Analysis of Testosterone in Serum using LC/Triple Quadrupole MS/MS

Application Note

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Abstract

A rapid method for the quantification of total testosterone in serum was developed using the Agilent 1200 Series Rapid Resolution LC (RRLC) system with the Agilent 6430 Triple Quadrupole LC/MS in atmospheric pressure chemical ionization (APCI) mode. A two dimensional LC approach was used to minimize matrix interferences and ion suppression, while reducing the time required for analysis.

Excellent linearity of quantification ($R^2 > 0.99$) was obtained in the range of 5 to 2000 ng/dL, with analysis times of only 4.5 minutes, and day-to-day precision was below 10%. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/dL and 5 ng/dL respectively, in 5% bovine serum albumin.

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Introduction

Testosterone exerts both anabolic and androgenic effects on the human body. Anabolic benefits include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturations.

This application note describes a method for the rapid, sensitive and accurate determination of total testosterone in serum using the Agilent 6430 Triple Quadrupole LC/MS System, with 2-D heart-cutting LC and APCI MS mode. The use of two dimensional, heart-cutting LC reduces matrix effects and ion suppression, with no interferences observed in a large number of serum samples, while reducing the time required for analysis in clinical research.

Experimental

Reagents and Standards

Stock solutions of testosterone and d3 deuterated testosterone (Sigma and Medical Isotopes, respectively) were prepared at 10 µg/mL in methanol (Fisher Scientific) and stored at ≤ 5°C. Calibration standard solutions were prepared from these stock solutions at 5, 10, 100, 500, 1000, and 2000 ng/dL in 5% bovine serum albumin.

Instruments

This method was developed on an Agilent 1200 Series Rapid Resolution LC (RRLC) system coupled with an Agilent 6430 Triple Quadrupole LC/MS with APCI. The instrument conditions are listed in **Table 1**.

Sample Preparation

Serum samples were prepared using a precipitation method:

1. 0.1 mL of working internal standard (d3 testosterone) was added at 100 ng/dL in H_2O acetonitrile to each test tube containing 0.1 mL of serum.
2. 0.4 mL of acetonitrile was added to each tube.
3. Samples were centrifuged at 3000 RPM for 10 minutes.
4. The clear layer of each sample was transferred to a second test tube.
5. The solvent was evaporated at 50°C (for about 10 minutes).
6. 0.1 mL of water:methanol (50:50) was added to each tube and vortexed.

Table 1. LC and MS Conditions

LC Run Conditions				
Column	Loading: ZORBAX SB C-8, 2.1 x 15 mm, 3.5 µm (P/N 871700-906) Analysis: ZORBAX Eclipse Plus C-18, 2.1 x 50 mm, 1.8 µm (P/N 959741-902)			
Column temperature	50°C			
Injection volume	10 µL			
Autosampler temperature	6°C			
Needle wash	Flush port (50:25:25, IPA:MeOH:H_2O, 5 sec)			
Mobile phase	A = H_2O + 0.1% formic acid B = methanol + 0.1% formic acid			
Analysis time	4.5 min			
Flow rate	0.5 mL/min			
Gradient pumps	Pump 1 (Loading)		Pump 2 (Analytical)	
	Time (min)	**%B**	**Time (min)**	**%B**
	0.0	45	0.0	45
	1.5	45	1.7	45
	2.2	80	1.9	80
	3.2	80	3.0	80
	3.5	45	3.7	45
			4.0	45
Columns valve timing	**Time (min)**	**Position**		
	0.0	1		
	0.9	2		
	2.2	1		

MS Conditions	
Ion mode	Positive, APCI
Drying gas temperature	300°C
Vaporizer temperature	300°C
Drying gas flow	4 L/min
Nebulizer pressure	20 psi
Capillary voltage	4500 V
Corona current positive	2 µA
MRM acquisition	Q1 peak and Q2 peak widths = 1.4 *m/z* and 0.7 *m/z*, respectively
Delta EMV	500 V

Chromatography

Heart-cutting was performed using a two dimensional LC approach in which a loading column was positioned in front of the analysis column. Pump 1 applied the sample, via an Auto Liquid Sampler (ALS), to the C-8 loading column with the valve in position 1 (**Figure 1A**). Testosterone was separated from many potential interfering substances on the loading column. Just before the testosterone eluted from the loading column, the valve was switched to position 2 in which the C-18 analysis column was used to further separate the testosterone from interfering substances (**Figure 1B**). Once the testosterone peak was on the analytical column, the valve was returned to position 1 (**Figure 1C**). Any remaining components on the loading column were eluted to waste, and it was then equilibrated and loaded with the next sample. The testosterone was eluted from the analytical column to the triple-quadrupole LC/MS, and the column then equilibrated to be ready for the next analysis. The mobile phases, gradients for each pump, and timing of the valve positions are shown in **Table 1**.

