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Development of a High-Accuracy IDMS Method for 19-Norandrosterone in Urine for the Certification of a Reference Material

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Abstract

A high-accuracy exact-matching isotope dilution mass spectrometry (IDMS) method for 19-norandrosterone (19-NA) in human urine was developed for the certification of a reference material (CRM NMIA X002). The CRM was prepared at the 2 ng/mL level for the total of the free and glucuronide forms of 19-NA (expressed as the free steroid). The method was developed for the certification of this CRM and optimised specifically for the matrix of the CRM. The method development included investigation of all potential analytical biases. The uncertainty of the reference method was rigorously investigated with the achieved expanded uncertainty being less than 4% at the 95% level of confidence. All measurements were made as mass fraction (ng/g) to provide higher accuracy.

Introduction

In July 2003, a World Anti-Doping Agency (WADA) funded project was commenced at NMI for the production of a urine-matrix reference material containing close to 2 ng/mL 19-NA and development of a high-accuracy reference method for its certification. The measurand for the method was the total of the free and glucuronide forms of 19-NA, in order to conform with the WADA requirements for reporting 19-NA [1]. IDMS is considered to be a potential primary method of measurement [2] and the appropriate use of a primary ratio method such as IDMS should ensure that the value assigned to a CRM will be traceable to the SI and have a very well-defined measurement uncertainty. The developed IDMS method was based on a published GC/HRMS procedure [3] with each component of the method optimised for analysis of the specific urine matrix of the CRM which was being produced and certified. In addition, the purity of 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.

Experimental

Chemicals and reagents: All solvents and reagents were of analytical grade. All aqueous solutions and buffers were prepared using 18 MΩ.cm water (Milli-Q, Millipore). β-Glucuronidase from *Escherichia coli* was from Roche Diagnostics (Mannheim, Germany), N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was from Sigma-Aldrich, Milwaukee, USA. Synthetic certified reference materials for 19-norandrosterone (19-NA), 19-norandrosterone glucuronide (19-NAG), 19-norandrosterone sulfate, d_4-19-norandrosterone (d_4-19-NA), d_4-androsterone glucuronide and trenbolone were obtained from the Chemical Reference Materials Team, NMI (Sydney, Australia). A portion (25 μg) of d_4-19-norandrosterone glucuronide was obtained from the German Sport University (Cologne, Germany).

GC/HRMS Method

IDMS blend preparation: Freeze-dried urine CRM NMIA X002 was reconstituted with water (20.00 g), gently inverted to dissolve the solid, then heated at 40°C for 30 min and equilibrated to room temperature. A portion (4 g) of the reconstituted material was then taken and the internal standard d_4-19-NA added (0.4 g, 20 ng/g solution in 0.1 M carbonate buffer). Calibration blends were prepared by adding the free steroid 19-NA (0.4 g, 20 ng/g solution in 1,2-dimethoxyethane) and d_4-19-NA (0.4 g, 20 ng/g solution in 0.1 M carbonate buffer) to water (4 g). Some calibration blends were also prepared with the glucuronide form of the steroid for comparison of the two standards.

Hydrolysis and extraction: Blends were adjusted to pH 6 using phosphoric acid (1.5 M), 50 μL of β-glucuronidase was added and the mixture incubated at 40ºC for 1 hour. To each blend, carbonate buffer (pH 9.8, 20%, 500 μL) and NaCl (1 g) was then added and the solution extracted with hexane (3 mL) three times. All three extracts were combined and evaporated to dryness under nitrogen at 50°C.

Fractionation by HPLC: The residues were redissolved in a mixture of water/methanolic trenbolone solution (retention time marker for HPLC). Portions (80 μL) were fractionated using a Waters Nova-Pak 125 × 3.9 mm RP C18 column at 1.00 mL/min, solvent programme:

67% methanol for 7 min, to 100% methanol at 8 min, 100% methanol for 3 min, to 67% methanol at 12 min, 67% methanol for 3 min. The fraction eluting between 4:00 to 5:45 min was collected and evaporated to dryness under nitrogen.

Derivatisation and GC/HRMS analysis: Residues were silylated by addition of 250 μL TMIS reagent as described in [3] and were analysed using a MAT 95 HRMS with HP 6890 gas chromatograph: injection volume 2 μL; column DB5ms 30 m x 0.25 mm x 0.25 μm film; inlet 280ºC pulsed (60 psi, 1 min) splitless; helium carrier gas constant flow 0.9 mL/min; temperature programme 180°C hold 1 min, then 40°C/min to 270°C, hold 5 min, then 40°C/min to 290°C, hold 3 min; MS transfer line 280ºC; ion source 220ºC; electron impact positive ions; filament 1.0 mA; electron energy 70 V; resolution 1400; selected ions 405.30, 409.32, 420.33, 424.35. The retention time for derivatised 19-NA was approximately 7 min with elution in the isothermal region of the GC temperature program.

