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Direct measurement of serum free testosterone by ultrafiltration followed by liquid chromatography tandem mass spectrometry

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ABSTRACT

Background: Currently there is no reliable method suitable for routine measurement of serum free testosterone (FT).

Aim: To develop such a method involving liquid chromatography tandem mass spectrometry (LC-IDMS/MS) that directly detects and quantifies the FT present in serum.

Methods: Ultrafiltrate testosterone obtained from 0.5 mL of serum was partially purified by liquid/liquid extraction and quantified using an Agilent 1200 Series HPLC system coupled to an API 5000 mass spectrometer equipped with an atmospheric pressure chemical ionization ion source. Using split samples serum free testosterone was compared between direct ultrafiltration (UF) coupled LC-MS/MS, analogue FT immunoassay, free testosterone calculated from mass action equations (cFT) and with equilibrium dialysis (ED) coupled LC-MS/MS.

Results: Total imprecision determined over twenty runs was <6% at 67 pmol/L and 158 pmol/L FT. The dynamic response was linear up to at least 2500 pmol/L while physical LLOQ (18 % CV) equaled 16 pmol/L. The UF method agreed poorly with analogue immunoassay (correlation coefficient 0.667; bias −81%), somewhat better against cFT when total testosterone was determined by immunoassay (correlation coefficient 0.816, bias 21%) and still better yet against cFT when total testosterone was determined by LC-MS/MS (correlation coefficient 0.8996, bias 10%). Agreement was closest with ED method (correlation coefficient 0.9779, bias 2.4%).

Conclusion: We present a relatively simple UF coupled LC-MS/MS definitive method that measures serum free testosterone. The method is relatively fast, reliable and is suitable for the routine clinical laboratory practice.

Introduction

Testosterone in the blood circulates in three forms—tightly bound to sex-hormone binding globulin (SHBG), loosely bound to albumin and unbound (free testosterone (FT)). Only the free fraction, amounting to 1–2% of the total, is able to penetrate the cell membrane to interact with the androgen receptor to regulate the expression of androgen-responsive target genes [1]. Because of this, free testosterone is considered the most physiologically relevant fraction. However, FT is rarely measured in routine clinical practice since it is more technically difficult to determine for its very low concentration and similar structure molecule interference than either total testosterone or the indirect measures of the free fraction expressed in terms of the bioavailable testosterone (albumin-bound fraction), androgen index (total testosterone modulated by the SHBG-bound fraction) or mass action calculation (total testosterone and both protein bound fractions) [2].

Although total testosterone accurately measured frequently suffices [3-5], it is inherently less reliable than the direct measure of the free fraction because many factors including aging, obesity, pregnancy, testosterone/estrogen treatment, and polycystic ovary syndrome, affect the amount and affinity of the binding proteins, SHBG in particular, thereby leading to a mismatch between total testosterone and the free fraction. When this occurs, the total testosterone becomes inconsistent with the clinical status of the patient [6,7]. For example, a cohort study of American men over 65 years of age [8] and a cross-sectional analysis of Australian men over 70 years of age [9] have shown that the level of total testosterone remains relatively stable with age while the amount of free testosterone declines. The mismatch

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arises because SHBG increases as the men age. A similar relationship by a cross-sectional population-based study, this time between total testosterone and bioavailable testosterone, was seen in southern Californian men aged 50 to 89 years [10]. These studies suggest that FT is significantly more informative than total testosterone in investigating the androgen status of aging men [2]. Other studies have indicated that FT is to be preferred for the work up of androgen excess in girls and women, gonadal failure in girls, disorders of sexual development and puberty in boys and in monitoring the response to hormone treatment [3,11,12].

Several approaches have been used to measure FT in the circulation. The most reliable method physically separates the protein-bound from the free testosterone prior to quantifying the latter either through indirect measurement involving radioactively labeled tracer or direct measurement. The physical separation has traditionally been carried out by equilibrium dialysis (ED), a tedious technique for routine clinical practice [3]. Also problematic, tracer impurities can cause substantial errors when radioactively labeled tracer is used to indirectly quantify the free fraction. A second approach has sought to calculate the free fraction from the amount of total testosterone, the binding capacity of SHBG and albumin and the affinity constants of albumin and SHBG for testosterone. The calculated FT (cFT) usually correlates well with FT measured by the reference equilibrium dialysis method, but is highly dependent on the accuracy of the total testosterone, SHBG and albumin quantification [3,13,14]. The final approach, most widely used in clinical labs but fraught with inaccuracy, has utilized analogue-based immunoassay to estimate the free fraction. Unfortunately, estimates by this approach reflect total testosterone levels more closely than they do the free fraction [6,15].

