

Quantitative Analysis of Testosterone in Serum by LC-MS/MS

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Key Words

- TSQ Quantum Ultra
- Clinical Research
- LC-MS/MS
- Transcend TLX-2

Introduction

Testosterone is the major androgenic hormone. It is responsible for the development of the male external genitalia and secondary sexual characteristics. In females, its main role is as an estrogen precursor. In both genders, it also exerts anabolic effects and influences behavior. In men, testosterone is secreted by the testicular Leydig cells and, to a minor extent, by the adrenal cortex. In premenopausal women, the ovaries are the main source of testosterone with minor contributions by the adrenals and peripheral tissues. After menopause, ovarian testosterone production is significantly diminished. Testosterone production in testes and ovaries is regulated via pituitary-gonadal feedback involving luteinizing hormone (LH) and, to a lesser degree, inhibins and activins.

Most circulating testosterone is bound to sex hormone-binding globulin (SHBG), which in men also is called testosterone-binding globulin. A lesser fraction is albumin bound and a small proportion exists as free hormone. Historically, only the free testosterone was thought to be the biologically active component. However, testosterone is weakly bound to serum albumin and dissociates freely in the capillary bed, thereby becoming readily available for tissue uptake. All non-SHBG-bound testosterone is therefore considered bioavailable.

For adults, the normal values for testosterone are 240-950 ng/dL for males and 8-60 ng/dL for females.

Goal

To develop a sensitive, quantitative LC-MS/MS assay for testosterone in serum.

Experimental Conditions/Methods:

Chemicals and Reagents

Testosterone standard was purchased from Steraloids, Inc. in the powder form and is stored at room temperature. The internal standard, Testosterone 16,16,17-d₃, was purchased from CDN Isotopes in the powder form and is also stored at room temperature. Bovine serum albumin and PBS buffer were purchased from Sigma-Aldrich and stored in a refrigerator. Bovine serum was used because human serum with undetectable levels of testosterone was not commercially available.

Sample Preparation

0.025 mL deuterated stable isotope internal standard (d₃-testosterone) is added to a 0.1 mL serum sample as internal standard. Protein is precipitated from the mixture by the addition of 0.25 mL acetonitrile. The testosterone and internal standard are extracted from the resulting supernatant by an on line extraction. This is followed by conventional liquid chromatography and analysis on a tandem mass spectrometer equipped with a heated nebulizer ion source.

Calibration Curve Standards Preparation

A standard stock solution of 1 mg/mL of testosterone was prepared in methanol. Standard spiking solutions of testosterone in methanol/water at concentrations of 1000 ng/mL and 100 ng/mL were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 100 mL of 5% BSA in 0.01M PBS (pH 7.4) to prepare calibration standards at the following concentrations: 5 ng/dL, 10 ng/dL, 20 ng/dL, 50 ng/dL, 100 ng/dL, 200 ng/dL, 500 ng/dL, 1000 ng/dL, and 2000 ng/dL. The standards were processed with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at -20 °C.

HPLC

HPLC analysis was performed using the Transcend TLX-2 System (Thermo Scientific, San Jose, CA). The 0.1 mL samples were injected onto a 4 x 2 mm C18 Guard cartridge that served as an extraction column. The analyte was directly transferred from the extraction column and focused onto the 33 x 4.6 mm analytical column which was packed with 3 micron particles. Loading and Eluting Mobile phase A was water. Loading phase B was methanol. Loading phase C was a solution containing 45% acetonitrile, 45% isopropanol, and 10% acetone which is used to wash the extraction column. Eluting Mobile phase B was a 50/50 solution of water and acetonitrile. The appropriate gradients and flow rates are described in Table 1.

MS/MS

MS/MS analysis was carried out on a TSQ Quantum Ultra™ triple stage quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) probe (Thermo Scientific, San Jose, CA).

Time (min)	Loading Flow (μL/min)	Loading A%	Loading B%	Loading C%	Eluting Flow (μL/min)	Eluting A%	Eluting B%
0.00	1.0	100			0.5	60	40
0.67	1.0	90	10		0.5	60	40
1.67	0.20	90	10		0.3	60	40
1.77	0.15	20	80		0.6	60	40
2.77	1.00	20	80		1.00		100
5.02	2.00			100	1.20		100
6.02	2.00	100			0.80	60	40

Table 1: HPLC Method

The MS/MS conditions were as follows:

Ion Polarity: Positive Ion Mode
Vaporizer Temperature: 525 °C
Capillary Temperature: 350 °C
Discharge Current: 5.0 uA
Sheath Gas Pressure (N₂): 35 units
Auxiliary Gas Pressure (N₂): 10 units
Scan Type: Unit Resolution
Scan Time: 0.050 s

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision	Tube Lens
Testosterone	289.201	97.118	23	113
Testosterone	289.201	109.114	25	113
Testosterone IS	292.216	97.111	21	92
Testosterone IS	292.216	109.097	26	92

Table 2: List of SRM transitions and their parameters

Results and Discussion

Representative-SRM chromatograms for Testosterone at 5 ng/dL and 200 ng/dL are shown in Figures 1 and 2, respectively. Clearly identifiable and quantifiable peaks were observed.

