Research Article   **Open Access**

Determination of Serum Testosterone Levels with Liquid Chromatography-Isotope Dilution Tandem Mass Spectrometry and Comparision with Other Immunoassays

Celik HT[1]*, Serdar MA[2], Abusoglu S[4], Sezer S[1], Ozdemir S[1], Ozdemir O[3], Soydas R[1], Bakir F[1], Gungor Torun O[1], Turhan T[1] and Yildirimkaya M[1]

[1]Department of Biochemistry, Ankara Numune Training and Education Hospital, Ankara, Turkey
[2]Department of Biochemistry, Gulhane Military Medicine Academy Hospital, Ankara, Turkey
[3]Department of Obstetrics and Gynecology, Ankara Numune Training and Education Hospital, Ankara, Turkey
[4]Biochemistry Department, Ankalab Laboratories, Ankara, Turkey

Abstract

Background: In clinical laboratories, Determination of serum testosterone levels in routine is challenging. Reliable analysis of bioavailable testosterone is difficult.

Methods: After liquid-liquid extraction, Shimadzu Prominence LC unit coupled to API 3200 mass spectrometer with atmospheric pressure chemical ionisation was used to quantify serum testosterone levels. Serum testosterone results taken from tandem mass spectrometry were compared with Roche, Beckman and Abbott commercial automated testosterone immunoassays

Results: In LC-MS/MS method, linearity for testosterone was 0.005-15.915 ng/mL. Recovery was 97-108% for 0.06-16 ng/mL concentration range. Intraday and inter-day CVs were 6.3,9,7; 2,2,8.7 and 1.3,0.3 for low (0.969 ng/mL), intermediate (4.816 ng/mL) and high (8.084 ng/mL) testosterone concentrations, respectively. There was a good correlation between commercial immunoassays. In method comparision, agreement was closest with Roche and LC-MS/MS method (r=0.9670).

Discussion: In contrast to commercial testosterone immunoassays, LC-MS/MS method is a reliable, simple and useful method for clinical laboratory practices.

Keywords: Testosterone; LC-MS/MS; Comparision; Automated immunoassays

Introduction

In adrenal cortex, placenta and gonads, testosterone is produced from the cholesterol precursor. Mitochondria and smooth endoplasmic reticulum have a major function for this production. Testosterone has three main forms in circulation: Tightly bound to sex hormone binding globulin (SHBG), bound loosely to albumin and free fraction. The free fraction is about 1-2% of total testosterone and this free form is active through the target gene expression [1].

Determination of testosterone levels is useful for investigation of androgen-producing tumors, anti-androgen therapy in prostate cancer and testicular functions in men [2]. It is also an informative marker for congenital adrenal hyperplasia and sex assignment in newborns [3]. For women, testosterone concentrations in circulation accounts 10-15% of men. Testosterone has physiological properties like maintaining bone mineral density, mood, libido and measurement of serum testosterone concentrations gives valuable clinical information for clinical conditions like hirsutism, alopecia, acne, oligo-amenorrhea presenting with hyperandrogenism [4]. For determining sex steroids, radioimmunoassay (RIAs) is inexpensive and requires minimum sample preparation. Although RIA kits which have extraction procedures or directly measuring testosterone levels compare well with LC-MS/MS and GC-MS, results obtained from studies performed with these kits do not always compare well with LC-MS/MS due to testosterone binding globulins and structurally similar molecules in serum [5]. Because of less interference, GC-MS and LC-MS are taken into consideration as gold standard method [6].

For excessive production or deficiency, measurement of serum or plasma testosterone is performed using automated immunoassays in most clinical laboratories. Commercial immunoassays are rapid, cost-effective and simple. Because of these properties they become widely used in clinical quantification of steroids. But there is a technical difficulty for measurement of testosterone especially in low concentrations [7]. Mostly used techniques measuring testosterone have some disadvantages like low precision, cross-reactivity, limited linear range, poor correlation. Relatively low circulating testosterone concentrations especially in children and women lead to limited precision, unacceptable cross-reactivity and result with positive bias [8].

For these reasons we described performance characteristics and established LC-MS/MS method to our laboratory for clinical practice.

Materials and Methods

Study design

Patients: Blood samples were taken among the patient samples sent to the laboratory at Ankara Numune Education and Training Hospital,

Ankara, Turkey, and centrifuged at 3000 g for 4 minutes to prepare three different serum pools. The first pool was prepared from female patients(aged between 20-30 years) who had low levels of testosterone aged between 20-30 years, second was from high levels of testosterone from men. Third was from intermediate levels of testosterone from geriatric men patients aged over 65 years. These serum pools divided into 20 aliquots and stored at -20°C until analysis. Serum testosterone levels were performed from prepared pools four times a day (Two times in the morning, two times in the afternoon). These study plan was designed for 20 days. Institutional review board approval was obtained from Ankara Numune Education and Training Hospital, Turkey.

