Application Note LCMS-114

Rapid Analysis of Low Level Testosterone in Serum with the Bruker EVOQ Elite LC-TQ

Abstract

A rapid and sensitive method for the quantification of total testosterone in serum was developed using the Bruker Advance UHPLC coupled to the EVOQ Elite LC-TQ mass spectrometer system. Excellent sensitivity, linearity and dynamic range were obtained with a limit of detection (LOD) of 0.1 ng/dL, $R^2 > 0.999$, and greater than four orders of dynamic range (0.25 ng/dL to 5000 ng/dL). The total run cycle time was 5 minutes.

Introduction

Testosterone concentrations in adult females and children (male and female) are an order of magnitude lower than adult male testosterone concentrations and require a sensitive and specific analytical method to accurately determine their concentrations. LC-MS/MS is becoming the analytical method of choice over immunoassays due to its sensitivity, specificity and accuracy for analysis of total testosterone in serum. This research method used a modified Deborah French[1] extraction method for sample preparation. High speed centrifugation along with transferring a controlled amount of 0.85 mL extracted supernatant minimized the contamination of the injection solution with the polar matrix.

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LC-MS/MS	

Material and Reagent

Testosterone and testosterone-2,3,4-$^{13}C_3$ (Internal Standard) were purchased from Cerilliant (Round Rock, Texas, USA). Human Serum (MSG3000) was from Golden West Biologicals, Inc. (Temecula, California, USA). Human serum containing testosterone at levels of 10 ng/dL, 100 ng/dL, and 250 ng/dL were from UTAK Laboratory Inc (Valencia, California, USA). Female Serum was purchased from LEE Biosolution (St. Louis, Missouri USA). Pooled Normal Human Serum was purchased from Innovative Research (Novi, Michigan, USA). HPLC grade water and HPLC grade methanol were purchased from VWR international (Visalia, Caloifornia, USA). All other chemicals were analytical reagent grade.

Method

Following the simple extraction using high purity hexane/ethyl acetate reagent, the Bruker Advance UHPLC coupled to the Bruker EVOQ Elite TQ system equipped with an heated ESI source was used to separate, detect and quantify testosterone in serum. The MRM ion transitions of m/z 289→97 (collision energy (CE) 21V) and m/z 289→109 (CE 21V) were used as quantifier and qualifier ion, respectively for testosterone. The transition of m/z 292.3→112 (CE 23V) was used for the stable labeled internal standard. The scan times for both testosterone and internal standard were 100 ms. The MSG3000, testosterone-free Human Serum, was used as matrix for preparing calibration standards.

Experimental

Calibration Curve Standard Preparation

Calibration A: Testosterone was spiked in human serum MSG3000 for calibration solutions at levels of 0.1 ng/dL, 0.25 ng/dL, 2.5 ng/dL, 5 ng/dL, 25 ng/dL, 100 ng/dL, 500 ng/dL, 2500 ng/dL, and 5000 ng/dL and a triplicate solution for concentration of 0.5 ng/dL, 50 ng/dL, and 1000 ng/dL was prepared as QC standard.

Calibration B: 10 ng/dL, 100 ng/dL, and 250 ng/dL (from UTAK Laboratory Inc)

Sample Preparation

A 0.20 mL of serum or calibration standard in a 2 mL microfuge tube was spiked with 10 µL of 1.0 µg/dL internal standard and vortex-mixed for 5 s and incubated at room temperature for 5 min. To this 2 mL microfuge tube, 1.0 mL of ethyl acetate:hexane, 10:90, v:v was added and vortex-mixed for 1 min and left at room temperature for 2 min. then centrifuged at 14,500 rpm for 3 min. A 0.85 mL of supernatant was transferred into a new 2 mL microfuge tube and dried by SpeedVac at 45 ˚C for 20 min.

The residue was dissolved in 100 µL of solvent (methanol: water, 50/50, v:v) and transferred into an autosampler vial with a 300 µL fused insert and analyzed by LC/MS. Sample preparation time was about 30 min and LC/MS run time was 5 min.

Chromatography

Instrument	Bruker Advance™ UHPLC system
Analytical column	YMC-Pack Pro C18 RS, 3 µm, 50mm × 2.0 mm I.D.
Column Temperature	40 ˚C
Injection volume	20 µL (100 µL Loop)
Flow rate	300 µL/min
Mobile Phase A	2 mM ammonium formate, 0.1% formic acid (FA) in water
Mobile Phase B	2 mM ammonium formate, 0.1% FA in Methanol
Gradient conditions	0.0 min, 50% B
	0.2 min, 50% B
	2.0 min, 95% B
	3.0 min, 95% B
	3.1 min. 50% B
	5.0 min. 50% B

MS Parameters

Instrument	Bruker EVOQ Elite
Voltage (Positive)	4000 V
Cone Gas Flow	15 units
Cone Temperature	300 ˚C
Heated Probe Gas Flow	40 units
Heated Probe Temperature	450 ˚C
Nebulizer Gas Flow	55 units
Exhaust Gas	on
q2 pressure	1.5 mTorr (Argon)

Results and discussion

Calibration A: The LC method used a five-minute cycle time with a testosterone retention time of 2.63 minutes (Fig 1). The blank shows no interference of the analysis at LLOQ level. The assay was sensitive (LOD 0.1 ng/dL, S/N 12) and linear (R2 >0.999). The response factor RSD for testosterone concentration ranged from 0.25 ng/dL to 5000 ng/dL was 11.0% (Fig 2).

