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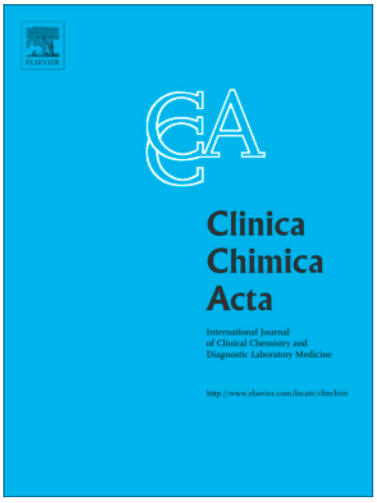

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The calculated and the rapid equilibrium dialyzed human serum free testosterone by LC-MS/MS and their performances in PCOS diagnosis

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Abstract

Objective: To compare the calculated and the rapid equilibrium dialyzed (ED) human serum free testosterone (FT) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to explore their performances in polycystic ovary syndrome (PCOS) diagnosis.

Methods: A rapid ED LC-MS/MS method for serum FT was established and validated for linearity, lower limit of the measuring interval (LLMI), imprecision, trueness, stability, dilution, matrix specificity and carryover. The validated ED LC-MS/MS (ED-FT) was compared with calculated LC-MS/MS method from Vermeulen's formula (cFT) for FT measurement in 139 PCOS patients and 100 healthy controls. The performances of total testosterone (TT), ED-FT and cFT by LC-MS/MS in PCOS diagnosis were investigated with the same cohorts.

Results: The linearity range of ED-FT was 1.74 - 890 pmol/L, with a LLMI at 1.74 pmol/L. The intra-assay and inter-assay imprecision were < 3.8% and < 5.9%. The trueness was

acceptable with recoveries of 92.9% - 108.2%. The equilibrium dialysis time was 4 h. The two FT methods displayed systematic and proportional differences and cFT showed significant positive deviations compared to ED-FT. Receiver operating characteristic curve analysis proved that ED-FT outperformanced in PCOS diagnosis with the an area under the curve at 0.973, sensitivity of 89.93% and specificity of 96.00%.

Conclusions: This study established a rapid, accurate and sensitive ED LC-MS/MS method for serum FT. ED-FT is optimal compared to TT and cFT by LC-MS/MS in PCOS diagnosis.

Keywords

Free testosterone; Equilibrium dialysis; LC-MS/MS; Polycystic ovary syndrome

1 Introduction

Testosterone, an androgen, plays a vital role after puberty in spermatogenesis and follicle development, regulates muscle development and fat distribution, and red blood cell production [1]. In females, testosterone is primarily produced and secreted by adrenal gland and ovaries. The testosterone levels in polycystic ovarian syndrome (PCOS) patients are usually elevated compared to healthy females, resulting in endocrine and reproductive problems [2].

Most circulating testosterone is tightly bound to sex hormone-binding globulin (SHBG) or loosely bound to albumin (ALB), orosomucoid, and corticosteroid-binding globulin. Merely 1-4% of circulating testosterone is unbound or free. Only unbound or free testosterone (FT) could enter the cell to exert biological effects [3]. Total testosterone (TT) is the sum of the bound and FT concentrations in the circulation. It is susceptible to changes in SHBG concentrations under the influence of gender, age, weight, metabolic syndrome, polymorphisms in the SHBG gene, and some medications [4]. Since FT is not affected by SHBG, it is an ideal indicator to assess the biologically active testosterone status. The accurate measurement of circulating FT is crucial and has been used in PCOS [5] diagnosis.

The extremely low serum FT concentration poses significant challenges for measurement techniques. Currently, most laboratories indirectly determine serum FT concentrations by calculating different mathematical models using TT, SHBG, and ALB (cFT) concentrations [6]. However, there were significant variations between the results of the various models. The direct serum FT measurement methods include two processes: separation of FT from the serum matrix and quantification of the separated FT. The techniques to separate FT from the serum matrix include equilibrium dialysis (ED) [7,8], ultrafiltration [9], and direct tracer analog immunoassays [10]. However, the direct tracer analog method should be avoided due to its inaccuracy [11]. The problem of nonspecific binding during ultrafiltration would also affect the accuracy [12]. A standardized ED method is recommended to effectively separate FT from the serum matrix. Quantification of FT in the dialysate was previously measured by immunoassays. However, immunoassays suffer from poor specificity due to cross-reactions

with other structurally similar steroids [13]. With the rapid development of liquid chromatography-tandem mass spectrometry (LC-MS/MS), the ED separation combined with LC-MS/MS has become the "gold standard" for quantifying FT [4].

Recent published ED LC-MS/MS methods for serum FT [7-10, 14] could be further improved.In these methods, the time required for the ED process ranged from 5 to 24 hours, and the lower limit of measuring interval (LLMI) ranged from 2.4 to 16 pmol/L. Lengthy ED time affects the turnaround time for result reporting. Serum FT concentration can be as low as 1% of serum TT [15]. The lower limit of female serum TT reference interval could be as low as 0.29 nmol/L [14]. Thus, to accommodate measuring female serum FT, the LLMI for an LC-MS/MS method would ideally be lower than 3 pmol/L. Therefore, a rapid and sensitive ED LC-MS/MS method for FT is required for clinical use.

This paper reports the development and validation of an ED LC-MS/MS serum FT method with improved ED procedure and sufficient sensitivity to accommodate female samples. The improved method was evaluated for its clinical performance in diagnosing PCOS and compared with the performance of TT and cFT.

