

# A-043: A FAST, NOVEL AND ANALYTICALLY SENSITIVE CLINICAL RESEARCH METHOD FOR THE ANALYSIS OF FREE TESTOSTERONE USING UPLC-MS/MS



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## BACKGROUND

Clinical research of free testosterone in human serum has long been considered challenging, partly due to the inherent low concentrations (<3% of total testosterone is free). Furthermore, issues relating to variability in results obtained from equilibrium dialysis, the “gold standard” method, have been noted due to technical challenges in addition to lack of control over temperature, pH and shifts in equilibrium.

Here we describe a new LC-MS/MS method for the analysis of free testosterone in serum for clinical research.

## METHODS

### Materials

- Testosterone and its internal standard, testosterone-<sup>13</sup>C<sub>3</sub>, were purchased from Merck Life Science (Dorset, UK).
- Calibrators were prepared in 52.75 mM HEPES buffer at pH 7.40, over 1-500 pg/mL (3.47-1734 pmol/L).
- Tri-level Quality Controls (QCs) for precision testing were prepared in-house using MSG4000 stripped serum (Golden West Biologicals, CA, USA) and pooled and individual male serum samples (BioIVT, UK).

### Equilibrium Dialysis and Liquid-Liquid Extraction

- 200 µL serum was placed into the sample, chamber of a Rapid Equilibrium Dialysis (RED) insert in a reusable base plate (both Thermo Fisher Scientific, UK)
- 400 µL 52.75 mM HEPES buffer, adjusted to pH 7.40, was added to the buffer chamber device and the plate sealed with sealing tape.
- The plate was mixed in a temperature calibrated orbital shaker for 2 hours at 800 r.p.m. at 37°C
- 300 µL of diasylate was transferred to a 2 mL microcentrifuge tube.
- 30 µL of a testosterone-<sup>13</sup>C<sub>3</sub> internal standard at a concentration of 1000 pg/mL in methanol was added, then the tube was capped and mixed.
- 1.5 mL of methyl tert-butyl ether (MTBE) was added and the tube mixed, then centrifuged at 16100g for 2 minutes.
- 1.3 mL of the top layer was transferred to a clean, 2mL 96 well sample collection plate (p/n: 186002482), and dried under nitrogen at 40°C.
- Samples were reconstituted in 70 µL mobile phase A:mobile phase B 50:50 (v:v).

### LC-MS/MS Parameters

- Injection was performed using a Waters ACQUITY™ UPLC™ I-Class FL System. Separation was performed using an ACQUITY BEH™ C<sub>18</sub> Column with mobile phases of 0.2mM ammonium fluoride(aq) in water and methanol, over a run time of 4.5 minutes.
- MRM mode on a Waters Xevo™ TQ Absolute Mass Spectrometer was used to detect free testosterone (ESI+) and its internal standard.

## An Accurate, Analytically Sensitive Free Testosterone in Serum Analysis with Fast Equilibrium Dialysis Method for Clinical Research



Link to Application Note

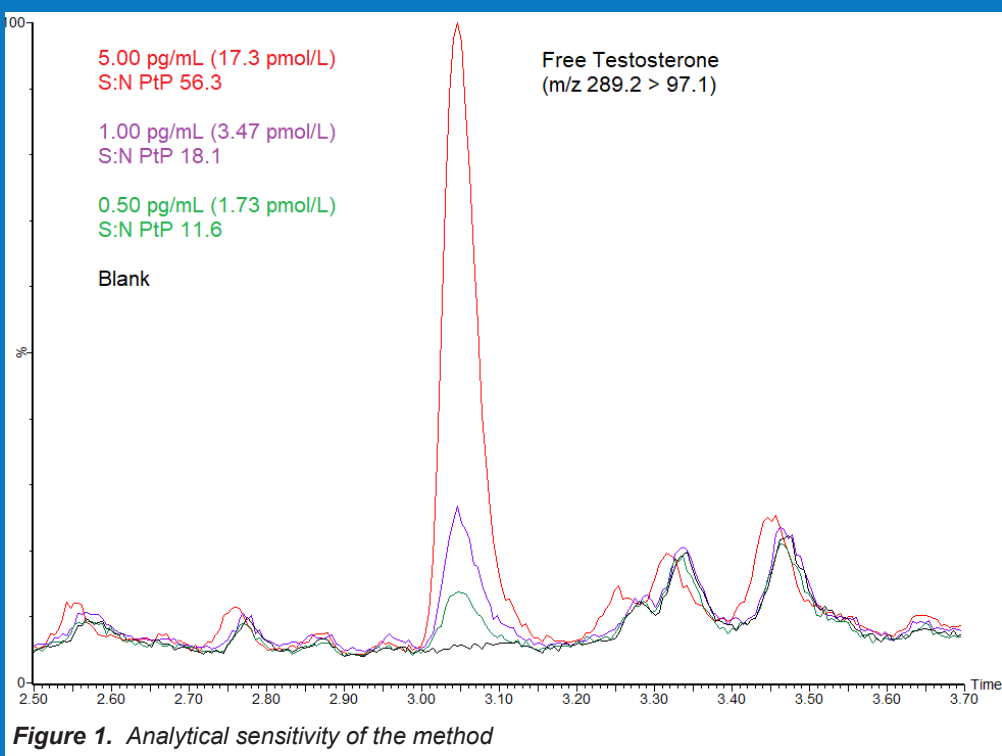


Figure 1. Analytical sensitivity of the method

## CONCLUSION

A clinical research method for the analysis of serum free testosterone has been developed using the Waters Xevo TQ Absolute Mass Spectrometer