Materials

Testosterone (1 mg/mL), d[5]- testosterone and Phenomenex Luna C8 HPLC column 50x2.1 mm (5 µm) were purchased from Sigma-Aldrich (St. Louis) and Agilent (USA), respectively. HPLC grade water, methanol, methyl tertbutyl ether (MTBE) and heptane were purchased from Merck (Darmstadt, Germany).

Sample preperation: After centrifugation at 3000 g for 4 minutes, 250 µL sample, standart and control were taken into eppendorf tubes. 3 µL internal standart was added to all samples (calibrator,control samples, patient samples). Testosterone was extracted by adding 1000 µL methyl tertbutyl ether (MTBE) to mixture, vortex-mixed for 2 minutes and centrifuged at 3000 g for 5 minutes. The upper organic layer was evaporated under nitrogen gas at 45°C. Then 500 µL 80% methanol-water and 1000 µL heptane were added,vortex-mixed for 2 minutes and upper layer was transferred to clean glass tube for evaporation under nitrogen gas at 45°C. 100 µL 50% methanol-water was added, vortex-mixed for a few seconds then transferred to autosampler vials for LC-MS/MS analysis. 30 µL was used as injection volume.

LC-MS/MS analysis: Determination of serum testosterone was performed with API 3200 mass spectrometer according to the method previously described by Chen et al. [7] in Ankalab Laboratories (Ankara,Turkey). Briefly, HPLC was conducted using Shimadzu Prominence LC unit in lineer gradient mode at a flow rate 1 mL/min through an Phenomenex Luna C8 column employing a mobile phase consisting of methanol-water (20/80, v/v) for 1 minute, increasing 100 % methanol over 5 minutes and maintained at 100% methanol for 2 minutes then reequilibrate for 2 minutes at methanol-water (20/80, v/v). API 3200 (Applied Biosystems/Sciex, Concord, ON, Canada) mass spectrometer coupled with an atmospheric pressure chemical ionization source was operated in the positive ion mode at 5 kV and 400°C source temperature. Testosterone and d[5]-testosterone were detected 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 data acquisition, peak area integration and calculation of analyte concentrations from standard curves. Statistical analysis was carried out by EP Evaluator 8 program (RHOADS, USA).

Results

Linearity

For determination of linearity, a commercial calibrator (Beckman Coulter) which has same properties with serum matrix was used. Linearity and mean values were calculated from serial dilution of the highest calibrator (16;8;4;2;1;0.5;0.25;0.125;0.06;0.03 ng/mL) (Figure 1).

Even when the concentration is 0,01 ng/mL, linearity is not affected (Table 1, Figure 2).

For accuracy and linearity test, "pass" criteria is defined as mentioned in testosterone assay performance goals [9].

Precision

Intra-day and inter-day precision of method was evaluated for different testosterone levels (low, intermediate and high). Intra-day CVs were 6,3%, 2.2% and 1.3% for 0,969 ng/mL (low level), 4,816 ng/mL (intermediate level) and 8,084 ng/mL (high level) testosterone concentrations, respectively. Inter-day CVs were 9,7%,8,7% and 0,3%
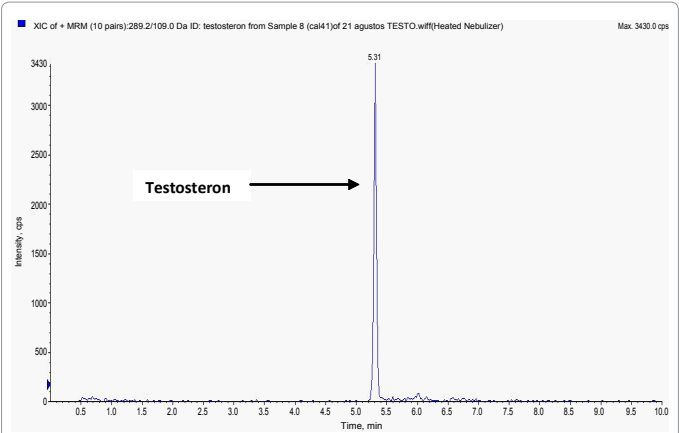

Figure 1: LC-MS/MS ion chromatogram of testosterone extracted from a patient sample.