2 Materials and methods

2.1 Measurement of ED-FT by LC-MS/MS

2.1.1 Materials

The testosterone standard (purity > 98.3%)used for preparing the calibrators was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Testosterone standard solution (1 mg/mL, purity > 99.0%) used for preparing in-house quality control (QCs) samples, and internal standard (IS) solution (testosterone-2,3,4-13C3, 100 μg/mL) were purchased from Cerilliant (Texas, USA). Hypergrade LC-MS LiChrosolv® acetonitrile and methanol (MeOH), and gradient grade LC LiChrosolv® isopropyl alcohol, methyl tertbutyl ether (MTBE), and formic acid were purchased from Merck-Millipore (Darmstadt, Germany). LC-MS grade water was purchased from Fisher Scientific (Pennsylvania, USA). HEPES, magnesium sulfate heptahydrate, calcium Chloride Dihydrate, potassium chloride, sodium chloride, and Urea were purchased from Sigma-Aldrich (Missouri, USA). potassium dihydrogen phosphate and sodium hydroxide were purchased from Guangzhou Jinhua Da Chemical Reagent (Guangdong, China). The Rapid Equilibrium Dialysis (RED) Device inserts (8kDa MWCO) and base plate were purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA).

2.1.2 Preparation of ED buffer, calibrators, IS solution, and QC samples

ED HEPES buffer

The ED HEPES buffer (52.8 mmol/L) was adapted from Van Uytfanghe's method [16]. 0.200 g/L KCl was added instead of NaN3. After adjusting the pH value to 7.40 ± 0.01 at room temperature by 1.0 M NaOH, the buffer was aliquoted and stored at -70 °C.

Internal standard (IS)

The IS working solution was at 3.5 nmol/L of IS in 30%(v/v) acetonitrile.

Standard (STD) preparation

Ten calibrators were prepared by diluting testosterone standards in ED-HEPES buffer solution, ranging from 1.74 to 890 pmol/L.

QC preparation

Three levels of dialysate QC (DQC) samples were prepared at 6.94, 52.0, and 451 pmol/L to monitor the performance of the LC-MS/MS procedure by spiking different amounts of testosterone standard into the ED-HEPES buffer. DQC samples did not go through the ED procedure.

Another 3 levels of serum QC (SQC) samples were prepared to monitor the performance of the overall ED-LC-MS/MS method. A healthy adult male serum pool was prepared by collecting serum samples from 20 male volunteers. Similarly, a healthy adolescent serum pool was also prepared. The serum QC samples were prepared by mixing different adult and adolescent serum pool proportions.

2.1.3 Separation of serum FT by equilibrium dialysis

After equilibrating at room temperature for 0.5 h, 400 µL of SQCs or serum samples were pipetted into the left side of the RED device, and 400 µL of ED-HEPES buffer was added into the device's right side. The RED device was kept inside an orbital shaker for 5 h at 37 ℃. 300 µL dialysate was transferred into a new centrifuge tube containing 50 µL of the IS working solution. The sample was mixed with the Biocomma® BCM2500 multi-function mixer (Biocomma Limited, Guangdong, China) for 3 min at 1600 rpm. Afterward, 1500 µL of MTBE was added to the same tube and mixed for 5 min at 1600 rpm to extract FT. Following centrifugation at 9296×g for 5 min at RT, the tube was kept under -70 ℃ for 1 h. The MTBE fraction was transferred to another clean tube and evaporated under a stream of nitrogen gas at 60 ℃. The residue was dissolved in 70 µL 35% MeOH and was transferred to an injection vial for LC-MS/MS analysis.

Quantitation of serum FT in dialysate by LC-MS/MS

The analysis was on a Shimadzu LC-30AD liquid chromatography system coupled with a SCIEX Triple QuadTM 7500 mass spectrometer with electrospray ionization in positive mode. The separation was carried on a Phenomenex Kinetex C18 column (2.6 µm, 2.1 × 100 mm), with the column temperature at 30 ℃. The injection volume was 50 µL, and the sample injector temperature was 8.0 ℃. The mobile phases consist of 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B). The mobile-phase gradient was programmed as in Table 1. The multiple reaction monitoring transitions and tuning parameters are listed in Table 2. The data were collected and analyzed using the Analyst® software (SCIEX, version 1.6.2)and

MultiQuant (SCIEX, version 3.0.3).

2.1.4 Method validation

The method validation included the evaluation of linearity, dilution, imprecision, trueness, specificity, carryover, matrix effect and stability according to guidelines established by the Clinical and Laboratory Standards Institute C62-A document [17].

Linearity, LLMI and LOD

The linearity was assessed by 10 calibrators with concentrations at 1.74, 3.48, 6.95, 13.9, 27.8, 55.6, 111, 223, 445, and 890 pmol/L. Two aliqouts were measured for each calibrator for three days. For linear regression analysis, the calibration curve was constructed by plotting the FT/IS peak area ratio (y) against the FT concentration (x), at a weighting of 1/X. The curves were considered linear when the correlation coefficient (r2) of all curves was greater than 0.99.

The limit of detection (LOD) was defined as the concentration of standard solution maintaining a signal-to-noise ratio (S/N) of 3:1. Then, three standard solutions with a concentration close to LOD were used to evaluate the LLMI and each concentration was performed in 10 times. The LLMI was defined as the concentration of standard solution produced a CV ≤ 20% and a recovery rate ranging from 85% to 115% while maintaining a S/N of 10:1.