Recently, Van Uytfanghe et al. [16,17] reported a reference method for FT that separated the protein-bound and free fractions by ultrafiltration (UF) instead of by equilibrium dialysis. This was attractive insofar as ultrafiltration is inherently faster and less technically demanding than equilibrium dialysis. However, the solid phase purification and the GC-MS detection used by Van Uytfanghe et al. is cumbersome and time consuming which makes it difficult for routine clinical testing. Previously we reported a LC-MS/MS procedure [4] for the measurement of serum total testosterone. Here we describe a new method using UF coupled with our testosterone LC-MS/MS procedure for the measurement of FT offering further improvements in analytical sensitivity, convenience and decreased sample requirement. A split sample comparison against analogue immunoassay, cFT and ED coupled LC-MS/MS is also presented.

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 μm) 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). The HEPES buffer used for UF and ED (52.75 mmol/L, pH 7.4) contained 5.265 g/L NaCl, 0.224 g/L KH_2PO_4, 0.275 g/L $MgSO_4 \cdot 7H_2O$, 12.570 g/L HEPES, 0.3 g/L urea, 0.275 g/L $CaCl_2 \cdot 2H_2O$, 0.9 g/L NaOH, and 0.520 g/L NaN_3 (Sigma-Aldrich, St. Louis, MI, all analytical reagent grade) [14]. Centrifree® ultrafiltration devices (Millipore, Tullagreen, Ireland, Cat #4104) with Ultracel® YM-30 regenerated cellulose membrane (cutoff 30 kDa) were used for the UF procedure. For ED, Micro DispoDialyzers with 5 kDa cutoff (Harvard Apparatus, Saint-Laurent, Québec, Canada, Cat #74-0717) were used.

Sample preparation

Ultrafiltration and sample extraction

0.5 mL of serum was diluted with 0.5 mL of HEPES buffer. After equilibrating at room temperature (RT) for 5 min, the mixture was transferred into a Centrifree® UF device and immediately centrifuged at 1800 g, 25 °C in a fixed angle rotor for 1 h. Testosterone-2,2,4,6,6-d_5 (40 fmol) was added to 0.5 mL of ultrafiltrate and the mixture vortex-mixed for 5 s, then incubated for 5 min at RT. Testosterone was extracted with 1 mL of MTBE. The MTBE fraction was evaporated under a stream of nitrogen gas at 40 °C and the residue was re-dissolved in 1 mL of 90% methanol to which 1 mL of heptane was added. After shaking, 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 70 μL of 50% methanol and a 50 μL aliquot was analyzed by LC-MS/MS.

Equilibrium dialysis

0.1 mL of serum was added into a Micro DispoDialyzer and dialysed against 0.5 mL HEPES buffer in a borosilicate culture tube (12×75 mm) at 37 °C overnight (16 h). Testosterone-2,2,4,6,6-d_5 (40 fmol) was added to 0.4 mL of dialysate and the mixture vortex-mixed for 5 s then incubated for 5 min at RT. Subsequent organic solvent extraction was the same as described above.

LC-MS/MS

LC-MS/MS measurement was the same as reported recently [4]. Briefly, HPLC was conducted using an Agilent Technologies 1200 series system in linear gradient mode at a flow rate of 0.85 mL/min through 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 1 min. An API 5000 (Applied Biosystems/Sciex, Concord, ON, Canada) mass spectrometer equipped with an atmospheric pressure chemical ionization source was used and operated in the positive ion mode. Testosterone and d_5-testosterone were detected and quantified at the ion-transitions of m/z 289.2→109.1 and 294.2→113.2, respectively. Analyst software (version 1.4.2) was used to control the system, mediate data acquisition, integrate peak-area and calculate the concentration of unknowns against a standard curve derived from calibrators analyzed within the same analytical run.