- Using 200µL plasma, analytical sensitivity of 0.5 pg/mL can be achieved.
- Excellent reproducibility and repeatability of ≤8.4% across QC samples and a strong agreement with an external quality assurance scheme has been demonstrated.
- A fast equilibrium dialysis step of just 2 hours can be used without compromising performance.

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## RESULTS

### Linearity, Analytical Sensitivity and Carryover

- A linear fit was established over 0.5-650 pg/mL for free testosterone.
- $r^2 \geq 0.995$  was determined on each occasion over the measuring range of 1—500 pg/mL.
- The analytical sensitivity of the method was assessed by extracting and quantifying 10 replicates of low concentration samples in pH 7.40 52.75 mM HEPES buffer over 5 days (n=50). The Lower Limit of the Measuring Interval (LLMI, ≤20%CV precision and ≤15% bias) was determined at a concentration of 0.5 pg/mL (Figure 1 shows analytical sensitivity of the method).
- No significant carryover was observed from 500 pg/mL into subsequent blank samples.

### Matrix Effects

- Matrix effect investigations were evaluated in MSG4000 stripped serum at 2 concentrations.
- Normalized matrix factor calculations, based on the analyte:internal standard response ratio demonstrated absence of ion suppression or enhancement, with mean matrix factors in the range 1.03-1.07.

### Precision

- Low, mid and high concentration serum pools, at 2.79, 8.80 and 134 pg/mL respectively) were analyzed in replicates of 5, on 5 occasions (n=25), to assess repeatability and total precision.
- Reproducibility and total precision was determined to be ≤8.4% CV across the range.

### Interference Testing

- Potential interference from endogenous compounds (albumin, bilirubin, creatinine, cholesterol, triglycerides and uric acid) was assessed.
- A substance was deemed to interfere if a recovery range of 85-115% was exceeded; recoveries ranged from 87.3-114.5%.

### Accuracy

- Analysis of male free testosterone EQA samples (NEQAS, UK, n=45, range 50.1-268.6 pg/mL) was performed to assess agreement to the scheme's all-laboratory trimmed mean (ALTM).
- Deming analysis provided a fit of  $y = 0.947x - 2.869$  and Bland-Altman agreement a mean bias of -7.62% (Figure 2), indicating a small negative bias but strong agreement overall.

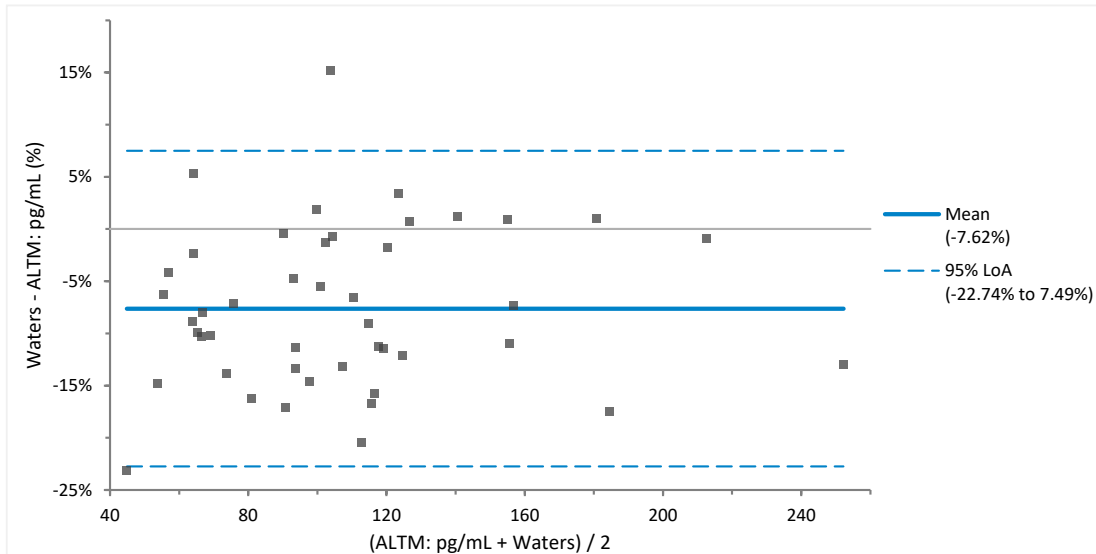


Figure 2. Bland-Altman agreement between Waters LC-MS/MS results and ALTM for UK NEQAS male free testosterone samples