Effect of dilution

Two pooled serum samples, one collected from 5 healthy females and the other from 5 healthy male donors, were used to investigate the effects of dilution on serum FT using the ED-LC-MS/MS method. The female and male serum pools' serum FT concentrations were 22.7 and 223 pmol/L, respectively. Before equilibrium dialysis, the pooled samples were diluted with the proportions of 90%, 80%, 70% and 60% (v/v) with 0.9% normal saline. Additionally, one serum pool at 139 pmol/L collected from males was diluted at 2× or 5× using HEPES buffer respectively after the ED procedure. Five aliquots at each concentration was processed and measured in parallel. The concentration of the undiluted sample was regarded as the reference target value. The effect of dilution was acceptable as recoveries between 85% and 115%.

Imprecision and Trueness

The intra- and inter-assay imprecision were assessed using the low, medium, and high levels of both SQC and DQC samples. It was calculated from 20 replicates for each QC level (intra-assay) and 2 replicates for each QC level per day for 10 consecutive days (inter-assay). The imprecision of each concentration should be <15% CV, and ≤ 20% CV for LLMI.

Trueness was determined by the recovery testing using low, medium, and high DQCs (n=3×3), evaluated as the ratio of the calculated spiked amounts against the actual spiked amount of the standard solution. The recoveries should be between 85% and 115%.

Matrix Specificity and Carryover

After injecting the STD solution containing IS and the SQC containing IS, the HEPES buffer as the double blank sample was injected for LC-MS/MS analysis to observe whether there were interference peaks near the target peak. Each sample was injected once. Background peaks should be absent or < 20% of the peak area for the analyte at the LLMI or ≤ 5% of the IS at the expected retention time.

The low and high DQCs were used for carryover testing. It was assessed by running DQCs in a sequence of low-high-low (C1-C3-C1) concentrations for triplicate. The ratio of (C3mean-C1mean)/C1mean ≤ 20 % was defined as acceptable.

Matrix effect

The matrix effect has been evaluated in LC-MS/MS method for TT, and the results were acceptable as shown in sT4.

Stability

The low, mid and high SQCs were used to evaluate the stability of processed serum samples and the serum samples after ED. The dialysate or extracted FT after reconstitution were stored at 2-8 ℃ and analyzed at 0, 24, 48, and 72 h, respectively. Triplicates were measured at each time point. The samples measured at 0 h was used as the target value and the CVs of samples measured at 24, 48, and 72 h were calculated. The CV of each sample should be within 2.77 times the average CV of all three levels of SQC in inter-assay imprecision testing.

2.1.5 Optimization of the ED equilibration time

To optimize the ED equilibration time, two pooled serum samples were prepared by collecting samples from healthy male and non-PCOS female volunteers, aged 18-40 years, among our laboratory staff. The samples were dialyzed for 1.5, 3, 4, 5, 6, and 9 h in triplicates. After extraction, the FT concentrations were determined using LC-MS/MS.

2.2 Measurement of serum FT in PCOS patients

The study was approved by the Medical Ethics Committee of Peking Union Medical College Hospital (PUMCH) (approval number: HS-3445). Healthy subjects and PCOS patients aged 18-40 years were enrolled in PUCMH. Informed consent was obtained from all individuals included in this study. The inclusion criteria for healthy controls were females with normal body mass index (18.5≤BMI< 24) and normal blood pressure < 140/90 mmHg, no medical history of anti-hypertensives or hormonal treatment, and no smoking and/or alcohol habits. Females who were pregnant, breastfeeding, or taking oral contraceptives were excluded. PCOS patients were diagnosed according to the revised Rotterdam criteria[18] by reproductive endocrinologists in PUCMH. None of the women had endocrine or systemic disorders that may affect reproductive physiology, including hyperprolactinemia, Cushing's syndrome,androgen-secreting tumors, congenital adrenal hyperplasia, and thyroid dysfunction. For all subjects, blood samples were collected at between 8:00 am and 10:00 am after fasting for 8 hours between days 3 and 7 after spontaneous or progestogen-induced bleeding episodes.

All samples were submitted to Guangzhou Kingmed Center for Clinical Laboratory Co., Ltd. for analysis. Serum FT concentrations were measured using both the ED LC-MS/MS method and the calculated method. The comparison between the two methods was performed among the total enrolled subjects, healthy controls and PCOS patients. Furthermore, the performances of TT, cFT and ED-FT for PCOS diagnosis were investigated.

2.3 Calculation of cFT

The cFT was calculated by Vermeulen's formula [19] based on TT, SHBG, and ALB levels. Serum SHBGwas measured using IMMULITE 2000 SHBG kit (Siemens Healthcare Diagnostics Products Limited, Llanberis, UK),. Serum ALB was measured using Albumin Gen.2 assay (Roche Diagnostics GmbH, Mannheim, Germany). Analytical performance of the reagent kits was verified according to the manufacturer's performance specifications. Serum TT was measured using the Shimadzu LC-20AD liquid chromatography system (Shimadzu, Tokyo, Japan) and a SCIEX Triple QuadTM 5500+ mass spectrometer (SCIEX, California, USA). The TT method was validated (Supplementary material 1).

2.4 Statistical analysis

Statistical analysis was performed on MedCalc (Ostend, Belgium, version 15.2.2). The correlation of the two methods were studied by Spearman correlation analysis. Passing-Bablok regression analysis and Bland-Altman plot analysis were applied to analyze the agreement between the methods. ROC analysis was used to evaluated the performances of analytes in PCOS diagnosis. Significance was defined as $P < 0.05$.