Analytical performance of the UF-LC-MS/MS method

The effect of the temperature at which ultrafiltration was conducted on recovery was assessed at 4 °C, 25 °C and 37 °C using four different patient samples. Non-specific adsorption of FT to the ultrafiltration membrane was assessed by subjecting aliquots of ultrafiltrate obtained from five different patient sera to a second round of ultrafiltration through a new membrane and then comparing FT in the once-filtered and twice-filtered ultrafiltrates. Ion suppression was evaluated by infusing d_5-testosterone using a Harward auxillary pump and injecting a serum MTBE extract. The ion transition of d_5-testosterone (294.2→113.2) was monitored. No chromatographic dipping was observed near the testosterone retention time.

The limit of quantification (LOQ) of the UF-LC-MS/MS assay corresponding to a sensitivity at imprecision (CV) of 20% was determined. Five replicate measures of eight serial dilutions consisting of different concentrations of testosterone (ranging 1000, 500, 250, 125, 63, 31, 16, to 8 pmol/L) were conducted and CVs of different concentrations were used to generated a precision profile from which the sensitivity at CV 20% was calculated as described before [4]. The lower limit of quantification (LLOQ), which is a physical sample, was defined at 16 pmol/L (CV 18%) and was compared against the FT

determined from sera submitted by nineteen adult women, originally for the purpose of measuring total testosterone (ranging from 0.6 to 2.9 nmol/L), in order to assess if the LC-MS/MS assay based on UF was sufficiently sensitive for use in adult women.

Linearity was tested over the range 0–2500 pmol/L by diluting testosterone from a stock solution. Ten serial dilutions (2500, 1000, 500, 250, 125, 63, 31, 16, 8, 0 pmol/L) were prepared in acetonitrile.

Within-run (20 replicates) and overall imprecision (20 runs over 10 days) was determined using patient serum pools with free testosterone concentrations of 67 and 158 pmol/L.

Carryover was assessed in 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 [4]. The following equation: $k = (b1 - b3)/(a3 - b3)$ was used to calculate the carryover, *k*. Two separate pairs of high and low samples (FT of 159 and 71 pmol/L; 154 and 63 pmol/L) were used for the carryover experiment.

Recovery was assessed by spiking testosterone standard into two patient sample ultrafiltrates (35 and 86 pmol/L, respectively) at two levels (74 and 107 pmol/L, respectively). The measured concentration before spiking was subtracted from that after spiking to determine the difference which was compared to the known amount of testosterone added [18].

Potential analytical interference from hemolysis, lipemia and icterus was assessed initially at levels of 3 g/L hemoglobin, 50 mmol/L triglyceride and 800 μmol/L total bilirubin, and then at lower concentrations (0.75, 1.5, 2.25 g/L) of hemoglobin because 3 g/L interfered. Ultrafiltrate containing 158 pmol/L of testosterone was spiked with supra-physiological levels (50–100 times the upper normal limit of free hormone) of protein-free progesterone, 17-OH progesterone, 21-OH progesterone, aldosterone, dihydrotestosterone, estradiol and cortisol to determine whether interference from these structurally similar steroids could be ruled out under conditions of normal clinical practice. Blood from a single subject was also drawn by venepuncture into different types of evacuated collection tubes available from Becton-Dickenson and the FT assayed to assess the effect of tube composition (with or without separation gel, different anticoagulants) on FT recovery.

Method comparison

Serum specimens from 60 male adult subjects with free testosterone concentrations ranging from 49- 439 pmol/L were included in the comparison study. The UF-LC-MS/MS method was compared with analogue-based immunoassay, cFT estimated from mass action equations and direct ED-LC-MS/MS. The analogue immunoassay was conducted with the Coat-A-Count RIA purchased from Siemens Medical Solutions Diagnostics. Serum samples were measured in duplicate. A validated algorithm [19,20] available with online calculator (http://www.issam.ch/freetesto.htm) was used to determine cFT. SHBG was assayed by the Abbott Architect i2000 and albumin by the Abbott Architect c8000. Total testosterone was measured either by the Abbott Architect i2000 or according to our previously reported LC-MS/MS method [4]. cFT was separately calculated with each of these measures of total testosterone. The RIA and Architect automated assays were carried out according to manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out by Microsoft Excel and SPSS one-way ANOVA (SPSS 11.5, Chicago, IL). The dilution curve was compared to the best fitted line determined by linear regression analysis to assess linearity. LOQ corresponding to functional sensitivity was calculated from the best fitting power curve to the five lowest concentration data points of the precision–concentration profile. Bland and Altman regression plots were used to assess systematic bias between methods [21,22].