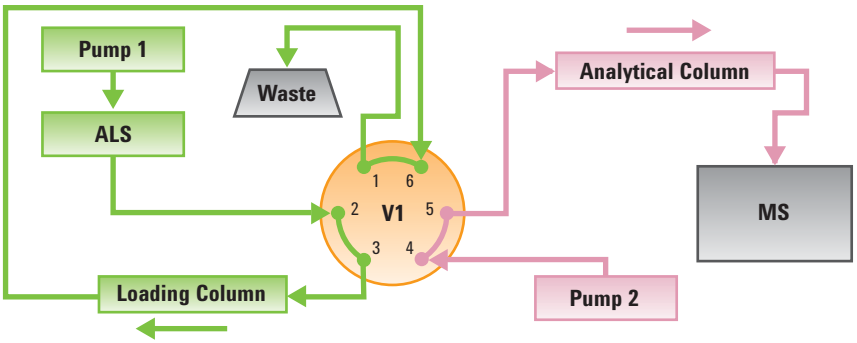
Analysis Parameters

The parameters used in the analysis of testosterone, as well as the deuterated internal standard (IS), are shown in **Table 2**.

Figure 1. Valve configurations for the 2-D heart cut analysis of testosterone. The chromatographic setup uses two pumps, one valve, and two columns to obtain rapid, high-resolution separation of testosterone from interfering substances. The Load Column is used for the first dimension separation, and the Analysis Column is used for the second dimension.
A.) First sample loading
B.) First sample analysis
C.) Subsequent sample loadings and analyses

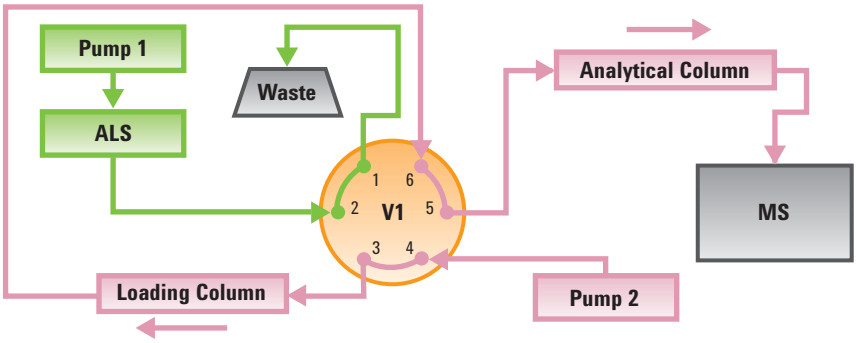
Figure 1. 2-D Heart Cutting LC

A. First sample loading



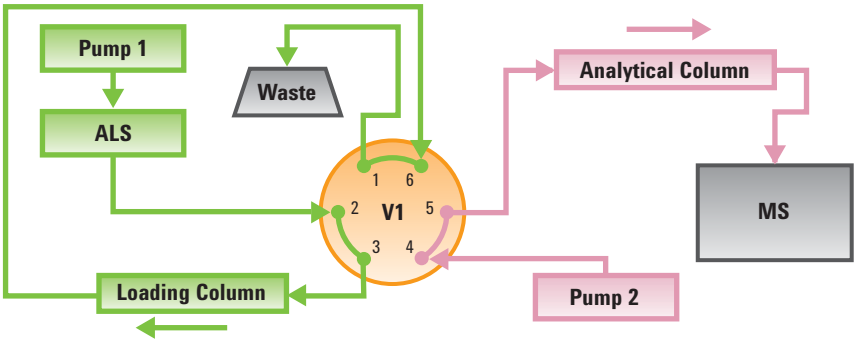
Valve Position 1
Loading column: injected with complex sample and unretained compounds are eluted to waste.
Analytical column: equilibrated and ready for analysis.