3 Results

3.1 Measurement of ED-FT by LC-MS/MS

Method validation

Linearity, LLMI and LOD

The linearity range was 1.74-890 pmol/L, with a good correlation of $r^2 > 0.999$. The LOD was 0.580 pmol/L, and the LLMI was at 1.74 pmol/L with a CV of 6.1%. The recoveries of the serum samples diluted in 0.9% of normal saline were within 96.3% to 111.6% (Table SS1 in supplementary material 2).

Effect of dilution

The recoveries of the diluted dialysate at 2× or 5× in HEPES buffer were between 93.1% to 105.5% (Table SS2 in supplementary material 2).

Imprecision and Trueness

The CVs of the intra-assay (n=20) and inter-assay (n=10×2) imprecision were all within ±15%

(Table 3). The trueness was acceptable, with recoveries of 92.9% to 108.2%.

Matrix Specificity and Carryover

Matrix specificity results showed no interference peaks near the signals of FT (Figure SS1 in supplementary material 2). Moreover, no carryover was observed, as the ratio of ($C3_{mean}$ - $C1_{mean}$) / $C1_{mean}$ was 1.0%.

Stability

The processed sample could be stored at 2-8 ℃ for 72 h with the CVs < 13.0% (Table SS3, SS4 in supplementary material 2).

Optimization of the ED equilibration time

The samples dialyzed for 1.5, 3, 4, 5, 6, and 9 h yielded mean concentrations (n=3) of 115, 163, 176, 165, 178, and 174 pmol/L for pooled male serum, and 15.1, 23.8, 24.9, 25.2, 25.4, and 25.8 pmol/L for pooled female serum. The FT concentration increased with time up to 4 h and plateaued from 4 to 9 h for both pooled serums (Figure SS2 in the supplementary material 2).

3.2 Comparison between the ED LC-MS/MS and the calculated LC-MS/MS method for FT

A total of 239 subjects (PCOS patients:139, healthy controls:100) were enrolled in the final study. The results of spearman rank correlation test, Passing-Bablok regressions analysis and Bland-Altman plot analysis between the two FT methods were shown in figure 1 and table 4. The Spearman's correlation coefficients varied from 0.942 to 0.983 (all $p < 0.0001$) between the two FT methods in total subjects and the two subgroups. The slopes, intercepts and 95% CI of biases were listed in table 4. The Passing-Bablok regressions analysis showed that systematic and concentration-dependent differences between the two FT methods were observed in three subjects groups. The Bland-Altman plot revealed that calculated LC-MS/MS method displayed positive deviations compared to ED LC-MS/MS method for FT quantification in total subjects and the two subgroups.

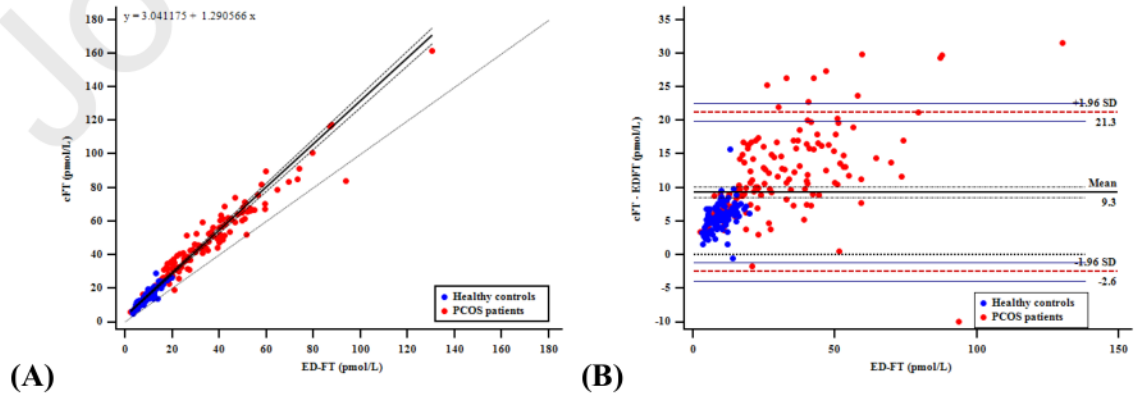
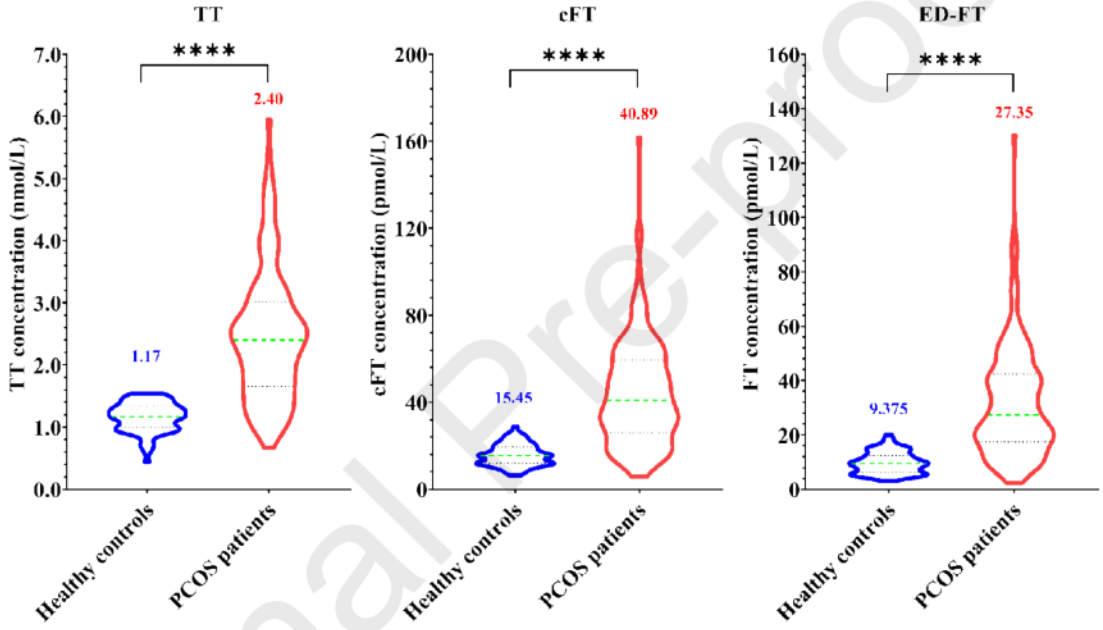