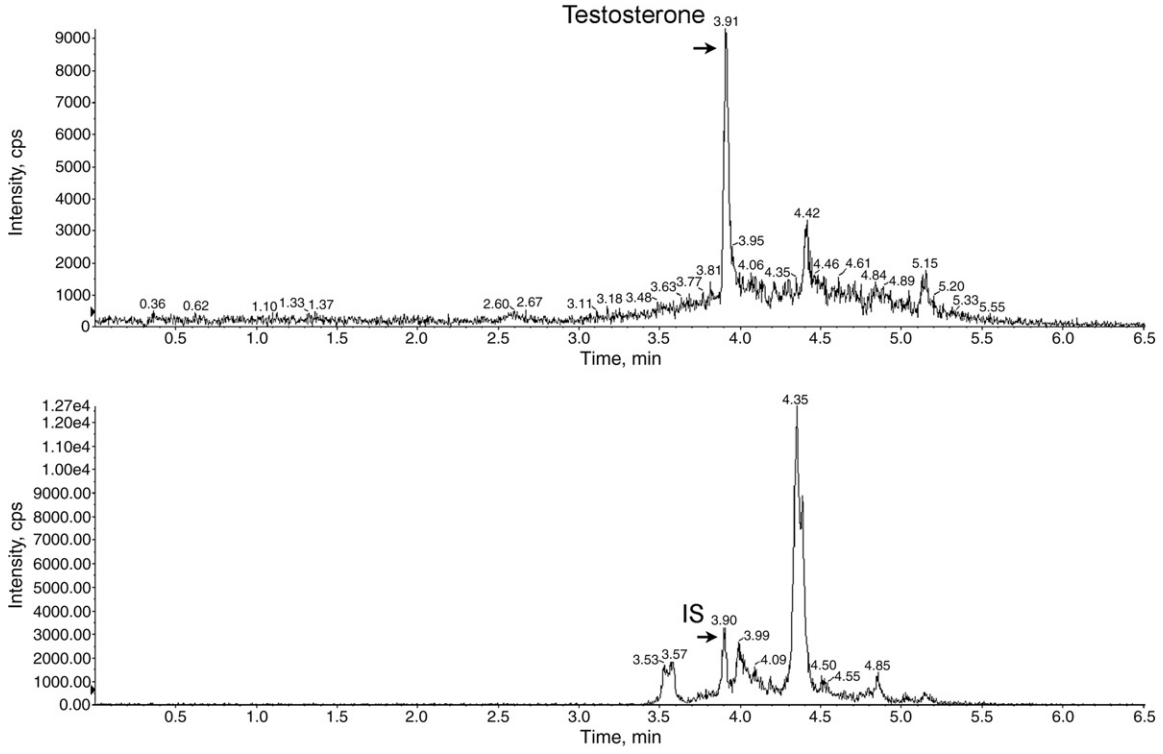

Fig. 1. Representative LC-MS/MS chromatograms of serum free testosterone from a male subject containing FT at a concentration of 252 pmol/L with the internal d5-internal standard added to the ultrafiltrate at a concentration of 80 pmol/L. The upper and lower panels show, respectively, the LC elution profile detected by MS/MS at ion transitions of 289.2 → 109.1 (used to identify and quantify testosterone) and 294.2 → 113.2 (used to identify and quantify d_5-testosterone, the internal standard). IS, internal standard.

Table 1
FT method accuracy and precision ($n=5$).

Calibrator	Spiked concentration (a, pmol/L)	Measured concentration (b, pmol/L)	Imprecision (CV, %)	Accuracy (b/a, %)
1	8	9	30	119
2	16	19	18	120
3	31	33	12	106
4	63	63	5.0	101
5	125	131	3.1	105
6	250	258	3.4	103
7	500	505	3.3	101
8	1000	1058	3.9	106

