Quantification of Testosterone in Serum by Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Clinical Research

Authors

Vasanta Putluri[1], Sriramya Donepudi[1], Feng Jin[1], Andre Szczesniewski[2], Vadiraj Bhat[2], Peter J Cook[1], Dolores J. Lamb[1], Arun Sreekumar[1], and Putluri Nagireddy[1]

[1] Dan L. Duncan Cancer Center, Department of Molecular and Cellular Biology, Alkek Center for Molecular Discovery, Baylor College of Medicine, Houston, TX, USA

[2] Agilent Technologies, Inc. Wood Dale, IL

Abstract

An ultra-sensitive quantitative analytical method is required for measuring the concentration of total and free testosterone found in men, and particularly the low levels found in women and children. Therefore, an analytical method was developed on an Agilent 6495 Ion Funnel Mass Spectrometer to quantify and characterize testosterone in serum, and to ascertain the most appropriate analytical method for laboratory use.

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Introduction

Testosterone (T) is an androgenic sex hormone that is responsible for the development of the male genitalia, and also has secondary sexual characteristics as the estrogen precursor in females. It exerts anabolic effects, and influences behavior in both genders. Circulating testosterone is bound to the sex hormone binding globulin (SHBG) while a small fraction is albumin-bound, and another small proportion exists as free hormone. The non-SHBG-bound free testosterone is the biologically active component since serum albumin-bound testosterone can dissociate freely.

Experimental

Chemicals and reagents

Human serum, used for matrix-matched calibrators, was from Golden West Biological Inc. (Temecula, CA). Standards and internal standards were bought from Sigma-Aldrich (St Louis, MO). Sample preparation and LC solvents were purchased from Burdick & Jackson (Morristown, NJ).

Sample preparation

Samples, calibrator, and quality control (QC) materials in matrix were prepared using the following procedure.

1. 100 µL of sample and 5 µL of 5 ng/mL internal standard was inserted into a tube.
2. 2 mL of HPLC grade 3:2 ethyl acetate:hexane was added to each tube, and vortexed for 2 minutes.
3. The organic layer was transferred to another clean tube, and 500 µL of 0.1 mol/L NaOH was added.
4. The organic layer was collected and dried down under nitrogen at room temperature.
5. Once dry, the samples were reconstituted in 100 µL of 70:30 water:methanol for analysis.

Data analysis

System control and data acquisition were performed by Agilent MassHunter Acquisition Software (B.07). Data were analyzed using Agilent MassHunter Quantitative Analysis Software (B.07).

LC Configuration and parameters

Configuration		
Agilent 1290 Infinity II high speed pump (G7120A)		
Agilent 1290 Infinity II multisampler (G7167B)		
Agilent 1290 Infinity II multicolumn thermostat (G7116B)		
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 mm (p/n 695775-902)	
Column temperature	50 °C	
Injection volume	10 µL	
Mobile phase A	0.1 % Formic acid in water	
Mobile phase B	0.1 % Formic acid in methanol	
Flow rate	0.3 mL/min	
Gradient	Time (min)	%B
	0.0	40
	5.0	70
	5.1	98
	7.0	98
	7.1	40

MS/MS Configuration and parameters

Configuration	
Agilent 6495 Triple Quadrupole LC/MS with Agilent Jet Stream	
MS/MS mode	MRM
Ion mode	Positive
Drying gas temperature	260 °C
Drying gas flow	11 L/min
Nebulizer pressure	30 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Capillary voltage	4,000 V
EMV	500 V
Nozzle voltage	2,000 V
Q1/Q2 resolution	0.7/0.7 Unit
Dwell time	80 msec
Fragmentor voltage	380 V
Cell accelerator voltage	3
Ion funnel low pressure RF	100
Ion funnel high pressure RF	110

Compound	Precursor ion	Quantifier ion	Qualifier ion	Collision energy (V)	RT (min)
Testosterone	289.2	109	97	28/24	2.455
Testosterone $^{13}C_3$	292.2	112	100	28/20	2.453

Results and Discussion

Linearity

Calibration curves gave mean of coefficient of determination (R^2) values greater than 0.998 for total T over the 1–1,000 pg/mL range using linear regression fitting and 1/x weighting, ignoring the origin (Figure 1). The limit of quantitation (LOQ) was determined to be 5 pg/mL, with a 10:1 signal-to-noise (S/N) determination.

Accuracy and precision

The study produced outstanding results, as shown by the summary in Table 1. Six levels of QC were analyzed five times in a single run for 4 days. Table 1 shows daily accuracies and overall precision. Accuracy for all QCs was 75–125 %, and %precision <13 %.

Method comparison

The LC/MS/MS analytical method was compared to a previously validated ELISA method to ensure accuracy of results. Twenty-five confidential patient samples were extracted and analyzed using the presented LC/MS/MS analytical method as well as the ELISA method. Table 2 presents the results (ng/dL) and percent difference between the two methods. The R^2 was >0.96, as shown in Figure 2.