Figure 1. Comparison between cFT by LC-MS/MS and ED-FT by LC-MS/MS in 239 total

subjects, 100 healthy controls as well as 139 PCOS patients. Passing-Bablok regressions (A) and bland-Altman plots (B). Red dots represent PCOS patients samples (n=139) and blue dots represent healthy controls samples (n=100).

3.3 TT, cFT, and ED-FT levels in healthy controls and PCOS patients

As shown Figure 2, the results obtained from Mann-Whitney test showed a significant difference ($P<0.05$) between healthy controls and PCOS patients was observed with TT (Median: 1.17 nmol/L VS 2.40 nmol/L), cFT (Median: 15.45 pmol/L VS 40.89 pmol/L) and ED-FT (Median: 9.375 pmol/L VS 27.35 pmol/L).



****: statistically significant, $P < 0.05$.

Figure 2. Mann-Whitney test for TT, cFT, and ED-FT levels in 100 healthy controls and 139 PCOS patients.

3.4 The performances of TT, cFT, and ED-FT in PCOS diagnosis

The diagnostic performances of TT, cFT and ED-FT for PCOS diagnosis were investigated using ROC analysis, as shown in Figure 3. ED-FT showed the greatest diagnostic performance with an area under the curve (AUC) of 0.973 at a cut-off value of 16.13 pmol/L while the AUC and cut-off value are 0.914 and 1.54 nmol/L for TT and 0.911 and 24.6 pmol/L for cFT respectively. ED-FT yielded an extraordinary specificity of 96.00% and sensitivity of 89.93%. The positive predict value for ED-FT in PCOS diagnosis is 96.89% (95%CI: 92.25 to 99.14), and the negative predict value is 87.27% (95%CI: 79.57 to 92.86).

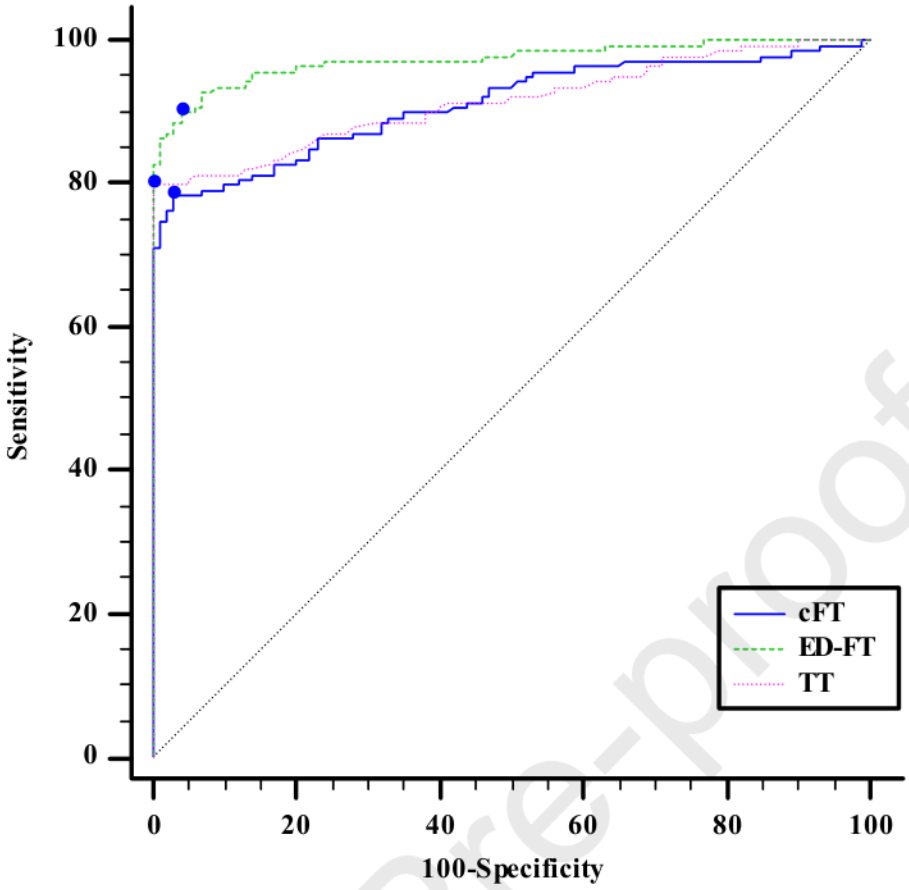


Figure 3. Receiver operating characteristic (ROC) analysis of 139 PCOS patients compared with 100 healthy subjects as control. ROC curves to analyze the diagnostic value of TT, cFT and ED-FT.

