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Determination of sex hormones in human serum and plasma using a LC/TQ medical device

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Introduction

Sex hormones are steroid hormones synthesized from cholesterol, and many are of great clinical importance. Considerable inaccuracy of testosterone and progesterone assays via immunoassay has been well documented due to poor specificity.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has become an alternative for steroid analysis in clinical routine diagnostics due to simplified sample preparation and increased specificity and accuracy compared to immunoassays.

In this study, we demonstrate a comprehensive LC/MS method which utilizes an Agilent LC/MS medical device composed of an Infinity LC coupled with a K6460 triple quad mass spectrometer for the determination of testosterone and progesterone in human serum and plasma. Excellent robustness, precision and accuracy were achieved on this platform. Wide dynamic range and good sensitivity with this LC/MS allows accurate quanfitication of these sex hormones at all concentration levels in the general population.

Figure 1. Agilent K1260-6460 Class I LC/MS system

Experimental

Experimental

Sample pretreatment: Liquid–liquid extraction was performed on 200 µL of serum (plasma) and 20 µL of 13C3-testosterone (500 ng/dL) using 1000 µL of 90:10 (v/v) hexane: ethyl acetate. The sample was vortexed and centrifuged at 4000 rpm for 10 min. The organic layer containing testosterone was pipetted off, dried under nitrogen and reconstituted with 60% methanol and water. 20 µL is injection onto LC-MS/MS.

HPLC Conditions

Agilent K1260 Infinity HPLC series binary pump, thermostatted column compartment

Infinity HPLC Column: Agilent InfinityLab Poroshell HPH-C18, 2.1 mm × 50 mm, 2.7 µm

Column temperature: 40 °C

Injection Volume: 20 µL

Autosampler Temperature: 4 °C

Needle Wash: Flush port (70%Methanol:30%Water) 3 seconds

Mobile Phase A: 0.4 mM ammonium fluoride in Water

Mobile Phase B: Methanol

Flow Rate: 0.4 mL/min

Gradient: 0min: 50%B; 3min: 98%B; 7min: 98%B; 7.1 min: 50%B.

Run time: 10 minutes

MS Conditions

K6460C triple quadrupole mass spectrometer

Ion mode:	AJS Positive Mode
Gas Temperature:	300 °C
Gas Flow:	10 L/min
Nebulizer:	45 psi
Sheath Gas Temperature:	350°C
Sheath Gas Flow:	11 L/min
Capillary Voltage:	3500V
Nozzle Voltage:	1500V
Q1/Q2 Resolution:	0.7 FWHM/0.7 FWHM
Dwell time:	50 msec
Delta EMV:	+200V
Testosterone MRM:	289.2>97.0; 289.2>109.0
Progesterone MRM:	315.3>109.2
Testosterone-13C3 MRM:	292.2>100.1

Linearity

Linearity in both serum and plasma were investigated. Greater than three orders of dynamic range was achieved.