Results

Fig. 1 shows the LC elution profile detected by MS/MS at the specific testosterone transition of 289.2→109.1 (upper panel) and d_5-testosterone (internal standard) transition of 294.2→113.2 (lower panel). Both testosterone and d_5-testosterone elute at 3.9 min from the LC column [4] as previously described. Testosterone and d_5-internal standard were present at 252 and 80 pmol/L, respectively, in the ultrafiltrate used to generate the LC elution profiles shown in Fig 1. The chromatograms show that compounds that would potentially interfere in the MS/MS signal (i.e. those with the same ion transition as testosterone and the d_5-internal standard) are adequately separated from the 3.9-min eluted fraction by the LC column. The chromatograms also show that the background noise is not excessive compared to the signals generated by expected clinical concentrations of free testosterone ($s/n=20$) and by the 40 fmol of internal standard ($s/n=28$) added to the 0.5 mL of ultrafiltrate, as specified in the UC-LC-MS/MS procedure. LC Column elution and reconditioning took a total of 6.5 min.

The effect of the temperature at which ultrafiltration is conducted on the amount of FT that passes through the filter is shown in Fig. 1 of the supplemental data. More testosterone passed through the filter as the temperature increased, about 10% more at 25 °C than at 4 °C with a further 40% increment at 37 °C. In this study, ultrafiltration was routinely conducted at 25 °C. The rationale for adopt this temperature will be discussed later.

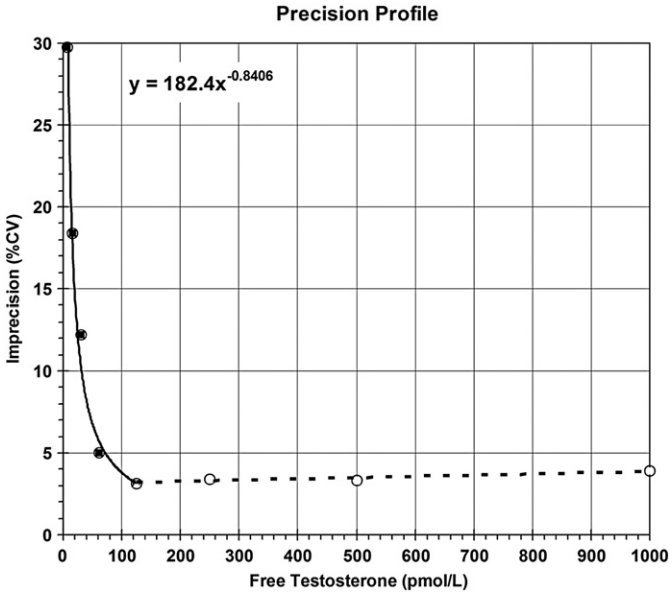

Fig. 2. The limit of quantification (LOQ) of the UF coupled LC-MS/MS assay corresponding to a functional sensitivity of 20% imprecision was determined as 14 pmol/L.

Table 2
FT recovery by spiking 2 patient sample ultrafiltrate with 2 levels of standard.

Sample	FT measured before addition [a] (pmol/L)	Amount of FT added to ultrafiltrate [b] (pmol/L)	FT measured after addition [c] (pmol/L)	Difference [c-a] (pmol/L)	% Recovery [(c-a)/b] *100
1	34.82	74.07	115.60	80.78	109.06
		107.14	154.94	120.12	112.11
2	85.52	74.07	157.51	71.99	97.19
		107.14	204.31	118.79	110.87

Serum FT did not appreciably bind to the ultrafiltration filter as shown in Table 1 of the supplemental data. Recovery was virtually the same whether the FT was centrifuged through one or through two unused ultrafiltration devices.

The LOQ based on a precision limit of 20% (sensitivity) was 14 pmol/L (Fig. 2). The physical LLOQ was defined at 16 pmol/L (CV 18%, accuracy 120%) (Table 1). The LLOQ did not lie substantially below the FT levels (Table 2, supplemental data) found in the majority of the sera submitted by nineteen adult women. The FT concentration in nine of these sera was less than the LLOQ (11–15 pmol/L). FT ranged from 17 to 53 pmol/L in the rest ten female samples.

The present method demonstrated a dynamic linear response up to at least 2500 pmol/L. The linear regression line fitted the data with a correlation of 0.9998 (Pearson's correlation coefficient squared).

Within-run and total precision using serum pools were, respectively, 3.6% and 5.5% at 67 pmol/L FT and 2.9% and 4.0% at 158 pmol/L FT.

