Project Review

"Improved detection of testosterone abuse"

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Testosterone is the most commonly reported of all banned substances with 1,124 cases in the WADA statistics for 2006. As testosterone is a natural hormone, detection requires analysis with highly specialised techniques.

A key component in identifying use of testosterone for performance enhancement is the ability to accurately determine the changes that occur in carbon isotope ratios. When an athlete takes synthetic testosterone it has a different $^{13}C/^{12}C$ ratio to that of the testosterone produced naturally. The measurement of these ratios is a complex technical task as the changes in ratio are very small and only specialised mass spectrometers can measure this change. A thorough review of the factors affecting accuracy in $^{13}C/^{12}C$ ratio results will be performed in this project to provide insight into the aspects of the methodology that contribute most to the measurement uncertainty. This will allow the methods to be further optimised to provide even greater accuracy and increase the probability of detection of doping.

Profiling of the concentrations of a range of compounds in the testosterone metabolic pathway is often used to provide important additional information in cases of suspected doping. Longitudinal studies are being used to detect abnormal changes in metabolites over time. For this to be effective however, it is imperative that the results being produced by different laboratories around the world are accurate and comparable over time. The NMI Australia, with a research grant from WADA, has produced a certified reference material (CRM) of human urine with accurately known values for testosterone and epitestosterone concentrations and T/E ratio. The certification will be extended to include the concentrations of those testosterone metabolites being used in profiling studies. These additional reference values traceable to the international measurement system will provide an unequivocal benchmark for key measurement parameters in the detection of testosterone abuse.

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Results and Conclusion

The first part of the project investigated opportunities to minimise the uncertainty associated with measurements of stable carbon isotope ratios of steroids related to testosterone in urine. A rigorous investigation of the inputs to a measurement uncertainty budget compliant with the JCGM Guide to Uncertainty in Measurement (GUM) 1 was performed. Optimised analytical method conditions were identified experimentally and a new instrument calibration method was developed that minimises measurement bias and facilitates uncertainty estimation. Using method validation data generated in this project, the combined uncertainty associated with results from the optimised analytical method employing this calibration approach was compared with that obtained when using the calibration method commonly employed in instrument software.

A new approach has been developed for the calculation of carbon isotope ratios when using GC-C-IRMS. The optimised approach uses a single point calibration rather than a linear calibration, and the measurement equation has been re-evaluated to avoid introducing a bias when the sample and internal standard 45R' ratios do not match. While unmatched sample and internal standard 45R' ratios do not introduce a bias when using this new approach, matching these ratios does improve the measurement uncertainty.

A number of factors in the sample preparation were identified that influence measurement uncertainty. These sample preparation steps were investigated and optimised to ensure the lowest possible measurement uncertainty:

- SPE of conjugated, unconjugated and derivatised steroids
- Hydrolysis of conjugated steroids
- LLE of unconjugated steroids
- HPLC of unconjugated steroids
- Derivatisation

Validation of the optimised method including investigations of ruggedness, reproducibility and accuracy to allow an estimate of measurement uncertainty associated with a typical result. In developing this estimate, the full measurement equation for calculation of the isotope ratio values was expanded to include factors representing any bias in each of the main terms and validation results were used to provide estimates of the potential size of such bias even when it was not detected.

Complete uncertainty budgets were prepared for the carbon isotope ratios of key analytes and their delta differences in line with the GUM1. Detailed calculation of the sensitivity coefficients for each of the terms of the both measurement equations was required. This was relatively simple for the new measurement equation but required the use of

mathematical software for the equation used by the IRMS instrument software. Uncertainty budgets were prepared for results from each calibration method for the $\delta^{13}C$ values of androsterone, etiocholanolone, 11-oxoetiocholanolone, 11β-hydroxyandrosterone, 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, 5β-pregnanediol, testosterone and dehydroepiandrosterone. They were also prepared for the differences between $\delta^{13}C$ values ($\Delta^{13}C$) for androsterone versus 11-oxoetiocholanolone; etiocholanolone versus 11-oxoetiocholanolone; androsterone versus 11β-hydroxyandrosterone; etiocholanolone versus 11β-hydroxyandrosterone; 5β-androstane-3α,17β-diol versus 5β-pregnanediol; and 5α -androstane-3α,17β-diol versus 5β-pregnanediol.

This allowed a comparison of the measurement uncertainty associated with results from the two calibration approaches. The uncertainties are very similar for the two calculation approaches, although the uncertainty when using the new equation is consistently lower.