## Project Review

### "Certified Reference Materials for accuracy in longitudinal monitoring for testosterone abuse"

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Testosterone is an anabolic steroid that is naturally present in everyone at various concentrations. Therefore it is not a simple task to determine whether it has been used by athletes for doping. A level of testosterone greater than four times that of its close analogue epitestosterone can point to steroid abuse in many subjects, but others have naturally high or low T/E ratios. Samples with high T/E ratios need further investigation by carbon isotope ratio mass spectrometry (IRMS) to confirm whether the steroid is from a natural or synthetic origin. This however is a relatively complex and time consuming technique.

Long-term monitoring of the T/E ratio and concentrations of related steroids over time can reveal unusual changes in metabolite levels and/or an atypical T/E ratio for a particular athlete that may be indicative of doping. For such longitudinal studies to be effective it is imperative that the results being produced by different laboratories around the world are accurate and comparable over extended periods of time. Reference materials of urine with concentration values traceable to the international system of measurement units (SI) are an excellent tool to verify the comparability of laboratory results.

The NMI Australia, with a research grant from WADA, has already produced a certified reference material (CRM) of human urine (NMIA MX005) with accurately known values for the concentration of testosterone and epitestosterone and a T/E ratio close to 4. The certification of this material is currently being extended to a range of other natural steroids related to testosterone. In this project, a pre-existing freezedried urine CRM (NMIA MX002) will become the basis of a second urine CRM with a different T/E ratio and concentration of these steroids. This will assist labs to maintain accuracy and comparability of results over a range of concentrations and T/E ratios.

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## Results and Conclusion

An existing freeze-dried urine certified reference material (NMIA MX002) with property values for the mass fraction and mass concentration of 19-norandrosterone glucuronide has been provided with further certification of the concentrations of testosterone and epitestosterone glucuronides, the testosterone/epitestosterone ratio and the concentrations of four important testosterone metabolites used in longitudinal profiling studies; 5α-androstane-3α,17β-diol glucuronide, 5β-androstane-3α,17β-diol glucuronide, androsterone glucuronide and etiocholanolone glucuronide. The provision of these additional property values traceable to the international measurement system will provide an unequivocal benchmark for key measurement parameters in the detection of testosterone abuse. The NMIA MX002 certified reference material is the second reference material certified at NMIA for testosterone, epitestosterone and the key metabolites of testosterone at a range of concentrations to assist laboratories in longitudinal profiling measurements and in the detection of testosterone abuse.

Two independent reference methods were employed to certify the mass fractions of 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, androsterone, etiocholanolone, testosterone and epitestosterone glucuronide conjugates in the urine CRM. The two independent reference methods employ different high-efficiency, two-dimensional HPLC clean-up techniques with determination by the complementary techniques of GC with high resolution mass spectrometry (GC/HRMS) and liquid chromatography with tandem mass spectrometry (LC/MS/MS). In both cases the primary ratio technique of isotope dilution mass spectrometry (IDMS) was employed with an exact-matching calibration protocol to minimise bias in quantification.

Studies have been conducted on the homogeneity and stability of the urine material during long-term storage, transport and use with respect to the mass fractions of testosterone, epitestosterone and the testosterone metabolites. Sample analysis was performed using both the GC/HRMS and LC/MS/MS reference methods to investigate possible measurement biases.