B. First sample analysis



Valve Position 2
Loading column: early-eluting contaminants eluted to waste.
Analytical column: target compound (testosterone) received from loading column.

C. Subsequent sample loadings and analyses



Valve Position 1
Loading column: elutes rest of components to waste and then is equilibrated and loaded with the next sample.
Analytical column: elutes target compound (testosterone) to MS and then is equilibrated to be ready for the next analysis.

Table 2. MS/MS Analysis Parameters

Compound	RT(min)	MRM Transition	Fragmentor Voltage	Dwell (ms)	Collision Energy (EV)
Testosterone	3.11	289.2 > 109 289.2 > 97	155	250	30
IS-Testosterone-d3	3.10	292.2 > 97	155	250	30

Results and Discussion

2-D Heart Cutting LC

Using two columns in a heart-cutting configuration reduced the matrix effect and ion suppression while shortening the analysis time. The valve engaged the analysis column shortly before the known elution time of the heart of the testosterone peak from the loading column (first dimension). The testosterone peak was then further separated on the analysis column (second dimension) to remove any remaining interfering substances (**Figure 1**). The end result was a clean chromatogram in which testosterone is the only major component (**Figure 2**).

Analyze testosterone with minimal interferences and matrix effects

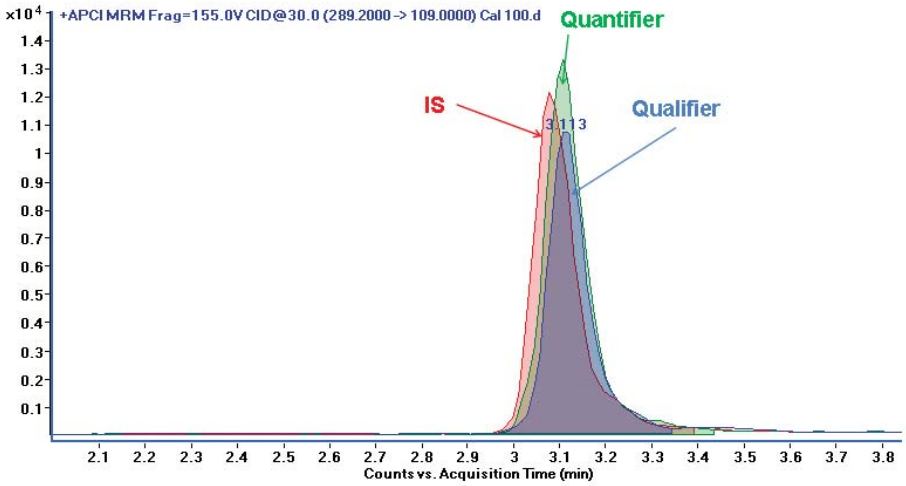


Figure 2. Extracted ion chromatograms of a representative serum sample containing testosterone as well as the internal standard, both at 100 ng/dL. The overlay of the internal standard (IS) (97 m/z), quantifier ion (97 m/z), and qualifier ion (109 m/z) peaks are shown.

Accurate, Reproducible Quantification

Calibration curves were constructed for the testosterone, using concentrations of 5, 10, 100, 500, 1000, and 2000 ng/dL of testosterone that were spiked into 5% bovine serum albumin, with each concentration determined in triplicates. **Figure 3** illustrates the excellent linearity across this wide dynamic range of 2.4 decades, with an R^2 value of 0.999. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/dL and 5 ng/dL, respectively. The LOD was defined as the lowest detectable amount of testosterone measured with a signal-to-noise ratio of 10 or better in all five values determined in a set of five separate analyses, each done in triplicate. The LOQ was defined as the lowest detectable level of testosterone measured within 20% of the target concentration in all five values determined in a set of five analyses, each done in triplicate. The day-to-day (inter-assay) coefficients of variation (CV's) for quantification of the calibration standards were also excellent, falling as low as 0.7%, and never exceeding 10% (**Table 3**).