	Assigned	N	Est	Mean	Residual	Linearity
0	0.01	1	0.0341	0.0110	-0.0231	Pass
1	0.03	1	0.0541	0.0400	-0.0141	Pass
2	0.06	1	0.0839	0.0650	-0.0189	Pass
3	0.125	1	0.1487	0.1150	-0.0337	Pass
4	0.25	1	0.2732	0.2550	--0.0182	Pass
5	0.5	1	0.5223	0.5150	-0.0073	Pass
6	1	1	1.0205	0.9700	-0.0505	Pass
7	2	1	2.0168	2.0000	-0.0168	Pass
8	4	1	4.0095	4.0600	0.0505	Pass
9	8	1	7.9948	7.9750	-0.0198	Pass
10	16	1	159655	15.9150	-0.0505	Pass

Table 1: Shows the values for 0,01-16 ng/mL testosterone concentrations.
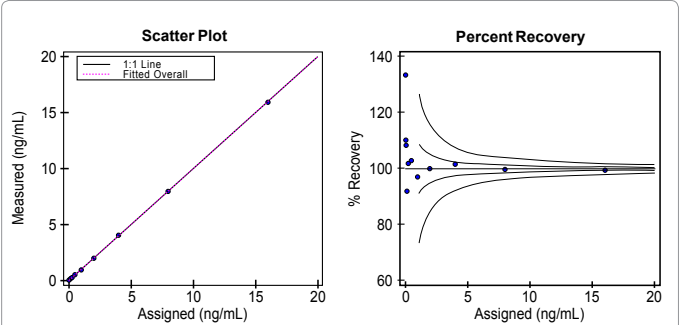

Figure 2: Linearty and recovery graphics for 0,01-16 ng/mL testosterone concentrations.

for 0,969 ng/mL (low level), 4,816 ng/mL (intermediate level) and 8,084 ng/mL (high level) testosterone concentrations, respectively.

Table 2 shows CV and SD values for testosterone concentrations.

Coefficient of variation for low testosterone concentrations is 6,7% and standart deviation is 0.062 (Table 2, Figure 3.1).

CVs and for intermediate and high testosterone concentrations are 2.6% and 2.8%, respectively. The standart deviation for intermediate and high testosterone concentrations are 0.107 and 0.229, respectively (Figure 3.2 and 3.3).

Method comparison

For method comparision, 60 patients (15 newly diagnosed

	Low		Intermediate		High	
	CV (%)	SD	CV (%)	SD	CV (%)	SD
Within run	6,3	0,062	2,2	0,107	1,3	0,106
Between run	6,7	0,065	2,6	0,123	2,8	0,229
Between day	9,4	0,091	8,7	0,420	0,3	0,02
total	13,2	0,128	9,3	0,450	3,1	0,253

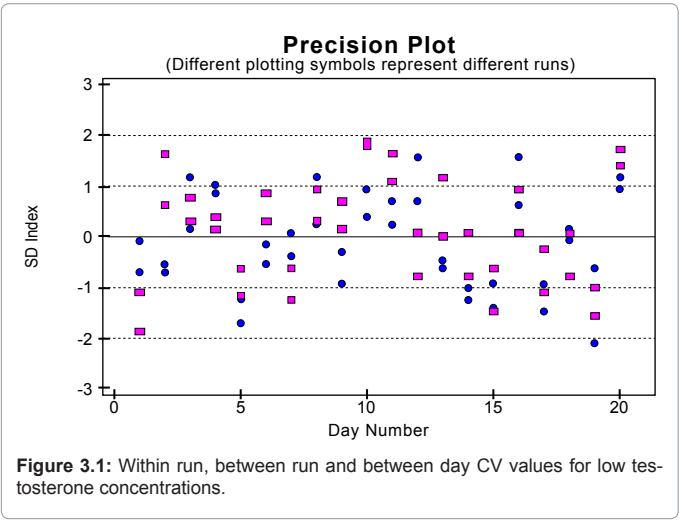**Table 2:** CV and SD values for testosterone.