4 Discussion and conclusion

Accurate measurement of FT level in human serum is essential and methodologically challenging [20]. The calculated free androgen index (FAI) was utilized as an alternative for FT, yet FAI is no longer recommended [21]. Keevil, et.al [22] investigated the consistency of FAI and ED-FT measured by LC-MS/MS, showing the FAI/ED-FT ratio was inconsistent and increased when SHBG concentration was below 30 nmol/L. PCOS patients commonly have obesity and insulin resistance, leading to lower SHBG levels [23]. Thus, utilizing FAI may result in inaccurate findings when investigating hyperandrogenism in these patients.

Although the direct ED LC-MS/MS method were considered the gold standard for FT quantification [4,9], the slow separation and equilibrium procedure and unsatisfactory LLMI limited its application. For the ED LC-MS/MS method presented herein, the assemble-free RED Device were applied and the optimal equilibrium dialysis time was shortened to 4 h, which makes it possible to use this method in routine clinical practice for rapid FT

measurement. Compared with the ED LC-MS/MS methods in previous articles (shown in table 5), our stable method is derivatization-free and the equilibration time is shortest, makes it operator friendly and time-saving. And this validated method is most sensitive with the LLMI at 1.74 pmol/L and a broad linearity ranged from 1.74 to 890 pmol/L. Additionally, the dilution validation proved that the sample was prepared well and extended the linearity to 4450 pmol/L, which is fairly enough for FT levels in females, children and males.

The Passing-Bablok analysis and bland-altman plot analysis indicated that even though cFT level was highly correlated with ED-FT level in total subjects, in PCOS patients as well as in healthy controls, there is a systematic overestimation of FT by calculated LC-MS/MS, which is in accordance with Fiers, et.al 's study [7]. The researchers found that cFT (using Vermeulen's formula) strongly correlates with ED-F(r=0.91) but systematically overestimates FT by 20% to 30%. This aligns with our results of Passing-Bablok regression analysis, which showed systematic overestimation of FT by cFT compared to FT by ED LC-MS/MS, with a regression equation slope exceeding 1, as shown in Figure 1(A). In this study, the systematic overestimation of FT by cFT was around 29%. And the FT overestimation is most significant in PCOS patients. Besides, cFT depends on the accurate determination of TT, ALB, and SHBG, meaning that three assays should be performed and more workloads with more imprecision. The Mann-Whitney test proved that the levels of TT and FT is significantly higher in PCOS patients compared to the healthy controls, which is as expected. The ROC analysis of 100 healthy controls and 139 PCOS patients demonstrated ED-FT is the best biomarker for PCOS diagnosis compared to TT and cFT.

Our study has several limitations. Despite our cohort comprised 139 PCOS patients and 100 healthy controls, this sample size may still be inadequate for detailed subgroup analysis, limiting the generalizability and extrapolation of our findings. Additionally, our study population was exclusively Chinese, potentially limiting the applicability of our results to PCOS patients globally or to otherracial and ethnic groups. The single-center design may restrict the universality of the results. Furthermore, we did not establish a reference interval for ED-LC-MS/MS for FT in this study, which could impact the clinical application of this method. However, our research team is actively recruiting reference subjects to address this limitation.

In this research, a rapid and accurate ED LC-MS/MS serum FT method was developed and validated. FT levels in serum samples collected form 100 healthy controls and 139 PCOS patients were measured by calculated LC-MS/MS and newly-developed ED LC-MS/MS method. The method comparison results showed significant differences between the two FT methods. And ROC analysis demonstrated ED-FT is the best biomarker for PCOS diagnosis.

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Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest

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Tables

Table 1. Mobile-phase gradient of liquid chromatography

Time (min)	A (%)	Flow rate (mL/min)
0.00	48.0	0.4
4.50	65.0	
5.80	65.0	
5.81	100.0	
6.50	100.0	
6.51	48.0	
8.00	48.0	

Table 2. Parameters of the mass spectrometer

Parameters	Values
Source temperature	500 ℃

Curtain Gas		45 psi	
Collision Gas		8.0 psi	
IonSpray Voltage		1.9 KV	
Gas 1 pressure		75 psi	
Gas 2 pressure		70 psi	
Entrance potential		10 V	
Dwell time		80 msec	
Analyte	**Transitions, m/z**	**Collision energy, V**	**Collision cell exit potential, V**
T(quantification)	289.2-97.0	29.5	11.5
T (confirmation)	289.2-109.0	35.0	6.5

T-$^{13}C_3$ (quantification)	292.2-100.0	32.0	6.0
T-$^{13}C_3$ (confirmation)	292.2-112.0	34.0	6.0

Table 3. The results of intra-assay (n=20) and inter-assay (n=10×2) imprecision of FT.

Sample	Intra-assay imprecision		Inter-assay imprecision	
	$\bar{x} \pm s$, pmol/L	CV, %	$\bar{x} \pm s$, pmol/L	CV, %
DQC	6.79 ± 0.209	3.1	6.48 ± 0.233	3.6
	48.3 ± 0.835	1.8	54.6 ± 1.43	2.7
	456 ± 10.4	2.3	425 ± 13.3	3.2
SQC	8.49 ± 0.311	3.7	9.00 ± 0.528	5.9
	45.1 ± 1.70	3.8	43.6 ± 1.92	4.4
	299 ± 9.89	3.4	295 ± 11.1	3.8

Table 4. Statistical parameters of the two FT methods comparisons.