Carryover was minimal as judged by two criteria. First, there was no detectable testosterone peak observed in methanol blanks injected immediately after serum samples containing 400 pmol/L of FT. Second, there was no consistent increase in the first compared to the third aliquot of a specimen with relatively low levels of FT when these sequentially followed three aliquots of a specimen containing substantially higher levels of FT. Specifically, carryover k was 0.09 for one high-low pair of samples (165, 149, 163 followed by 75, 73, 66 pmol/L) and −0.06 for the other (149, 152, 161 followed by 62, 59, 67 pmol/L). The percent carry over was −1% to 5% (Table 3, supplemental data).

Testosterone was added at two concentrations to two different serum specimens to assess recovery (Table 2). Recovery varied from 97% to 112 %. Interference in the assay from lipemia, icterus, hemolysis and added steroid compounds is shown in Table 3. Lipemia and icterus, even at the high concentrations tested, did not interfere, but hemoglobin when present at 3 g/L reduced recovery to 71%. Recovery improved to 82% at 2.25 g/L hemoglobin and 95% at 1.5 g/L hemoglobin until it was no longer impaired when hemoglobin equaled 0.75 g/L (104% recovery). None of the other steroids tested interfered in the FT assay, even when added to the serum ultrafiltrate

Table 3
Analytical interference.

Interferent	Amount added	[a]Recovery (%)
Progesterone	256 μmol/L	94
17-OH progesterone	30 nmol/L	102
21-OH progesterone	6 nmol/L	94
Aldosterone	2 nmol/L	97
Dihydrotestosterone	100 nmol/L	94
Estradiol	2 nmol/L	99
Cortisol	1 μmol/L	101
Hemoglobin	3 g/L	71
Bilirubin	800 μmol/L	102
Triglyceride	50 mmol/L	99

[a] Recovery was determined by FT measured before and after spike the neat serum ultrafiltrate containing 158 pmol/L FT, and from the average of three replicate analyses.