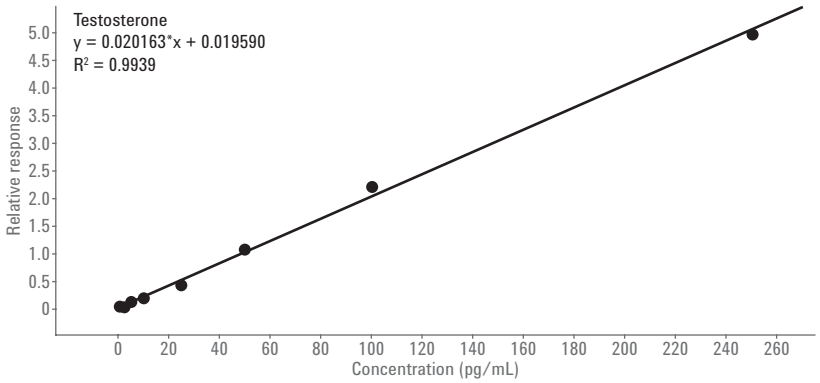

Figure 1. Selected calibration curves from 5–1,000 pg/mL testosterone.

Table 1. Accuracy and precision measurements of testosterone over 4 days.

	Day 1		Day 2		Day 3		Day 4		
Expected conc.	Calculated conc. (pg/mL)	Accuracy	Calculated conc. (pg/mL)	Accuracy	Calculated conc. (pg/mL)	Accuracy	Calculated conc. (pg/mL)	Accuracy	Precision (%)
25	31.3	125.2	27	108.1	26.5	106.1	25.7	102.9	9
50	46.7	93.4	42.6	85.2	39.3	78.6	45.5	91.1	7.6
100	74.6	74.6	93	93	75	75	94	94	12.8
250	232	92.8	229.9	92	202.8	81.1	223.3	89.3	6
500	442	88.4	479.7	95.9	593.9	118.8	498.6	99.7	12.9
1,000	1,036	103.6	1,015	101.5	986.1	98.6	1,008	100.8	2

Table 2. Testosterone correlations of serum samples between LC/MS/MS and ELISA.

Serum sample	LC/MS/MS Conc. (ng/dL)	Elisa value (ng/dL)	% Diff.
1	712.5366	689	103 %
2	688.1343	697	99 %
3	158.2041	165	96 %
4	107.0431	122	88 %
5	995.1148	1024	97 %
6	1734.5629	1582	110 %
7	560.4065	572	98 %
8	130.7686	147	89 %
9	63.4752	81	78 %
10	1484.4782	1228	121 %
11	71.49831763	73	98 %
12	722.5537	768	94 %
13	124.6216	136	92 %

Serum sample	LC/MS/MS Conc. (ng/dL)	Elisa value (ng/dL)	% Diff.
14	2010.3385	1586	127 %
15	32.0723	39	82 %
16	430.8323	429	100 %
17	884.159	899	98 %
18	330.8751	322	103 %
19	602.7057	885	68 %
20	1146.4567	981	117 %
21	1096.0418	1056	104 %
22	104.4476	73	143 %
23	94.3172	94	100 %
24	629.4994	640	98 %
25	410.7574	379	108 %

Instrument comparison

Two Agilent 6495 Triple Quadrupole LC/MS instruments were compared by analyzing the same samples on both instruments. The R^2 value was >0.96, as shown in Figure 3.

Conclusion

The data indicate that this extraction procedure and LC/MS/MS analytical method are able to carry out the required quantitation of low levels of total testosterone. One limitation was the presence of low level testosterone and other interfering compounds in the purchased matrix material, which impeded the ability of the analytical method to detect lower levels of testosterone. Excellent linearity of calibration curves was achieved, as well as reproducible, accurate, and precise data.

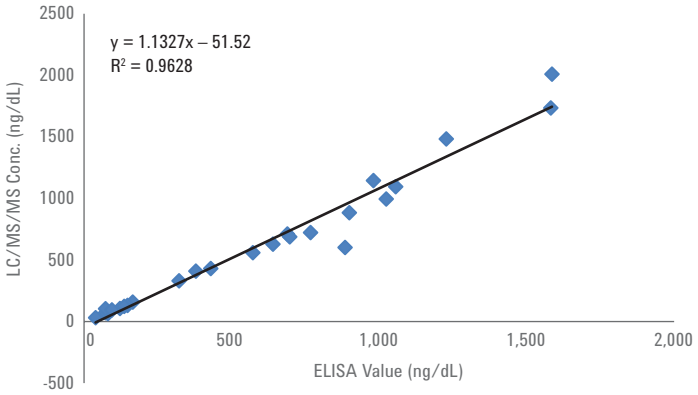

Figure 2. Testosterone correlations of serum samples between LC/MS/MS and ELISA.

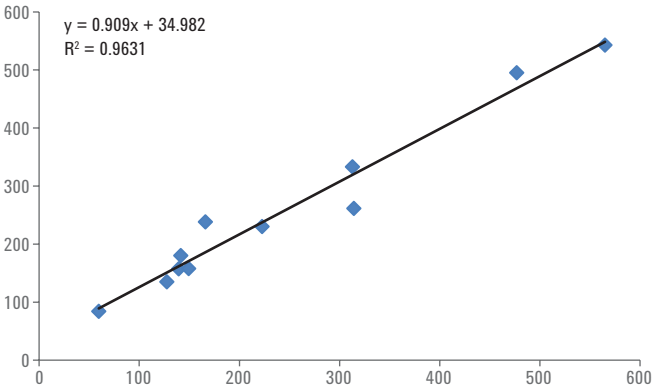

Figure 3. Testosterone correlations between two Agilent 6495 LC/MS/MS instruments.

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Published in the USA, July 13, 2017
5991-8209EN

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