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Tel: +44 (0) 2920 314750
E-mail: contact@weqas.com
Web: www.weqas.com

Weqas
Unit 6, Parc Tŷ Glas
Llanishen, Cardiff, UK
CF14 5DU

ID-LC/MS-MS Reference Measurement Method for Testosterone in Serum

Ducroq DH, Brett GL, Fraser HL, Thomas MA

Introduction

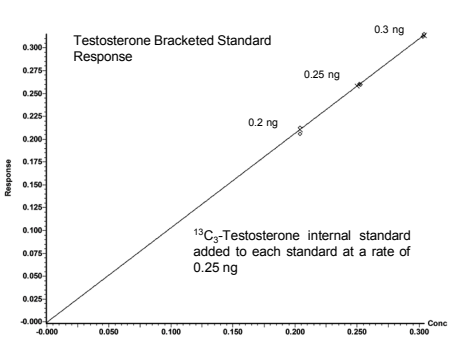
The traceability of laboratory results is a recognised need within quality standards based on ISO 15189 and ISO 17025. Where possible, higher order reference measurement methods are required to provide traceability to the SI unit for vitro diagnostic measurement results, ensuring the transfer of accuracy from definitive methods to routine methods.

A reference measurement method for testosterone based on a JCTLM listed method (C8RMP6)[1] using exact matching isotope dilution LC-MS/MS has been developed and validated within the Weqas Reference Measurement Laboratory. Traceability was assured by the use of NMI-M914 (Australian National Measurement Institute certified standard), with an accuracy matrix check using NIST 971.

Method

Gravimetric sample preparation used exact matching isotope dilution with a $^{13}C_3$-testosterone internal standard (0.25 ng of testosterone in samples and 0.25 ng internal standard). Ammonium acetate solution was added to each sample to disassociate steroids from their binding proteins. This was followed with liquid-liquid extraction of testosterone from the samples with ethyl acetate/hexane (60:40), with a second liquid-liquid extraction in hexane. The combined evaporated samples were reconstituted with water/methanol/formic acid (50:50:0.1). Samples were measured using a Waters Acquity UPLC I-Class coupled to a Xevo TQ-XS triple quadrupole mass spectrometer, with ESI in positive mode. The column of choice was an Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm column (Waters) with mobile phases of ammonium acetate (1 mM) and formic acid (0.1%) in (A) Water and (B) Methanol (60:40). The mass spectrometer was used in mixed reaction mode (MRM), monitoring ions m/z 289 (testosterone P), 97, 100 (testosterone D) and m/z 292 ($^{13}C_3$-testosterone P), 100, 112 ($^{13}C_3$-testosterone D) for each sample, control and standard. The ion pair ratio was converted to a testosterone concentration via reference to bracketed standard curves (fig. 1). Duplicate measurements of samples were made on three separate occasions. An MS scan was also carried out during analysis, to detect any potential contaminants.

Figure 1 Testosterone Bracketed Standards



Results

No major interferents were identified during the validation phase following a mass spectral scan of stock solutions at a concentration of 1 µg/mL (table 1). Where the observed masses were close to the monitored mass for testosterone, the retention time was observed to be sufficiently distant from that for testosterone.

Linearity was assessed by preparation of a series of samples in charcoal stripped serum ranging from 0.5 to 40 nmol/L. (fig. 2). The correlation coefficient (R^2) of 0.9999 and gradient for the line as 1.0121 indicate the method is linear across the measured range. The limit of detection (LOD) was assessed to be lower than 0.35 nmol/L (fig. 3), although with exact matching isotope dilution, increased volume of sample could be used to increase response signal for lower concentration samples.

Bias of certified reference material was also within accepted criteria for a mass spectrometry method for testosterone (table 2). The maximum imprecision was within published reference measurement criteria based on duplicate analysis of samples on 6 separate occasions. Internally prepared IQC material prepared by spiking certified testosterone (M914) into charcoal stripped serum also showed good reproducibility when measured in duplicate on 6 separate occasions.