The sensitivity and linear range well covers the testosterone concentrations in children and adults of both genders. The %RSD of QC standards at levels of 0.5 ng/dL, 50 ng/dL, and 1000 ng/dL were 1.7%, 1.4% and 2.0% (n=3) with accuracy of 115.0%, 103.4% and 106.3%, respectively (Table 1). The results for single female donor serum and pooled male serum were 24.1 ng/dL and 427.5 ng/dL, respectively. The testosterone test results are in the range for adult female and male.

The experimental accuracies for calibration B solutions at concentrations of 10 ng/dL, 100 ng/dL, and 250 ng/dL from UTAK Laboratory Inc were 103.0%, 120% and 120.9% respectively. The calibration B solution was used as method check standard initially. The results showed that the check standards were 10-30% higher. This lead to the suggestion that Calibration solution A was not qualified as a reference standard whereas Calibration B solution can be used as a reference standard.

Calibration B: The curve was linear (R2 was >0.999) and the response factor %RSD was 9.0% using UTAK calibration solution. The measured results for calibration A solution at 5 ng/dL, 50 ng/dL, 500 ng/dl, and 5000 ng/dL were 5.1 ng/dL (102%), 43.8 ng/dL (87.5%), 412.5 ng/dL (82.5%), and 4120.5 ng/dL (82.4%), respectively. The accuracies for the concentrations below 5 ng/dL were very low (they fall far outside the calibrated range). Using calibration curve B, the measured results, for single female donor serum and pooled male serum were 20.9 ng/dL and 344.2 ng/dL, respectively.

The measured results showed a difference of 20% using different calibration standards suggesting that the testosterone standards need to be qualified as reference standards before use. Both calibration curves are linear and have good response factor %RSD indicating the LC/MS system and method are suited for total testosterone analysis.

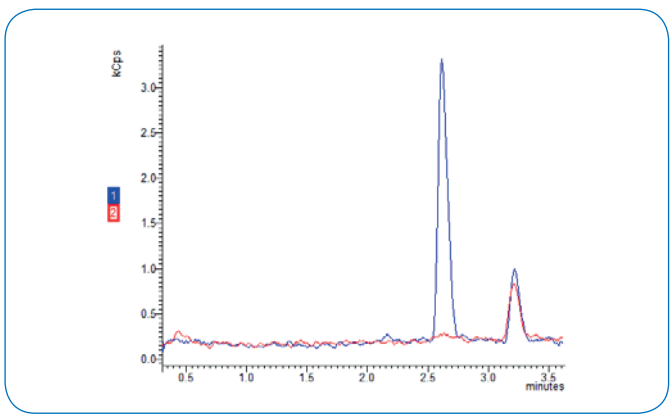

Figure 1. Detection of testosterone in human serum: red trace (blank) and blue trace (RT 2.6 min, 5 ng/dl testosterone).

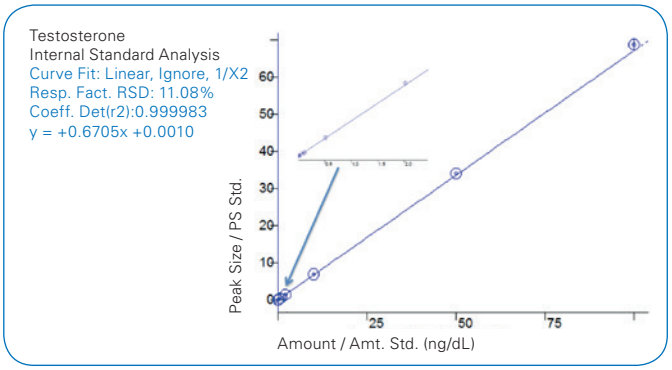

Figure 2. Testosterone calibration curve A, levels from 0.25 ng/dL to 5000 ng/dL with internal standard concentration of 50 ng/dL.

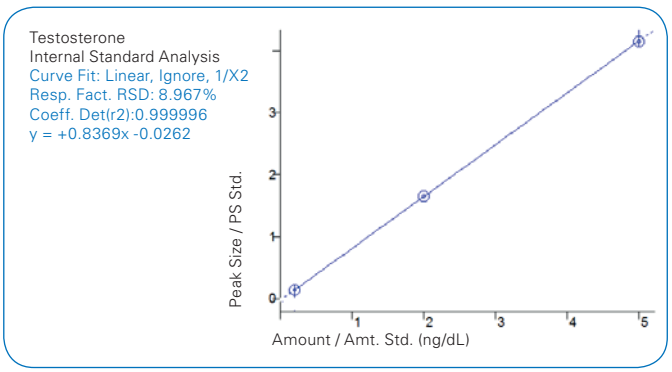

Figure 3. Testosterone calibration curve B, levels from 10 ng/dL to 250 ng/dL with internal standard concentration of 50 ng/dL.

Table 1. QC Results for the triplicate QC preparation standards

Testosterone in Serum (ng/dL)	Measured Amount (ng/dL)	Accuracy (%)	RSD (%)
0.5	0.58	115	
0.5	0.57	113	1.7
0.5	0.58	117	
50	52.7	105	
50	51.9	104	1.4
50	51.2	102	
1,000	1,052.4	105	
1,000	1,049.8	105	2.0
1,000	1,087.1	109	

Conclusions

The Bruker EVOQ Elite LC-TQ is well suited for the analysis of total testosterone in serum from low to high levels. The system is sensitive enough to detect sub ng/dL (0.1 ng/dL) levels of testosterone in serum and provides over four orders of dynamic detection range. This research method provides a rapid, simple, accurate and precise procedure for the rapid determination of testosterone in male and female adults and infants.

References

[1] Deborah French Development and validation of a serum total testosterone liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay calibrated to NIST SRM 971. Clinica Chinica Acta 415 (2013 109-117

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