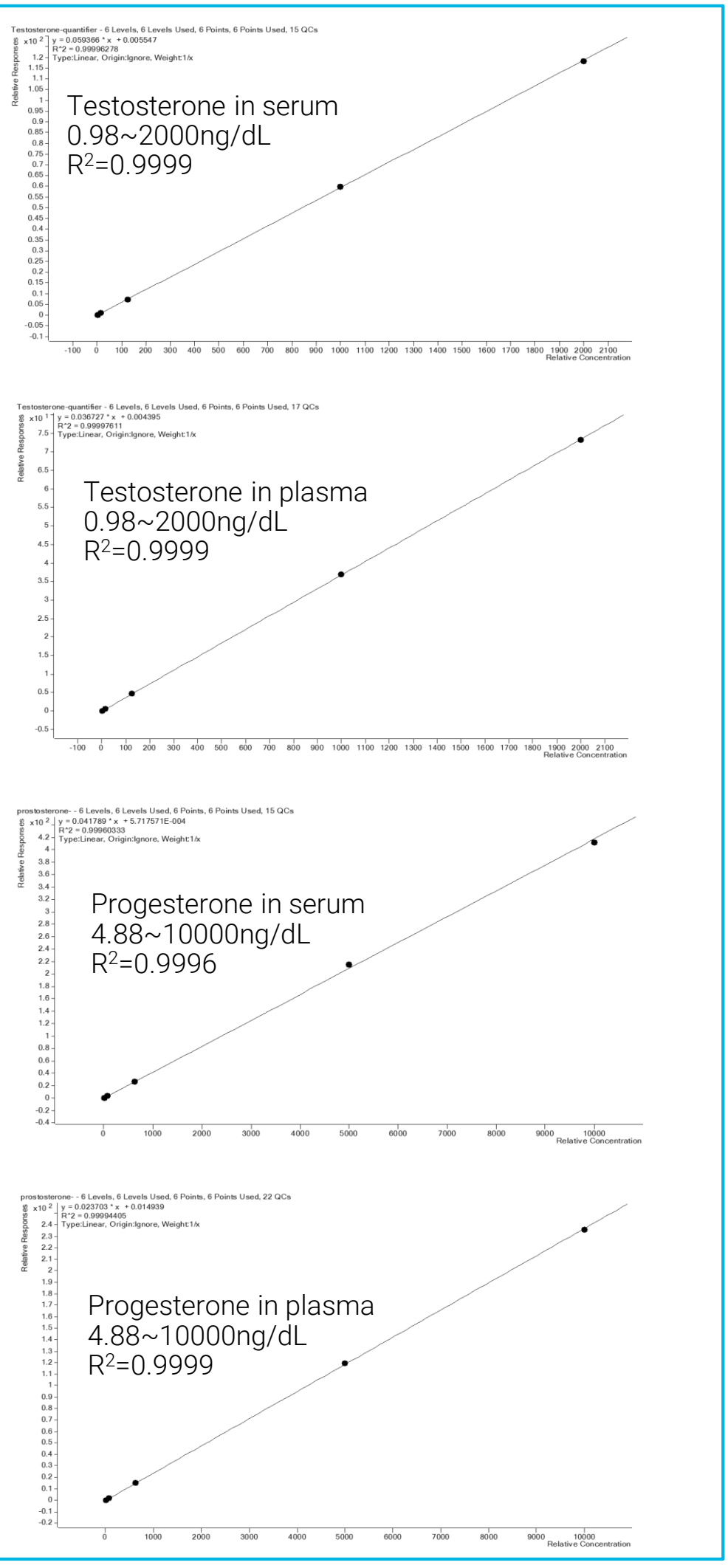
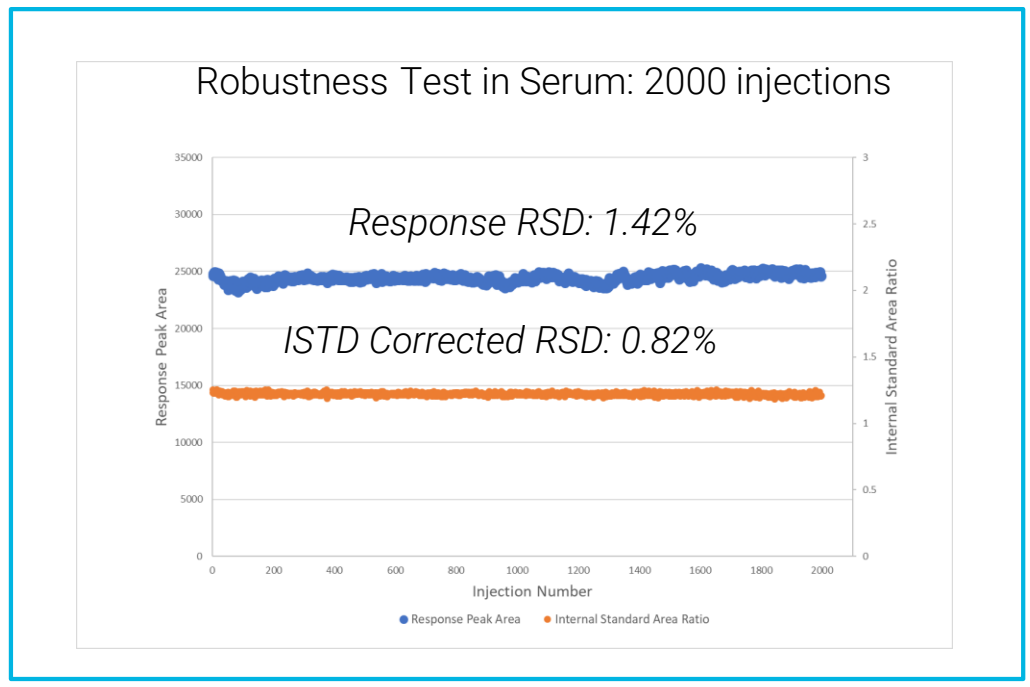


Figure 2. Calibration Curves. Weighting of 1/x was applied.