Table 3. Inter-Assay Precision of Quantification (Coefficient of Variation)

Concentration (ng/dL)	CV (%)
5	9.2
10	7.3
100	3.1
1000	0.7

Accurately quantify testosterone from 5 to 2000 ng/dL

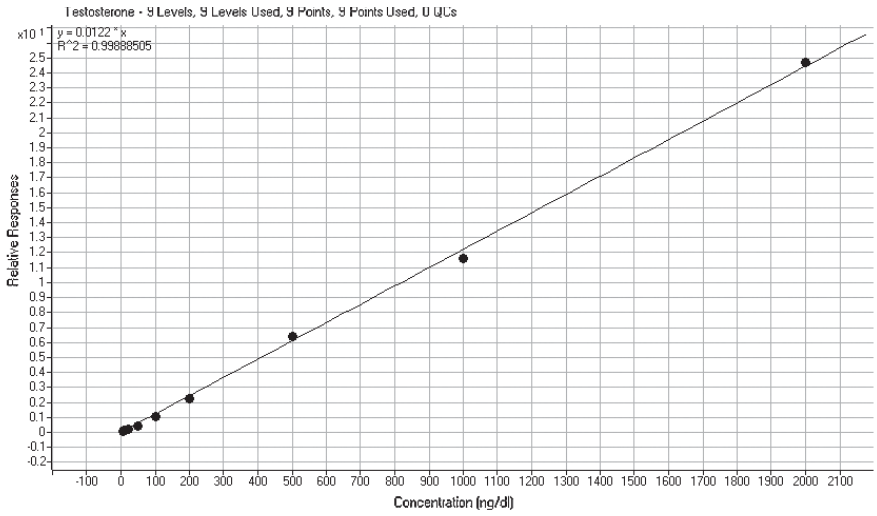


Figure 3. Calibration curve for testosterone in normal serum. The transition used for quantification was 289>97.

Verification using Real-World Samples

The method was verified with a set of 50 serum samples that had previously been quantified using another LC/MS/MS procedure. The correlation of quantification with the reference lab method was very good, giving an R^2 value of 0.978 (**Figure 4**), and no interferences were observed in any of the 50 samples. It should be noted that the method described in this application note is not a diagnostic solution for testosterone testing. Serum samples have been used in the verification of the method to illustrate its performance in the detection and quantification of testosterone.

Excellent correlation with a reference lab method

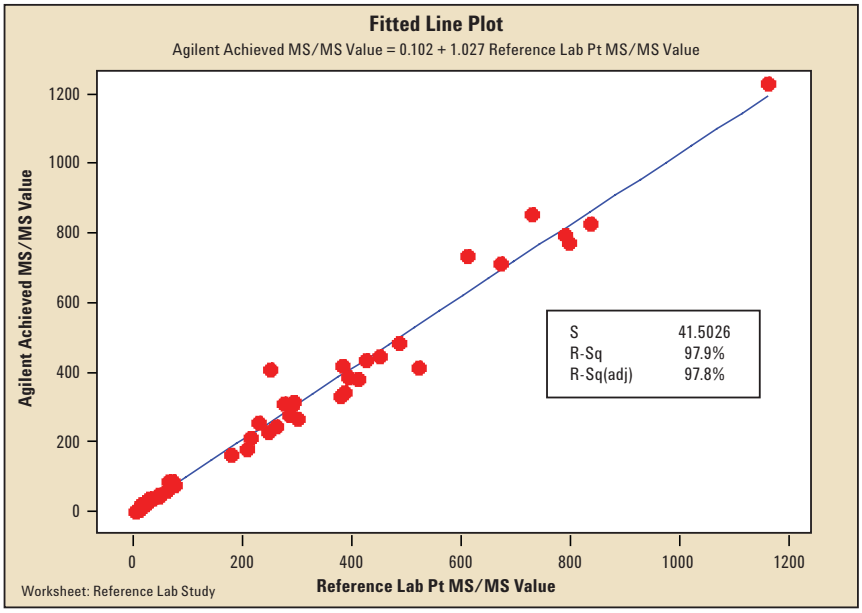


Figure 4. Correlation of testosterone levels measured with the Agilent method versus the values obtained by a reference lab, for 50 serum samples.

Conclusions

A method has been demonstrated for the accurate and reproducible detection of testosterone in serum across a wide dynamic range, using 2-D heart-cutting LC and triple-quadrupole LC/MS. The LOD was 2 ng/dL and the LOQ was 5 ng/dL. The 2-D heart-cutting LC approach minimized the matrix effect and ion suppression due to lipids and other biological compounds present in serum. Analysis was performed in less than five minutes.

Acknowledgements

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References

1. S. Bolour and G. Braunstein, "Testosterone Therapy in Women: A Review," *Int J Impot Res.* 17:399-408, **2005**.

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