Figure 3.1: Within run, between run and between day CV values for low testosterone concentrations.
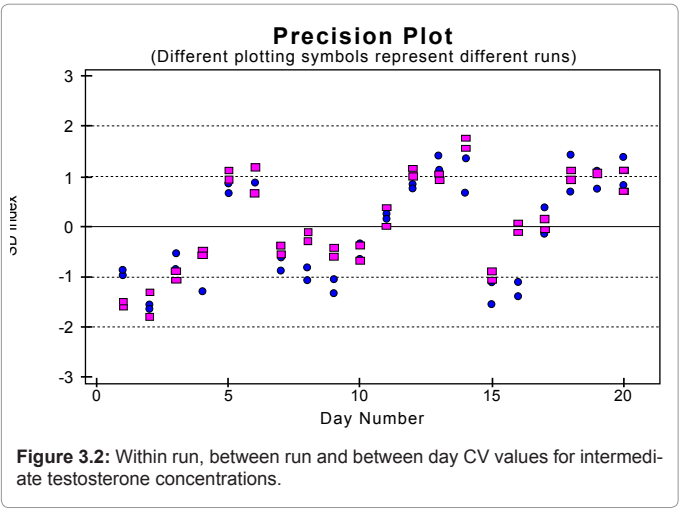

Figure 3.2: Within run, between run and between day CV values for intermediate testosterone concentrations.

polycystic ovary syndrome patients, 45 control) aged between 20-30 years were included in this study. 15 Controls aged between 20-30 and the last 15 were over 65 years. Serum samples were stored at -80°C until analysis.

Testosterone determination from all samples were performed by LC-MS/MS, Beckman Coulter Unicell DXI-800 (chemiluminesansce microparticule immunassay), Roche Modular Analytics E-170 (electrochemiluminesansce), Abbott Architect CMIA (chemiluminesansce microparticule immunassay).

In method comparision with EP Evaluator Release 8 CLSI EP 9 program correlation coefficents were 0,9483, 0,9221, 0,9670, 0,9742, 0,9722, 0,9894 for LC-MS/MS-Abbott, LC-MS/MS-Beckman, LC-MS/MS-Roche, Beckman-Abbott, Beckman-Roche and Abbott-Roche, respectively.

Recovery

Results for recovery and method comparision were analyzed from EP Evaluator Release 8 program are shown at Table 3, Figure 4.1, 4.2, 4.3 and 4.4 respectively.

Correlation coefficients were analyzed with Pearson correlation.

Discussion

Reference method for steroid measurement is isotope-dilution GC-MS. Published methods using LC-MS and GC-MS require high sample volumes [10,11]. Our study allows simple, useful analysis of low level of testosterone in women by short analysis time (10 minutes) and less sample volume (250uL).
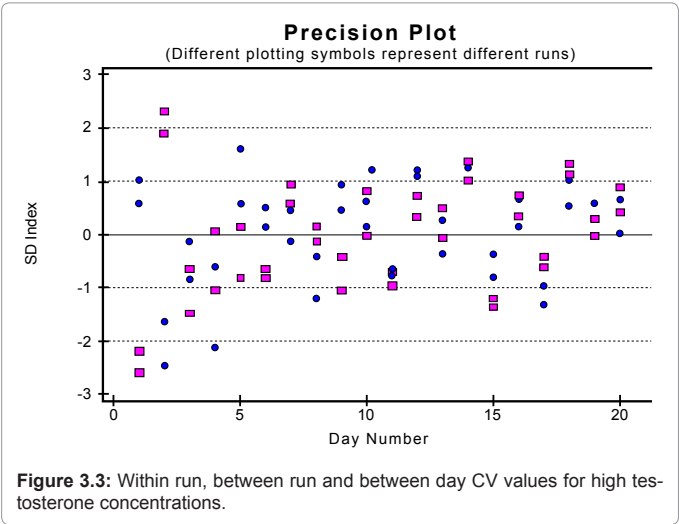

Figure 3.3: Within run, between run and between day CV values for high testosterone concentrations.

|	Assigned	N	Est	Mean	Recovery	Status
0	0.01	1	0.0341	0.0110	110.0	Pass
1	0.03	1	0.0541	0.0400	133.3	Pass
2	0.06	1	0.0839	0.0650	108.3	Pass
3	0.125	1	0.1487	0.1150	92.0	Pass
4	0.25	1	0.2732	0.2550	102.0	Pass
5	0.5	1	0.5223	0.5150	103.0	Pass
6	1	1	1.0205	0.9700	97.0	Pass
7	2	1	2.0168	2.0000	100.0	Pass
8	4	1	4.0095	4.0600	101.0	Pass
9	8	1	7.9948	7.9750	99.7	Pass
10	16	1	159655	15.9150	99.5	Pass