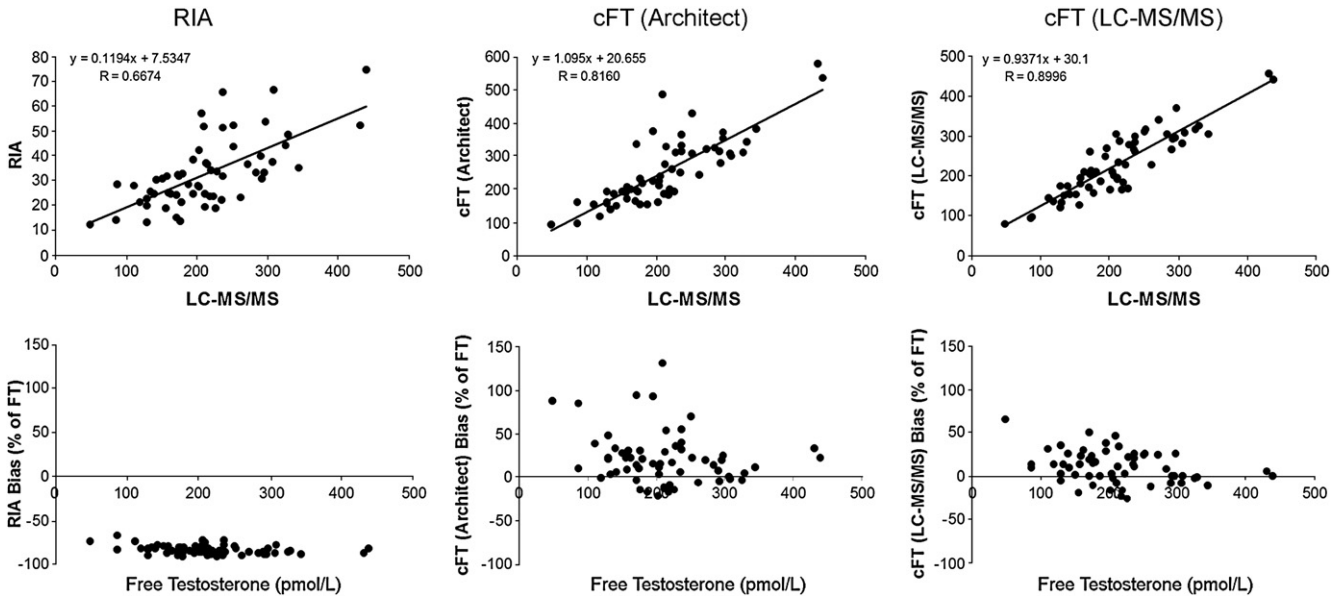

Fig. 3. UF-LC-MS/MS compared to analogue FT immunoassay (RIA) and to cFT estimated under two conditions—(1) with total testosterone determined by the Architect automated immunoassay and (2) with total testosterone determined by LC-MS/MS assay [4]. Sixty male specimens were included in the comparison. The upper panel shows the correlation of RIA and the two cFT estimates with UF coupled LC-MS/MS. The lower panel shows the corresponding bias plots. RIA, radioimmunoassay; cFT, calculated free testosterone.

at much higher concentrations than would be encountered clinically (Table 3). Table 4 in the Supplementary Data shows the comparison of FT from blood of the same subject collected into the variety of vacutainers shown. The difference was somewhat lower in the absence of anticoagulant and increased up to +25% when heparin or potassium EDTA were included in the collection tube. Blood was routinely collected into the SST vacutainer.

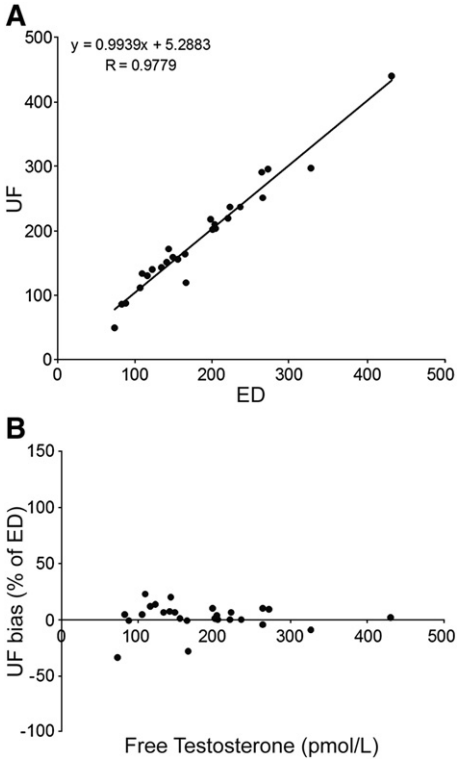

Fig. 4. Method comparison of UF coupled LC-MS/MS with ED coupled LC-MS/MS ($n = 26$). The upper panel shows the correlation plot of UF vs. ED. The lower panel shows plots of the bias between free testosterone measurements by UF and ED as a percentage of ED values (Y axis) against the ED measured FT concentration. UF, ultrafiltration coupled LC-MS/MS; ED, equilibrium dialysis coupled LC-MS/MS.

Fig. 3 shows the comparison results of the proposed UF coupled LC-MS/MS method with analogue FT immunoassay (RIA) and with cFT estimated under two conditions, first when the total testosterone was determined by the Architect immunoassay and second when the total testosterone was determined by our recently reported LC-MS/MS method. The following relationships were found for the sixty specimens tested: RIA = 0.1194 LC/MS/MS + 7.5, $R = 0.6674$, bias = −81%; cFT (Architect measure of total testosterone) = 1.095 LC/MS/MS + 21, $R = 0.816$, bias = 21%; cFT (LC-MS/MS measure of total testosterone) = 0.9371 LC/MS/MS + 30, $R = 0.8996$, bias = 10%. Fig. 4 shows the comparison of the UF method with ED for 26 adult male specimens tested. The two method agreed closely (UF = 0.9939 ED + 5.3, $R = 0.9779$, bias = 2.4%) pointing to the equivalence of ultrafiltration and equilibrium dialysis in separating protein bound from free testosterone.

Discussion

Reliable measurement of free steroid and thyroid hormones in the blood is inherently technically challenging and until recently has been difficult to achieve in routine clinical practice. However, the increasing sensitivity and ease of use of LC-MS/MS technology has made this an attractive, if not superior, alternative to immunoassay and when coupled to prior ultrafiltration (UF) or equilibrium dialysis (ED) to remove the protein bound fraction, a powerful tool to measure free hormone and drug levels. Several methods using just this technology have recently been published for the measure of free thyroid hormone and unbound antiretroviral drugs [23-25]. Although both UF and ED are acceptable as reference procedures to separate protein bound from circulating free ligands, UF is inherently better suited to the demands of the clinical lab because of its greater simplicity and speed, and accordingly, we chose to implement it in our proposed method. To the best of our knowledge, our proposed method is the first in the literature that directly measures FT by LC-MS/MS.