Robustness

2000 consecutive injections of testosterone in serum was done. Very low response RSD value at 1.42% and ISTD corrected RSD at 0.82% were obtained which demonstrates the excellent robustness of this LC/MS system.



Sensitivity

The mobile phase modifier was investigated to get better sensitivity of testosterone and progesterone. Testosterone responses were 12 fold higher in NH_4F than that in 0.1% FA while progesterone responses were improved 14 times in mobile phase with 0.4 mM NH_4F. See the results in Figure 2. Addition of NH_4F in mobile phase greatly improved the hormone sensitivity.

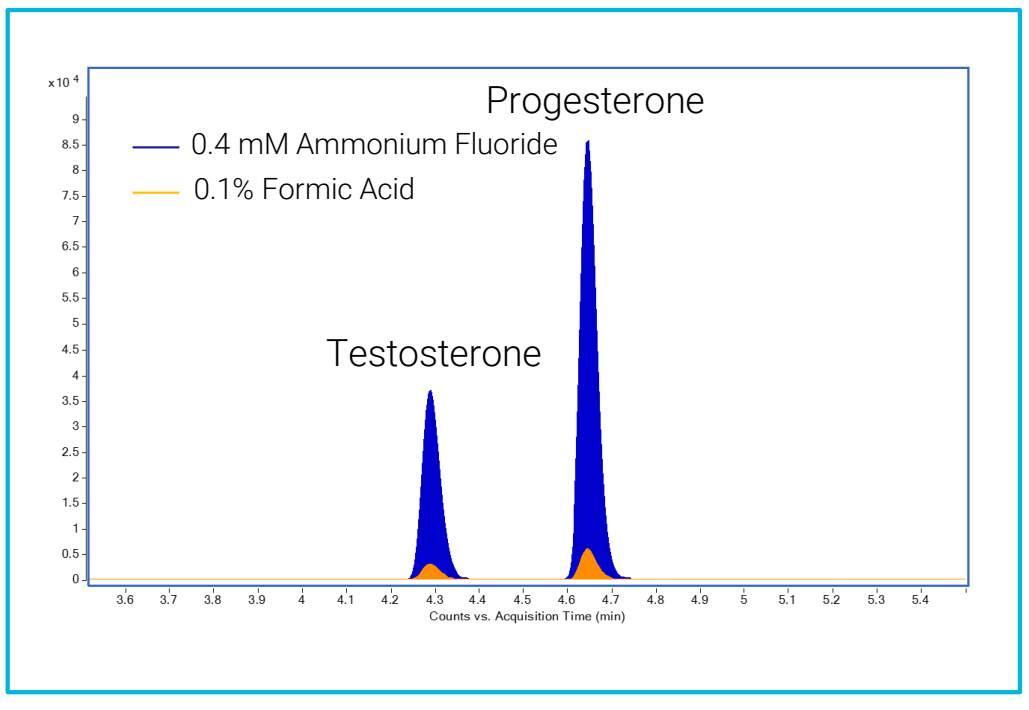


Figure 4. Response improvement using NH_4F as the mobile phase modifier instead of formic acid.

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Results and Discussion

Thus, low LOQ was achieved in both serum and plasma. See result in Figure 3. Criteria for determining LOQ: S/N>20, CV<20%; bias <20%.

We observed that a lower LOQ of progesterone in serum can be achieved compared to that seen in plasma, while testosterone sensitivity is comparable in both serum and plasma matrices.

