use. Finally, our method can detect using the same LC separating conditions other

Table 3
Comparison with four other MS methods measuring testosterone

	Our method	Taieb et al. [6]	Wang et al. [7]	Kushnir et al. [10]	Turpeinen et al. [12]
MS type	LC-MS/MS	GC-MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
L-L extraction	+	+	+	+	+
SPE	–	+	–	+	–
Derivatization	–	+	–	+	–
Sample vol. (mL)	0.25	1–2	2	0.1	0.25–0.5
Sample run time (min)	6.5	12	n/a	3	10
Functional sensitivity/LOQ (nM)	0.056	0.15	0.69	0.035	0.15
Within-run imprecision (CV %)	4.5 at 1.3 nM, 2.4 at 11.0 nM, 1.9 at 23.4 nM	3.6 at 2.67 nM, 2.8 at 17.6 nM	10.5 at 1.74 nM, 8.6 at 26 nM	6.2 at 0.42 nM, 2.3 at 7.2 nM	3.1 at 2.0 nM, 0.9 at 20 nM

circulating hormones such as cortisol, 17-hydroxyprogesterone, and vitamin D₂/D₃ metabolites (data not shown), thereby raising the possibility of generating a comprehensive steroid panel in one testing pass of the sample. Such a multianalyte panel would have enhanced clinical utility and lead to greater efficiency in applying LC-MS/MS technology in routine clinical laboratories.

In conclusion, we have developed a simple highly selective and sensitive method for the determination of serum testosterone. With minimal sample preparation, short LC-MSMS running time, reasonable throughput, superior specificity and sensitivity, this method will be able to replace immunoassays in clinical laboratories.

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