The UF-LC-MS/MS method described here compares favorably to the UF coupled GC-MS reference method recently published by Van

Uytfanghe et al. [16]. This method achieves equivalent LLOQ (16 pmol/L vs. 15–20 pmol/L) with less sample (0.5 mL vs. 1 mL serum), higher throughput (3.9 vs. 10.45 min LC elution), and no need for derivitization.

The Centrifree® ultrafiltration device is designed for the rapid separation of free ligands by the unhindered passage through the YM hydrophilic and nonabsorptive membrane (30 kDa cutoff) with a high degree of protein retention. This membrane was ideal in that it truly retained the protein present in the serum (less than 0.07 g/L, the limit of detection of our protein assay, in the ultrafiltrate) while not adsorbing FT (Table 1, Supplementary Data). Of note, the Centrifree® ultrafiltration device only worked when spun in a fixed angle rotor; swinging bucket rotors were unsuitable.

Soldin et al. [23] reported for their free thyroxine UF coupled LC-MS/MS assay that the temperature at which ultrafiltration was carried out influenced the concentration of free thyroxine in the ultrafiltrate, presumably through altering the equilibrium of bound and free species in the serum retentate. We also found that the temperature at which the ultrafiltration step was conducted also appreciably influenced FT recovery (Fig. 1, Supplementary Data). Generally UF at 37 °C generates about 40% more FT recovery than at 25 °C, however, we found that UF coupled LC-MS/MS at 25 °C agrees best with ED coupled LC-MS/MS which is conducted at 37 °C ($R = 0.9779$, bias = 2.4%). This finding is consistent with Soldin et al. [23] on their UF and LC-MS/MS method using the same UF device and similar LC-MS/MS instrument for free thyroxine, which was conducted at 25 °C eventually. Furthermore, working at 37 °C for UF is very impractical for a routine laboratory practice which requires pre-warm up sample, UF device, centrifuge (may take about an hour), and quick sample transfer. Given this, along with the convenience of running the centrifuge at room temperature, we adopted 25 °C as the temperature to conduct the ultrafiltration step.

Our proposed method is attractive in that it agrees closely with ED and LC-MS/MS which must be considered the gold standard, but is much faster and easier to run, and better suited for the routine clinical laboratory, than the latter. The ultrafiltration procedure requires approximately 1 h to complete while equilibrium dialysis requires at least 16 h to complete the dialysis step. The dynamic range of the procedure described here easily accommodates FT levels expected in the adult male, and lipemia, icterus and other steroids do not interfere, and imprecision is less than 6% at FT levels expected in the adult male. The limitation of the current LLOQ make the method is not sufficient for all female samples at low levels. However, for female test application, it is the high end of the range most clinical interested, i.e. hyperandrogenic conditions such as polycystic ovary syndrome. The 16 pmol/L LLOQ needs to be reduced by at least half to make the method suitable to measure with an imprecision of less than 20% the FT levels expected in many women. Reducing the functional sensitive by 50% might be achieved by increasing the serum ultrafiltrate from 0.5 to 1 mL or by adopting atmospheric pressure photoionization, but these will require additional studies to substantiate. Overall fraction of total testosterone concentration measured by the present UF-LC/MS/MS method is 1.72%, which is very close to the reported before as 1.87% by UF-GC/MS [17]. Further clinical studies are needed for the evaluation of clinical utilities of FT by this UF coupled LC-MS/MS method in subpopulations such as hypoandrogenic men and poly cystic ovary syndrome women.

Consistent with previous reports [17,26] that analogue-based FT RIA assay compares poorly with reference quality FT methods, we found the same when we compared the former to our UF and LC-MS/MS method (Fig. 3). Numerous previous reports and position statements [7,14,17,27] have indicated that analogue-based assays are unreliable and should not be used to measure FT.

In conclusion, we have developed a simple, rapid, highly selective and sensitive method that accurately determines serum-free testosterone. The minimal sample preparation, reasonable throughput and superior specificity and sensitivity may allow this method to serve both as a reference procedure and a routine method in the routine clinical laboratory practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.12.005.

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