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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: The aim of this work was to develop an analytically sensitive, rapid clinical research method for the analysis of free testosterone in serum using a small sample volume. Laboratories have found this difficult, partly due to the inherent low concentrations (less than 3% of total testosterone is free), technical challenges and variability in results have been noted with equilibrium dialysis, which is considered the gold standard.

METHODS: Testosterone certified reference material (Merck, UK) was used to create calibrators in 52.75 mM HEPES buffer adjusted to pH 7.4 to mimic the ionic environment of serum. Precision was evaluated using in-house QC materials prepared in serum (Golden West Biologicals, USA and BioIVT, UK). 200 μ L serum was incubated at 37°C with 400 μ L pH-adjusted 52.75 mM HEPES buffer for 2 hours, mixing at 800 r.p.m. with Rapid Equilibrium Analysis (RED) inserts and plates (Thermo Scientific™, UK). Testosterone- $^{13}\text{C}_3$ (Merck, UK) was spiked into resultant dialysate, then liquid-liquid extraction (LLE) was used to concentrate and clean the sample. The ACQUITY™ UPLC™ I-Class System with the Waters™ ACQUITY UPLC BEH C_{18} , 1.7 μ m, 2.1 x 100 mm Column using a water/methanol/ammonium fluoride gradient and the Waters Xevo™ TQ Absolute Mass Spectrometer were used for quantification. An accelerated protocol for equilibrium dialysis, using a 2-hour incubation and fast mixing step was used (4 hours or longer is more typical historically) though it is essential to control the pH of the dialysis buffer and, particularly, the temperature of the chamber.

RESULTS: No significant carryover or matrix effects were demonstrated with linearity from 0.5-650 pg/mL. Addition of high concentrations of endogenous substances (albumin, bilirubin, creatinine, cholesterol, triglycerides and uric acid) did not affect quantification. Analytical sensitivity investigations indicated the analytical sensitivity would allow precise quantification ($\leq 20\%$ CV and $\leq 15\%$ bias) at 0.50 pg/mL. Coefficients of variation (CV) for total precision and repeatability on 5 analytical runs for low, mid and high QCs at approximately 2.79, 8.80 and 134 pg/mL respectively were all $\leq 8.4\%$ (n=25). Accuracy was assessed by analyzing 45 male external quality assurance samples (NEQAS, UK)

ranging in concentration from 50.1–268.6 pg/mL and the Bland-Altman agreement showed a small negative bias of -7.6% compared with the all-laboratory trimmed mean (ALTM), though excellent agreement overall. When LC-MS/MS determined concentrations were processed against Vermeulen equation determined concentrations (total testosterone and SHBG concentrations were provided by the scheme, a concentration of 45 g/L albumin was assumed), again good agreement was shown by the Bland-Altman bias of -7.7%.

CONCLUSIONS: A novel clinical research method using a fast equilibrium dialysis procedure with LLE and LC-MS/MS analysis with only 200 µL of serum has been demonstrated for clinical research use. Sub pg/mL analytical sensitivity, excellent linearity and precision, with minimal matrix effects and a strong agreement with an EQA scheme were established.