The method precision was evaluated by analyzing patient sample pools at concentrations of 20 ng/dL, 50 ng/dL, 140 ng/dL and 1000 ng/dL. Intra-assay variability was determined by processing and analyzing twenty replicates of the lowest two QC sample pools. Inter-assay variability was determined by processing and analyzing two replicates of each QC sample pools in six different batches. Intra-assay and inter-assay precision results are displayed in Table 3 as % CV.

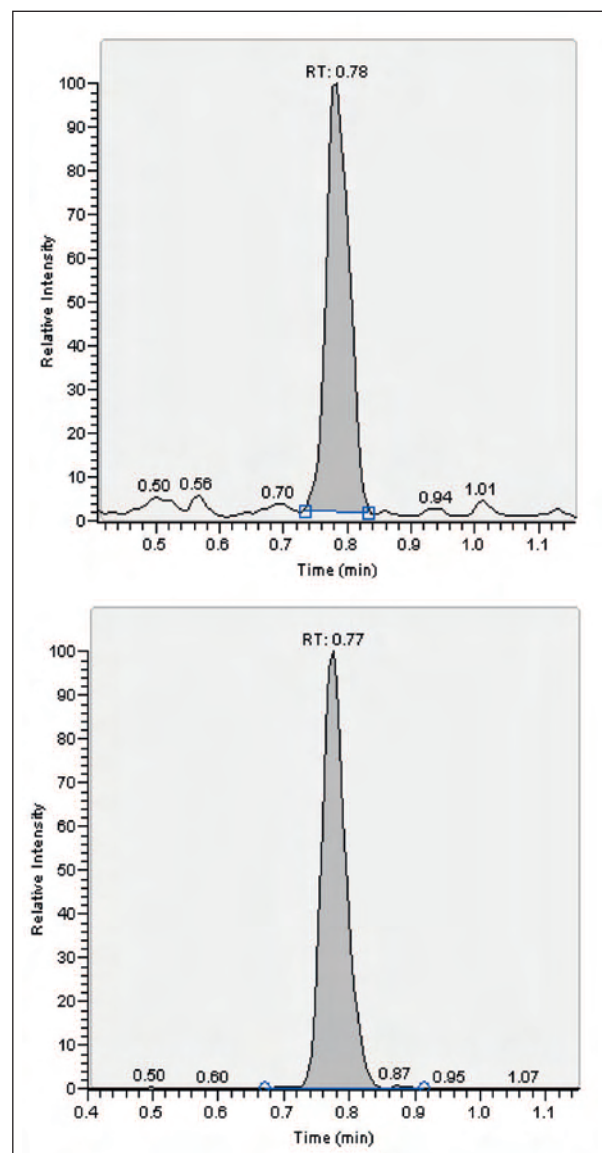


Figure 1: 5 ng/dL Testosterone standard with deuterated internal standard

	Precision	
	Intra-assay	Inter-assay
QC Level 1 (n=20)	8.43%	N/A
QC Level 2 (n=20)	7.33%	N/A
QC Level 1 (n=12)	N/A	15.70%
QC Level 2 (n=12)	N/A	12.29%
QC Level 3 (n=12)	N/A	2.91%
QC Level 4 (n=12)	N/A	5.71%