Group	Subjects	Spearman's correlation coefficient	Passing-Bablok regression	Bland-Altman (difference)

	number	R (95%CI)	P value	Slope (95%CI)	Intercept (95% CI)	95% CI of biases
Total subjects	239	0.983 (0.979 to 0.987)	<0.0001	1.2906 (1.2552 to 1.3213)	3.0412 (2.5458 to 3.4074)	9.3354 (8.5603 to 10.1105)
PCOS patients	139	0.974 (0.964 to 0.981)	<0.0001	1.2334 (1.1875 to 1.2846)	4.6702 (3.5193 to 5.6813)	12.0418 (10.9418 to 13.1417)
Healthy controls	100	0.942 (0.915 to 0.961)	<0.0001	1.3136 (1.2353 to 1.4116)	2.5895 (1.6408 to 3.3411)	5.5735 (5.1568 to 5.9902)

Table 5. Method parameters of reported ED LC-MS/MS method for FT quantification.

Method	Sample volume	Sample preparation	Equilibration（+Derivatization） time	Linearity/LLMI	Precision	Accuracy
Our method	400 μL	ED+LLE	4 h	1.74 - 890	intra-: <	92.9% -

				pmol/L	3.8 % inter-: < 5.9 %	108.2%
Chen, et.al [9]	100 µL	ED+LLE	16 h	16 - 2500 pmol/L	intra-: < 3.6 % inter-: < 5.5 %	97.19% - 112.11%
Rhea, et.al [8]	200 µL	ED+Derivatization+ LLE	overnight	2.5–2500 pg/mL (8.675-8675 pmol/L)	intra-: < 9.8 % inter-: < 15.3 %	--
Fiers, et.al [7]	Male: 500 µL Female: 1000 µL	ED+SPE	24 h	2.4 pmol/L	--	--
Schuijt, et.al [14]	1000 µL	ED+SPE	5 h	6 pmol/L	--	--

Huang, et.al [24]	300 µL	ED+Derivatization+ LLE	5 h	1.5-1000 pg/mL (5.2-3467 pmol/L)	intra-: < 8.2 % inter-: < 6.7 %	96.1%–108.1%

Abstract

Objective: To compare the calculated and the rapid equilibrium dialyzed (ED) human serum free testosterone (FT) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to explore their performances in polycystic ovary syndrome (PCOS) diagnosis.

Methods: A rapid ED LC-MS/MS method for serum FT was established and validated for linearity, lower limit of the measuring interval (LLMI), imprecision, trueness, stability, dilution, matrix specificity and carryover. The validated ED LC-MS/MS (ED-FT) was compared with calculated LC-MS/MS method from Vermeulen's formula (cFT) for FT measurement in 139 PCOS patients and 100 healthy controls. The performances of total testosterone (TT), ED-FT and cFT by LC-MS/MS in PCOS diagnosis were investigated with the same cohorts.

Results: The linearity range of ED-FT was 1.74 - 890 pmol/L, with a LLMI at 1.74 pmol/L. The intra-assay and inter-assay imprecision were < 3.8% and < 5.9%. The trueness was acceptable with recoveries of 92.9% - 108.2%. The equilibrium dialysis time was 4 h. The two FT methods displayed systematic and proportional differences and cFT showed significant positive deviations compared to ED-FT. Receiver operating characteristic curve analysis proved that ED-FT outperformanced in PCOS diagnosis with the an area under the curve at 0.973, sensitivity of 89.93% and specificity of 96.00%.

Conclusions: This study established a rapid, accurate and sensitive ED LC-MS/MS method for serum FT. ED-FT is optimal compared to TT and cFT by LC-MS/MS in PCOS diagnosis.

Figures for

The calculated and the rapid equilibrium dialyzed human serum free testosterone by LC-MS/MS and their performances in PCOS diagnosis

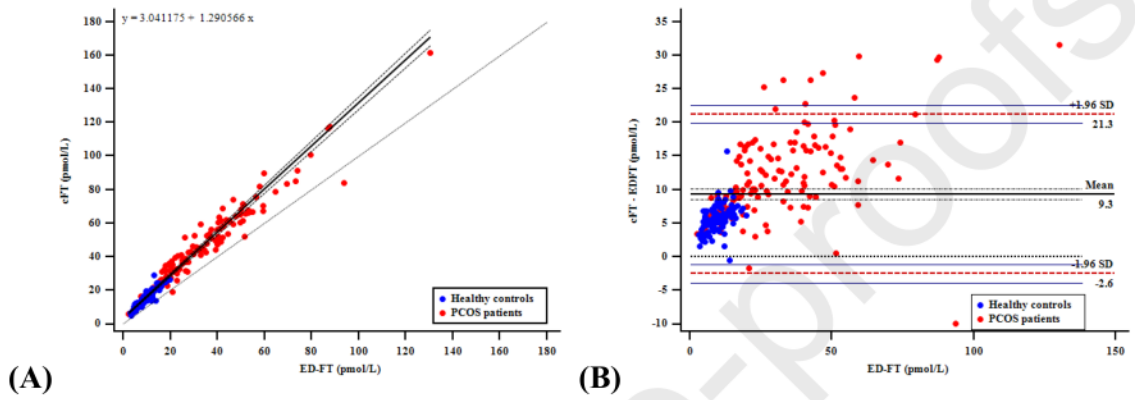
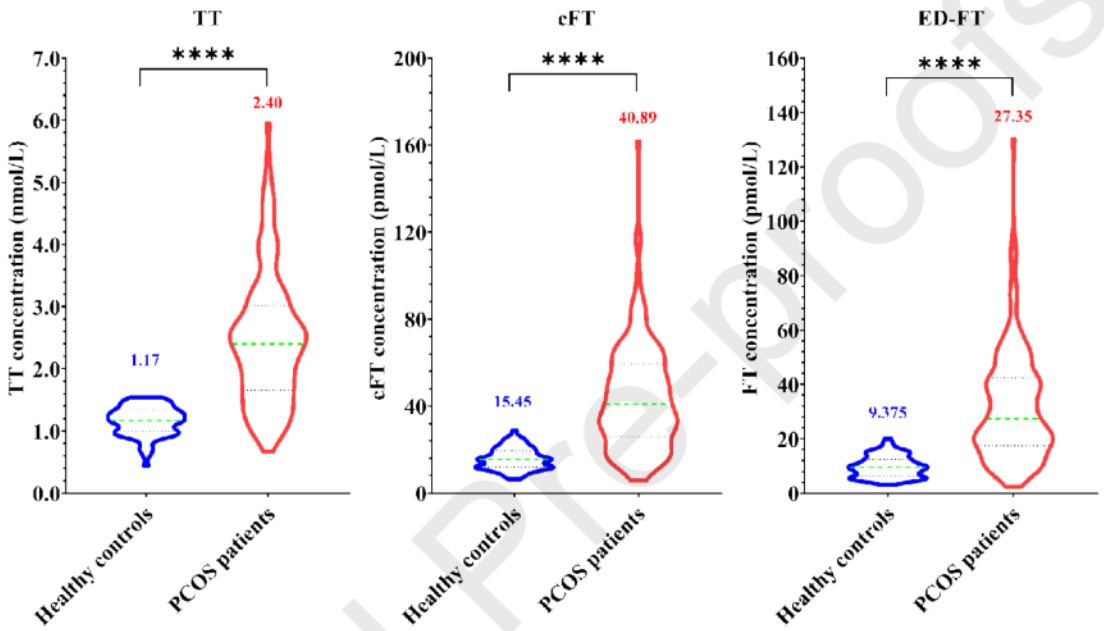