**Table 3:** Recovery for 0.01-16 ng/mL testosterone concentrations.
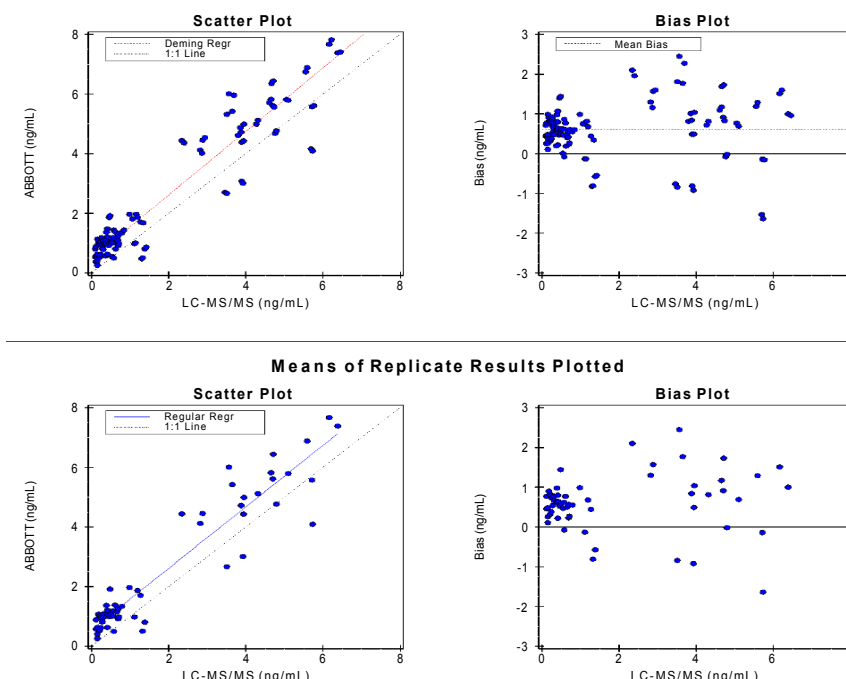
Figure 4.1: A. Distrubition of testosterone results with LC-MS/MS and Abbott Architect; B. Bias % of LC-MS/MS testosterone results; C. Mean values of testosterone results; D. Bias % of mean testosterone results
With regression analysis; equation is y= 0.4888+1.066x (R= 0.9483).
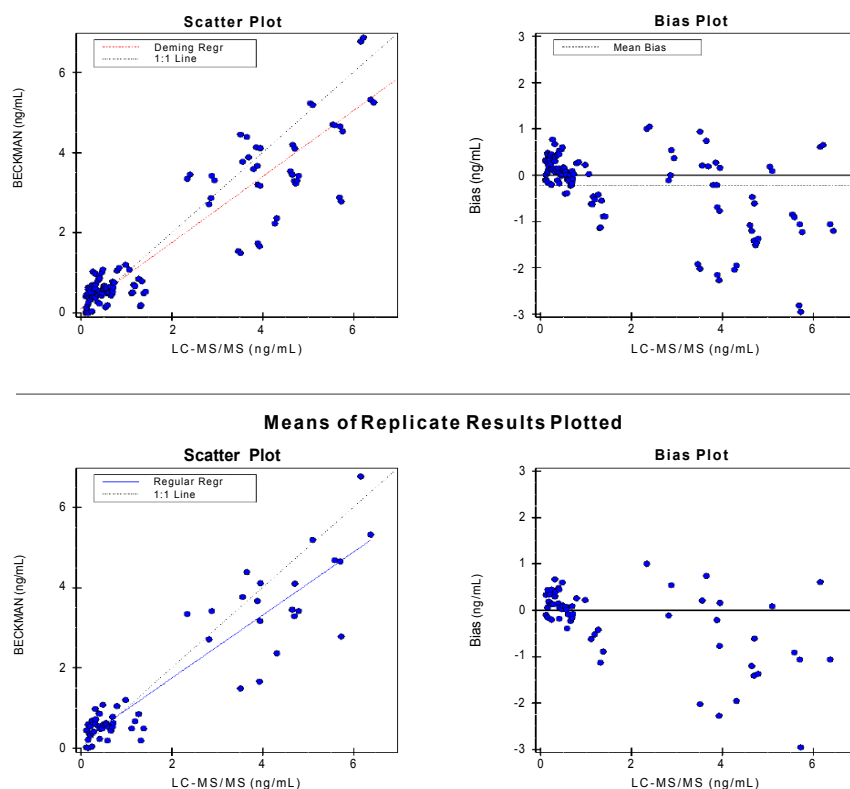
Figure 4.2: A: Distrubition of testosterone results with LC-MS/MS and Beckman Unicell DXI; B. Bias % of LC-MS/MS testosterone results; C. Mean values of testosterone results; D. Bias % of mean testosterone results.