Good performance of the method was observed within the IFCC RELA external quality assessment programme for Reference Measurement Laboratories. Data shown for RELA 2018 indicate measured values within the limits of equivalence (fig. 4) and comparable to other Reference Measurement Laboratories.

Table 1 Interference Data

Interfering compound	Retention time (mins)	Mass
Cortisol	N/A	362.4
Oestradiol	N/A	272.38
Progesterone	N/A	314.47
*4-Androstene-3,17-dione	2.72	286.4
4-Androsten-17β-ol-3-one	N/A	288.4
5-Androsten-3β-ol-17-one	N/A	288.4
5-Androsten-17β-ol-3-one	N/A	290.4
19-nor testosterone	N/A	274.4

*4-Androstene-3,17-dione shows a low level interference at high concentrations. At normal concentrations no interference is observed.

Figure 2 Testosterone Method Linearity

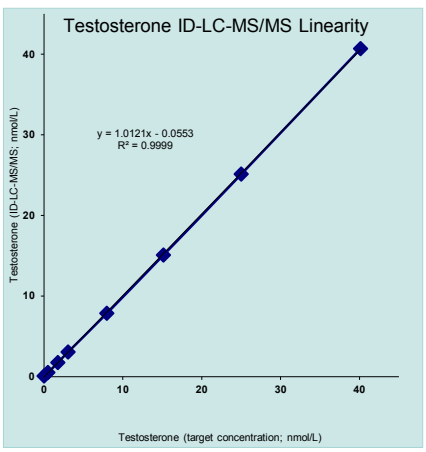


Figure 3 Limit of Detection

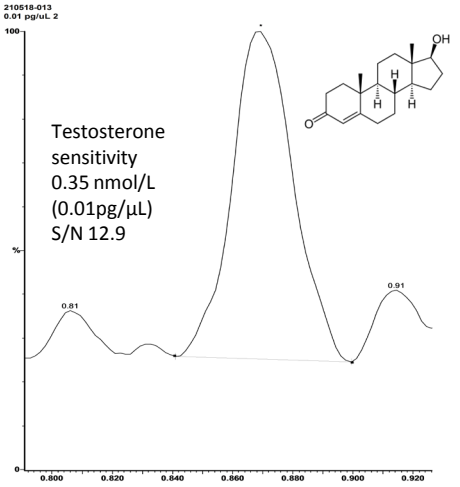
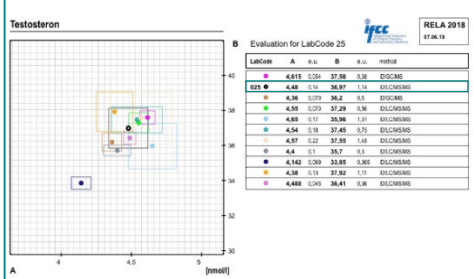


Table 2 Testosterone Reference Material Traceability and Reproducibility Data

Reference Material	Certified value (nmol/L)	Measured value (nmol/L)	N	% Bias	% CV
NIST 971 (female)	0.961 (0.939-0.983)	0.95	12	-0.86	1.18
NIST 971 (male)	22.31 (21.8-22.82)	22.12	12	-0.86	1.36
IQC Low	5.01 (4.85-5.17)	5.00	12	-0.13	1.86
IQC High	15.02 (14.54-15.50)	14.94	12	-0.55	1.62

Figure 4 RELA 2018 Performance (Lab 025)



Conclusions

A validated Reference Measurement Procedure has been developed and shown to be suitable for assigning values to human serum samples (EQA [PT], QC, standard and patient).This reference measurement method is being used to assign reference measurement values for all distributed samples within the Weqas Proficiency Testing Endocrine programme with an associated expanded uncertainty at a level of 95% confidence. The performance of routine testosterone methods can therefore be assessed relative to a gold standard method, ensuring traceability of field methods. Reference measurement data is known to be useful as an accuracy target in Proficiency Testing Programmes.

References
1. Botelho, JC et al Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry Candidate Reference Method for Total Testosterone in Human Serum Clin. Chem. 2013; 59(2):372-380