Table 3: Intra and Inter assay precision

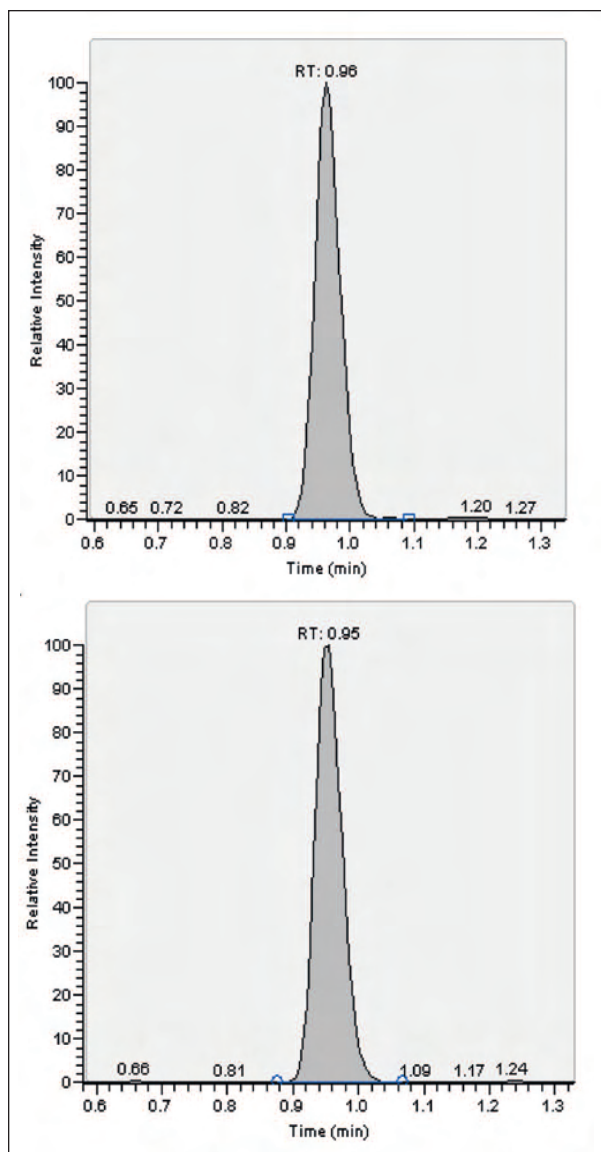


Figure 2: 200 ng/dL Testosterone standard with deuterated internal standard

SST Interferent Peak on Thermo Ultra

It had been observed in our clinical laboratory that specimens acquired with a serum separator tube had an interferent that eluted immediately in front of testosterone containing the same m/z transition. The presence of this interferent precluded baseline resolution and was very troublesome when integrating low levels of testosterone. Initially, this interferent was observed on the TSQ Quantum Ultra.

However, it was determined that by raising the capillary temperature of the TSQ Quantum Ultra up to 350 °C this interferent disappears without diminishing the testosterone response.

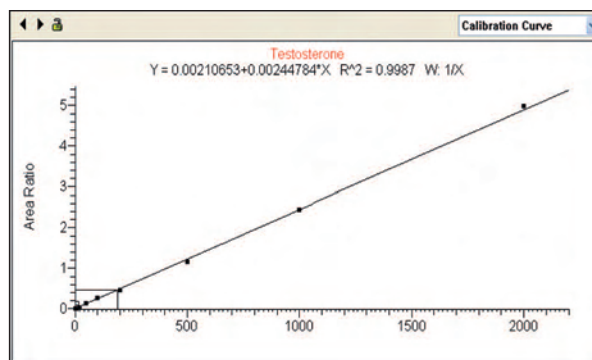


Figure 3: The linear fit calibration curve for testosterone. The calibration curve has R^2 values greater than 0.99, which indicate excellent linear fits over the dynamic range of 11-2000 ng/dL for testosterone. The LOQ value is 11 ng/dL with LOD values approximately 3 times lower.

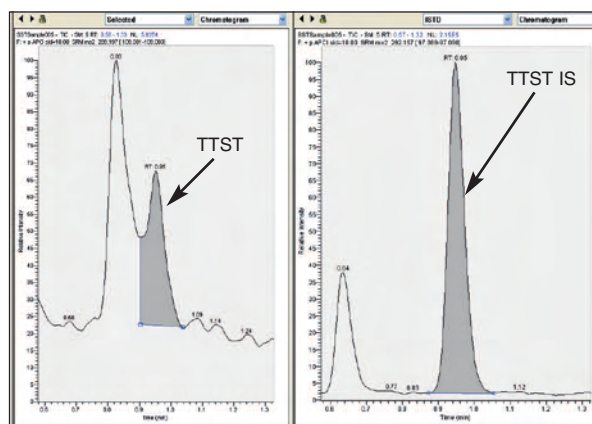


Figure 4: Testosterone sample injection with Interferent present from Serum separator Tube

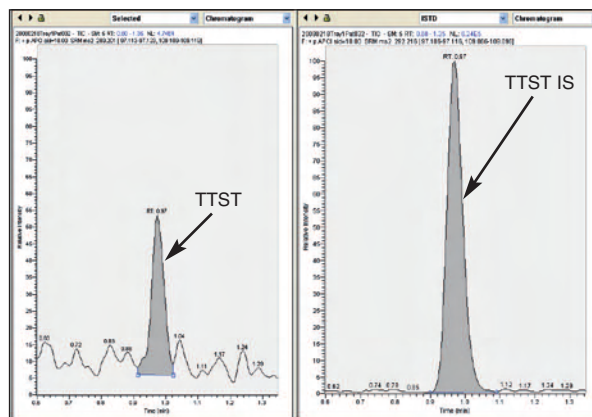


Figure 5: Testosterone sample injection with Interferent from Serum separator Tube eliminated

Conclusion

A fast, sensitive and reliable LC-MS/MS SRM method has been developed for the determination of testosterone in serum. Sample analysis was performed with a run time of 7 minutes with a quantitation limit of 11 ng/dL and a linearity range of 11-2000 ng/dL. The low intra-assay and inter-assay variability of the results demonstrates the reliability of the method.

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