With regression analysis; equation is y=0.122+0.822x (R= 0.9221).
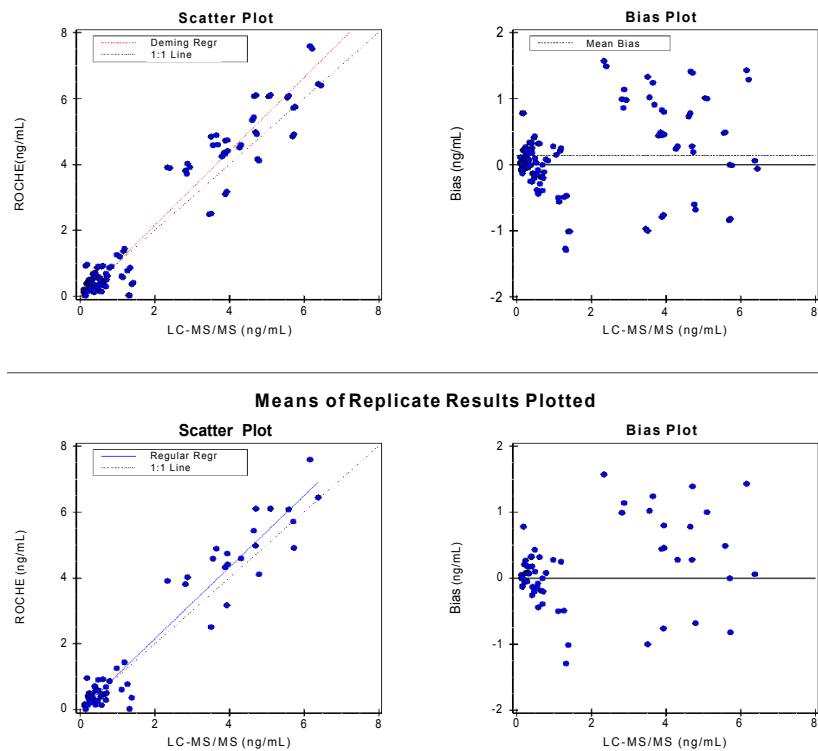
Figure 4.3: A. Distribution of testosterone results with LC-MS/MS and Roche E-170; B. Bias % of LC-MS/MS testosterone results; C. Mean values of testosterone results D. Bias % of mean testosterone results
With regression analysis; equation is y=-0.085+1.121x (R= 0.9670).
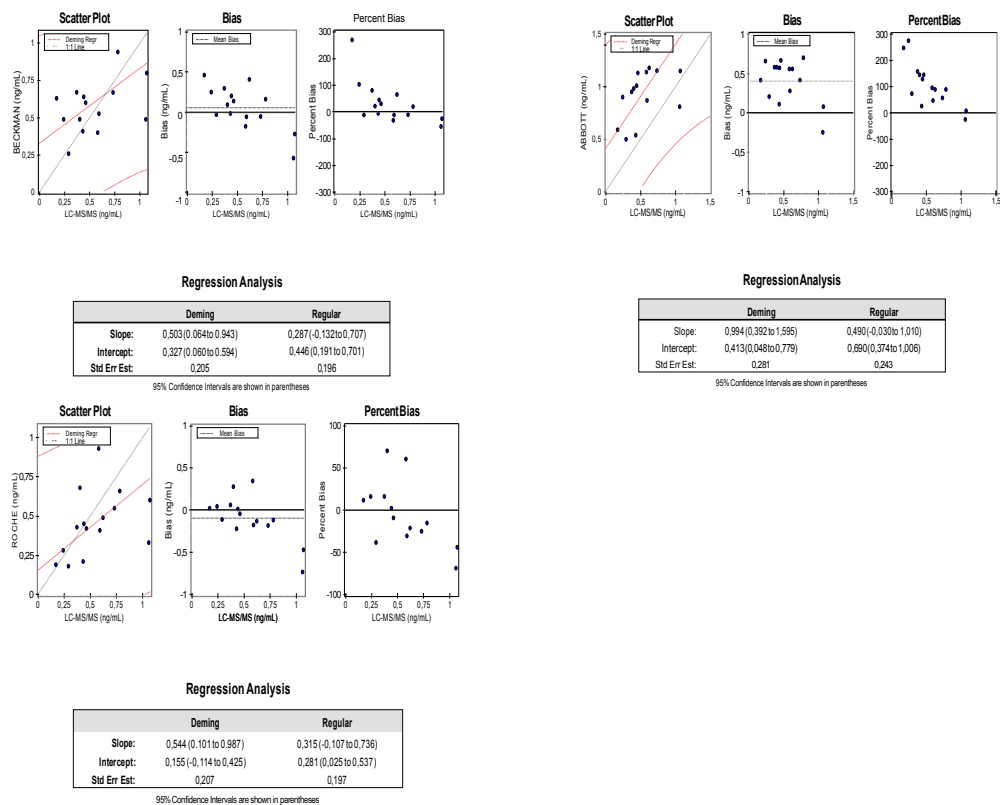
Figure 4.4: A. Comparision of PCOS group (<1 ng/mL) testosterone results with LC-MS/MS and commercial kits' results.