Figure 1. Comparison between cFT by LC-MS/MS and ED-FT by LC-MS/MS in 239 total subjects, 100 healthy controls as well as 139 PCOS patients. Passing-Bablok regressions (A) and bland-Altman plots (B). Red dots represent PCOS patients samples (n=139) and blue dots represent healthy controls samples (n=100).



****: statistically significant, $P < 0.05$.

Figure 2. Mann-Whitney test for TT, cFT, and ED-FT levels in 100 healthy controls and 139 PCOS patients.

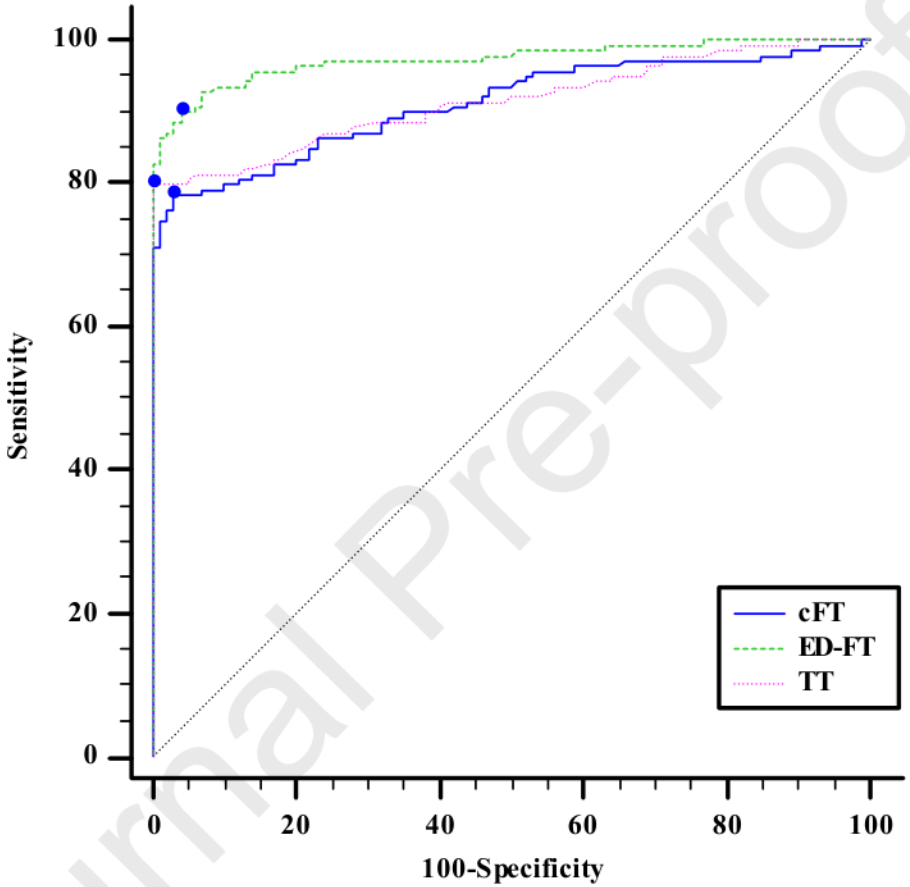


Figure 3. Receiver operating characteristic (ROC) analysis of 139 PCOS patients compared with 100 healthy subjects as control. ROC curves to analyze the diagnostic value of TT, cFT and ED-FT.

Highlights

- ✧ A rapid and sensitive ED LC-MS/MS method was developed for serum free testosterone to accommodate female samples.
- ✧ The validated method was compared with calculated method for free testosterone by LC-MS/MS in 139 PCOS patients and 100 healthy controls.
- ✧ Serum free testosterone by the validated ED LC-MS/MS showed a great diagnostic performance in PCOS diagnosis.

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