PROJECT REVIEW

"Development of Solution and Urine Matrix CRMs for the Detection of Steroid Doping"

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The aim of the project is to produce solution and urine matrix Certified Reference Materials (CRM5) certified for the presence of 19-norandrosterone, the main metabolite of the anabolic steroid nandrolone, at the level of 2 nanograms/ millilitre (ng/ml). This is the level above which a doping violation has occurred for a male athlete, as specified in the current ICC Medical Commission prohibited substance list.

The production and inclusion of CRMs of these types into routine testing procedures will serve the twin purposes of assisting laboratories in establishing the traceability of their measurement results and helping them to make more accurate estimation of the measurement uncertainty associated with their results. The matrix CRMs would specifically be useful for benchmarking the capabilities of laboratories and would also allow them to more readily detect and address bias in their analytical methods. Ultimately their production and use will result in greater confidence, particularly under potentially aggressive legal scrutiny, in these critical results. They would also assist research into new or improved methods for the qualitative and quantitative detection of doping with nandrolone.

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Results and Conclusions

The World Anti-Doping Agency (WADA) statistics show that nandrolone was the second most commonly abused steroid of those detected in 2004. A urine matrix CRM has thus been produced in conjunction with WADA for the major nandrolone metabolite, 19-norandrosterone. The material was prepared at the allowed cut-off level for 19-norandrosterone of 2 ng/mL. The exact measurand for the CRM was defined to link in with the requirements of the WADA technical document TD2004 NA "Reporting Norandrosterone Findings" and the measurand was thus defined as the total of the free and glucuronide forms of 19-norandrosterone.

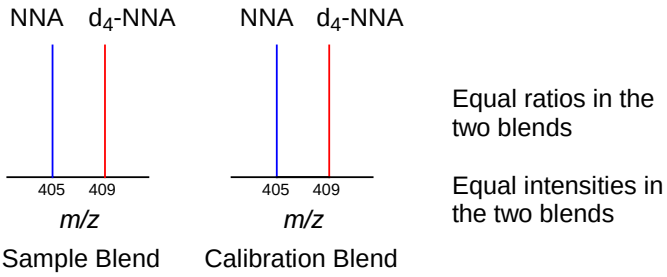
A freeze dried human urine, fortified with 19-norandrosterone glucuronide, was produced following ISO guides 34 and 35 and a high-accuracy isotope dilution mass spectrometry (IDMS) method was developed and used to certify the concentration of 19-norandrosterone in the reference material. Certification of the material included homogeneity testing of the 1,200 units produced and stability testing over the temperature ranges of –20 to 40°C. Results for 19-norandrosterone concentration from the within-bottle homogeneity testing of 30 units of the CRM, selected in a stratified random manner, had an RSD of 1.3%, indicating excellent agreement over the batch of 1,200 units. Stability testing of the material at its storage temperature of -20°C showed excellent stability over the 12-months of testing to date. The accelerated stability trial carried out at 4°C, 22°C and 40°C showed a change in level of the analyte only at the 40°C; at this elevated temperature the level had dropped by 25% after 12 months. Stability testing of the reconstituted urine, kept in its liquid form at 4°C for 4 weeks, showed no change in analyte level and therefore the freeze-dried material may be reconstituted and then refrigerated for later use.

The rigorous application of a primary ratio method such as IDMS should ensure that the value assigned to the CRM will be traceable to the SI and have a very well-defined uncertainty. A high-accuracy exact-matching isotope dilution mass spectrometry (IDMS) method for 19-norandrosterone (NNA) in human urine was developed. The developed IDMS method was based on a published GC/HRMS procedure [1]. However the various components of the method were specifically optimised for analysis of the specific urine matrix of the CRM which was being produced and certified. This included:

- optimisation of the hydrolysis step and measurement of the hydrolysis efficiency
- optimisation of clean-up of the hexane extract with HPLC fractionation employed
- optimisation of GC/HRMS conditions.

In addition, the calibration standards used for this project were rigorously investigated and standards of both the free and glucuronide forms of the steroid were used and compared. A confirmatory LC/MS/MS method was also developed to monitor the level of the glucuronide.

Exact-matching IDMS involves a one-point calibration procedure whereby the isotopically-labelled d_4-19-norandrosterone internal standard is added at the very beginning of the process to both the sample and calibration standard solution to create two blends.



The ratios of analyte to internal standard in each of the sample and calibration solution blends are matched to be equal and the instrumental intensities of all of the analytes are also matched. This technique minimises many of the systematic biases involved in high-accuracy MS measurements.

The uncertainty of the assigned value was thoroughly assessed with all analytical biases investigated and factors covering sample homogeneity and stability incorporated. The overall expanded relative uncertainty at the 95% confidence level was estimated as 8%, which should meet the needs of the WADA-accredited user community. The certified level of 19-norandrosterone (as the sum of the free and glucuronide forms of the steroid) in the CRM is certified as 2.13 ± 0.17 ng/g or 2.15 ± 0.17 ng/mL as mass fraction or mass concentration units.

References

1 W. Schanzer and M. Donike, "*Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites*", Anal. Chim. Acta, **275**, 23-48, 1993.

2 WADA Technical Document-TD2004NA. Reporting Norandrosterone Findings. 28 May, 2004.

3 L.G. Mackay, C.P. Taylor, R.B. Myors, R. Hearn and B. King, "High accuracy analysis by isotope dilution mass spectrometry using an iterative exact matching approach", Accred. Qual. Assur., **8**, 191-193, 2003.