There are differences between testosterone methods and assays measuring testosterone levels without extraction have high interassay variability [12]. Commercial assays directly measuring plasma or serum testosterone have a positive bias [13-15]. Because of low concentrations of testosterone in women and children and limited sample volume in pediatric population, it is especially important for samples taken from newborns and women [16]. Also difference between the commercial assays which use iodine-labeled testosterone was reported [12]. Radioimmunassays using tritium-labeled testosterone and a binding globulin use extraction and chromatographic seperation gives lower results in children serum [17] and concentrations are consistent with ID/GC-MS [13,18,19].

Intra-day and inter-day precision of method was evaluated for three levels of testosterone (low, intermediate and high), Intra-day CVs were 6,3%,2.2% and 1.3% for 0,969 ng/mL (low level), 4,816 ng/mL (intermediate level) and 8,084 ng/mL (high level) testosterone concentrations, respectively. Inter-day CVs were 9,7%,8,7% and 0,3% for 0,969 ng/mL (low level), 4,816 ng/mL (intermediate level) and 8,084 ng/mL (high level) testosterone concentrations, respectively.

These results are comparable with the other studies. Chen et al. [1] reported intra-day CVs as 4,5%, 2,4%, 1,5% and total CVs as 5,2%, 4,3%, 1,9% at 0,37 ng/mL (low level), 3,17 ng/mL (intermediate level) and 6,62 ng/mL (high level) testosterone concentrations, respectively.

Also, Ravinder [20] reported intra-day CVs as 11,6%, 5,3%, 2,5% and total CVs as 18,8%, 10,7%, 5,9% at 0,007 ng/mL (low level), 0,52 ng/mL (intermediate level) and 8,3 ng/mL (high level) testosterone concentrations, respectively.

Linearity of testesterone was 0,005-15,915 ng/mL in our study . Yu Chen, et al., Martin Blasco, et al. and Ravinder J. Singh [1,6,20] reported the linearity as 0, 01-23, 05, 0, 3-50, 0-10 ng/mL, respectively.

Recovery of testesterone was 97-100% for 0,06-16 ng/mL and 97-108% for 0,03 ng/mL testosterone concentration in our study. Kushnir et al. [21] Shiraishi et al. [22] Ravinder J. Singh [20] reported the recovery as 98%, 100-110%, 97% ng/mL, respectively.

Ogibene et al. [23] compared three non-isotopic RIA methods for determination of testosterone in adult population. Two methods give different results in the lower concentrations of dynamic range when compared with ID/GC-MS. Consistent method has limitations for measurement of testosterone in children and women.

Also, Jockenho et al. [24] reported commercially available testosterone kits give higher results at lower testosterone concentrations.

All commercial testosteron assays have a good correlation when compared with each other. However, they lack sensitivity and specifity in comparision with ID/GC-MS.

Roche Modular Systems E-170 testosterone assay has the best correlation in comparision with LC-MS/MS. We believe our method have advantages like short analysis time (10 minutes), minimum sample volume (250 uL).

Wang et al reported a similiar sample pretreatment as our study with a higher required sample volume (2 mL vs 250 uL) [7].

This method is useful for testosterone determination especially from women, children serum or late-onset hypogonadism mens' samples in clinical laboratories.

Some automated platform immunoassays are more accurate than others and the RIA is considered a more reliable immunoassay. However, the same holds for different LC-MS/MS methods. Validation between LC-MS/MS methods is necessary, preferably by comparison with a reference method.

Acknowledgments

References

1. Chen Y, Yazdanpanah M, Wang XY, Hoffman BR, Diamandis EP, et al. (2010) Direct measurement of serum free testosterone by ultrafiltration followed by liquid chromatography tandem mass spectrometry. Clin Biochem 43: 490-496.

2. Yeap BB (2009) Testosterone and ill-health in aging men. Nat Clin Pract Endocrinol Metab 5: 113-121.

3. Burtis CA, Tietz NW, Ashwood ER, Bruns DE (2006) Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. (4th edn), Elsevier Saunders, USA.

4. Lee S, Kwon S, Shin HJ, Park J, Lim HS, et al. (2010) Quantitative measurement of salivary testosterone in Korean adults by stable isotope-dilution liquid chromatographyelectrospray- tandem mass spectrometry. BMB repo 43: 761-765.