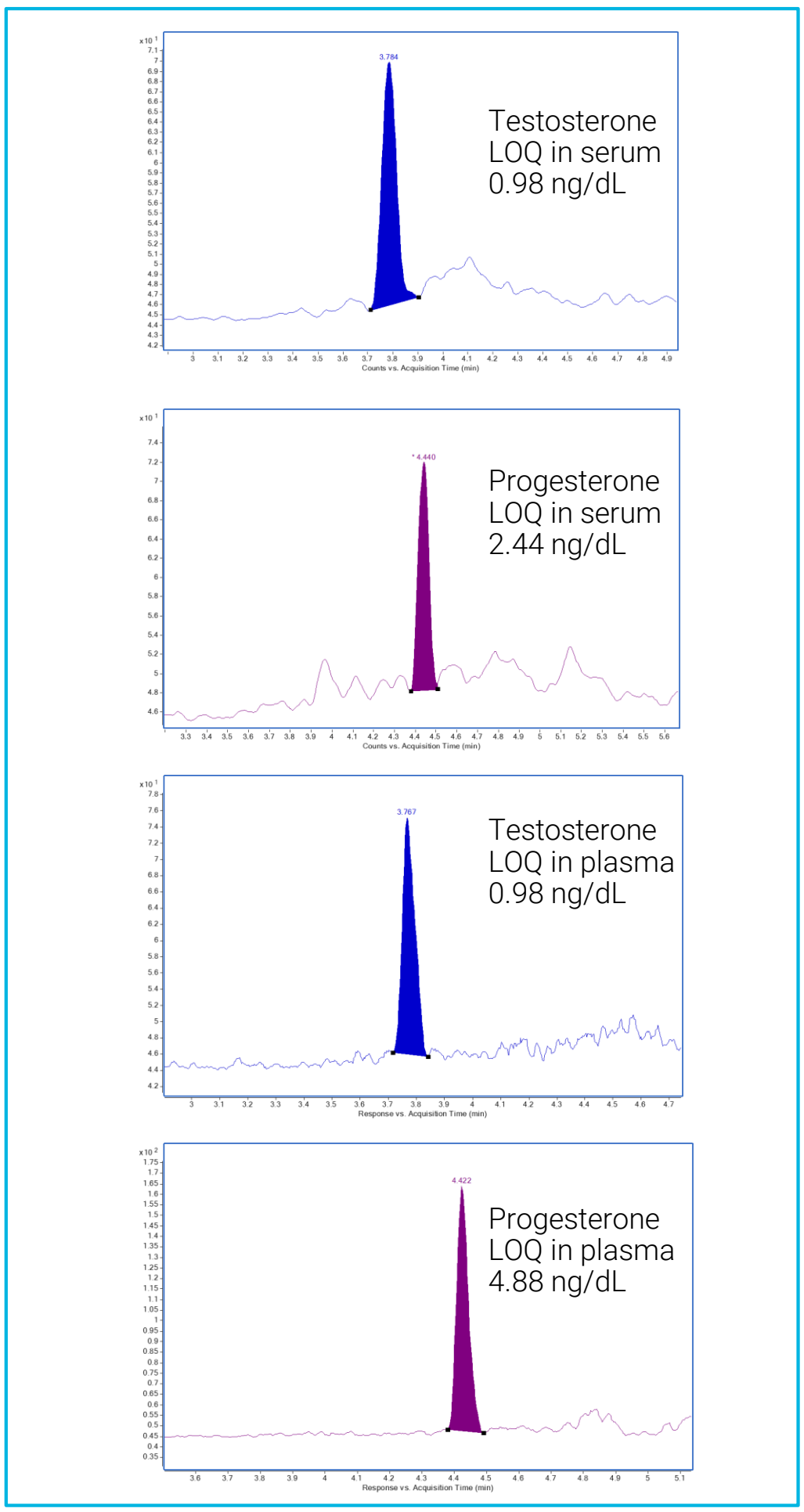

Figure 5. LOQ.(criteria: S/N>20, CV<20%, bias <20%)

Precision and accuracy

Precision and accuracy testing were established by running three levels of in-house QCs in five replicates. Both serum and plasma matrix were investigated. Results are shown in Table 1 below.

Table 1. Precision and Accuracy

Hormones in Serum	QC level	Measured value (ng/dL)	Accuracy %	CV% (N=5)
Testosterone	Low	3.8	97.0	5.5
	Medium	31.4	100.2	1.6
	High	499.3	99.8	2.3
Progesterone	Low	19.4	99.4	2.2
	Medium	160.7	102.8	1.5
	High	2619.0	104.8	2.3

Hormones in Plasma	QC level	Measured value (ng/dL)	Accuracy %	CV% (N=5)
Testosterone	Low	3.9	100.8	1.1
	Medium	31.2	99.8	1.3
	High	501.4	100.3	0.8
Progesterone	Low	19.8	104.3	1.4
	Medium	158.6	101.5	1.8
	High	2646.5	105.6	0.7

The intra-assay precision was found to have a CV%< 10% for both hormones in each matrix. The accuracy was less than 10% for all levels in either serum or plasma.

Conclusions

A robust and solid method was developed for the quantitation of testosterone and progesterone in both human serum and plasma using an Agilent LC/TQ medical device.

- Excellent linearity (>0.999) with greater than three orders of dynamic range is achieved in both matrices.
- Great robustness was observed in 2000 injections of serum testosterone sample which reached extremely low RSD at 0.82%.
- This LC/TQ platform also shows excellent accuracy and high sensitivity which is suitable for measuring sexual hormones across large reference interval in men, women and children.

For In Vitro Diagnostic Use.

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