5. Cawood ML, Field HP, Ford CG, Gillingwater S, Kicman A, Cowan D, et al. (2005) Testosterone measurement by isotope-dilution liquid chromatography–tandem mass spectrometry: validation of a method for routine clinical practice. Clin Chem 51: 1472-1479.

6. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H (2007) Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 92: 405-413.

7. Chen Y, Yazdanpanah M, Hoffman BR, Diamandis EP, Wong PY (2009) Rapid determination of serum testosterone by liquid chromatography-isotope dilution tandem mass spectrometry and a split sample comparison with three automated immunoassays. Clin Biochem 42: 484-490.

8. Blasco M, Carriquiriborde P, Marino D, Ronco AE, Somoza GM (2009) A quantitative HPLC-MS method for the simultaneous determination of testosterone, 11-ketotestosterone and 11-beta hydroxyandrostenedione in fish serum. J Chromatogr B Analyt Technol Biomed Life Sci 877: 1509-1515.

9. Kaplan LA, Pesce AJ (2010) Clinical Chemistry. (5th edn), Mosby/Elsevier, USA.

10. Kashiwagi B, Shibata Y, Ono Y, Suzuki R, Honma S, et al. (2005) Changes in testosterone and dihydrotestosterone levels in male rat accessory sex organs, serum, and seminal fluid after castration: establishment of a new highly sensitive simultaneous androgen measurement method. J Androl 26: 586-591.

11. Hsing AW, Stanczyk FZ, Belanger A, Schroeder P, Chang L, et al. (2007) Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. Cancer Epidemiol Biomarkers Prev 16: 1004-1008.

12. Boots LR, Potter S, Potter D, Azziz R (1998) Measurement of total serum testosterone levels using commercially available kits: high degree of between-kit variability. Fertil Steril 69: 286-292.

13. Fuqua JS, Sher ES, Migeon CJ, Berkovitz GD (1995) Assay of plasma testosterone during the first six months of life: importance of chromatographic purification of steroids. Clin Chem 41: 1146-1149.

14. Wudy SA, Wachter UA, Homoki J, Teller WM (1995) 17 alpha-hydroxyprogesterone, 4-androstenedione, and testosterone profiled by routine stable isotope dilution/gas chromatography-mass spectrometry in plasma of children. Pediatr Res 38: 76-80.

15. Wheeler MJ, Lowry C (1987) Warning on serum testosterone measurement. Lancet 2: 514-515.

16. Fitzgerald RL, Herold DA (1996) Serum total testosterone: immunoassay compared with negative chemical ionization gas chromatography– mass spectrometry. Clin Chem 42: 749-755.

17. Taieb J, Mathian B, Franc¸ Millot F, Patricot MC, Mathieu E, et al. (2003) Testosterone measured by 10 immunoassays and by isotope-dilution gas

chromatography–mass spectrometry in sera from 116 men, women, and children. Clin Chem 49: 1381-1395.

18. Lashansky G, Saenger P, Fishman K, Gautier T, Mayes D, et al. (1991) Normative data for adrenal steroidogenesis in a healthy pediatric population: age- and sex-related changes after adrenocorticotropin stimulation. J Clin Endocrinol Metab 73: 674-686.

19. Forest MG (1989) Androgens in childhood. Karger, Switzerland, 104-129.

20. Singh RJ (2008) Validation of a high throughput method for serum/plasma testosterone using liquid chromatography tandem mass spectrometry (LC–MS/MS). Steroids 73: 1339-1344.

21. Kushnir MM, Rockwood AL, Roberts WL, Pattison EG, Bunker AM, et al. (2006) Performance Characteristics of a Novel Tandem Mass Spectrometry Assay For Serum Testosterone. Clin Chem 52: 120-128.

22. Shiraishi S, Lee PW, Leung A, Goh VH, Swerdloff RS, et al. (2008) Simultaneous Measurement of Serum Testosterone and Dihydrotestosterone by Liquid Chromatography–Tandem Mass Spectrometry. Clin Chem 54: 1855-1863.

23. Ognibene A, Drake CJ, Jeng KY, Pascucci TE, Hsu S, et al. (2000) A new modular chemiluminescence immunoassay analyser evaluated. Clin Chem Lab Med 38: 251-260.

24. Jockenhovel F, Haase S, Hoermann R, Mann K (1996) New automate direct chemiluminescent immunoassay for the determination of serum testosterone. J Clin Ligand Assay 19: 138-144.

25. Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS (2004) Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 89: 534-543.