LC/MS/MS Method

IDMS blend preparation: Freeze-dried urine CRM NMIA X002 was reconstituted as described for GC/HRMS analysis. Sample blends were prepared by adding d_4-19-NAG (0.4 g, 200 ng/g in carbonate buffer) to the entire 20 mL of reconstituted urine CRM. Calibration blends were prepared by adding 19-NAG and d_4-19-NAG to either 20 g of blank urine or water. Blends were incubated at 40ºC for 30 min, equilibrated to room temperature and loaded onto a Strata-X SPE cartridge (200 mg, 6 mL, Supelco) pre-conditioned with water (6 mL), then methanol (6 mL). The cartridge was washed with water (6 mL) and aqueous methanol (5% methanol v/v, 6 mL) then air dried and eluted with methanol (4 mL). The eluate was evaporated to near dryness at 55ºC under nitrogen then reconstituted in aqueous ammonium acetate buffer (25 mM, pH 7, 200 μL). Extracts were analysed using a Micromass Quattro Micro MS/MS with a Waters 2795 LC. The LC column was a Phenomenex Luna 5μ C18 (2) 150 x 2 mm column with 1 mm C18 opti-guard column housed at 30ºC. The mobile phase was 50% 25 mM ammonium acetate at pH 7 and 50% methanol. MS conditions were positive electrospray, capillary 3.00 kV, source 120°C, desolvation temperature 450°C, cone gas 100 L/Hr, desolvation gas 400 L/Hr, collision gas pressure 5 x 10^{-3} mbar. MRM transitions were 470.2 > 259.2, 470.2 > 241.2, 474.2 > 263.2, 474.2 > 245.2 with dwell time 0.5 sec, cone voltage 15 V.

Results and Discussion

Exact-matching isotope dilution mass spectrometry

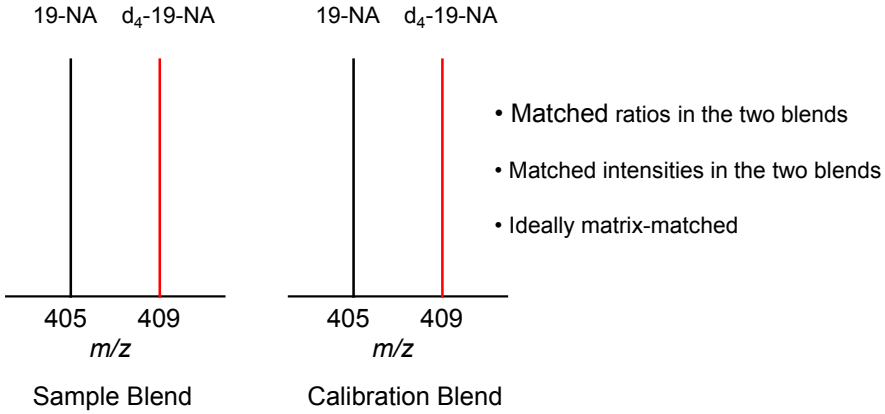
The analytical technique used as the basis of both the GC/HRMS and LC/MS/MS high-accuracy reference methods that were developed was exact-matching isotope dilution mass spectrometry [4]. The basic IDMS measurement equation for this is described in equation 1.

$$C_X = C_Z \cdot \frac{M_Y \cdot M_{Zc}}{M_X \cdot M_{Yc}} \cdot \frac{R_Y' - R_B'}{R_B' - R_X'} \cdot \frac{R_{Bc}' - R_X'}{R_Y' - R_{Bc}'} \qquad (1)$$

C_X = mass fraction of analyte in sample
C_Z = mass fraction of analyte in the standard solution added to the calibration blend
M_Y = mass of labelled internal standard solution added to sample blend
M_{Yc} = mass of labelled internal standard solution added to calibration blend
M_X = mass of sample added to sample blend
M_Z = mass of standard solution added to calibration blend
R'_Y = observed isotope amount abundance ratio in isotopically-labelled internal standard
R'_X = observed isotope amount abundance ratio in sample/calibration standard
R'_B = observed isotope amount ratio in sample/internal standard blend
R'_{Bc} = observed isotope amount ratio in standard/internal standard blend

Exact-matching IDMS involves a one-point calibration procedure whereby the isotopically-labelled d_4-19-NA internal standard is added at the very beginning of the process to both the sample and calibration solution to create two blends. The ratios of analyte to internal standard in each of the sample and calibration blends are matched to be equal and the instrumental intensities of all of the analytes are also matched. The sample and calibration blends are treated as similarly as possible and the calibration blend is subjected to exactly the same sample work-up (i.e. hydrolysis, extraction, HPLC fractionation etc.) as the sample blend. A summarised description of exact-matched blends is given in Figure 1. Ideally the calibration blend would also be matrix-matched in a urine matrix. In this case solution calibration blends were utilised with some confirmatory blends prepared in blank urine for comparison. These two types of calibration blend were shown to be in very good agreement and hence matrix-matching was not routinely employed.

Figure 1: Description of exact-matched sample and calibration blends for IDMS.



The technique of exact-matching minimises the effect of many of the systematic biases involved in high-accuracy MS measurements. It also simplifies the calculations necessary for determining the mass fraction of 19-NA in urine using Equation 1. Equation 1 is derived based on the assumption that the isotope ratio parameters are actual isotope ratios, rather than the instrumentally observed ratios. The use of the exact-matching approach allows the observed isotope ratios in the sample and calibration blends to be used as it is assumed that any biases on the ratios will cancel.

GC/HRMS methodology

The primary method developed at NMI involved the GC/HRMS analysis of the urine CRM. All aspects of the typical GC/HRMS analysis method [3] for 19-NA were examined and optimised for the specific matrix of this CRM. The main aspects investigated were the hydrolysis conditions, the clean-up conditions and the GC/HRMS conditions. An example of optimisation of the GC/HRMS conditions is the resulting GC program used for analysis. The GC temperature ramp was defined to ensure that the analyte elutes in the isothermal section of the program. This was desirable in order to obtain the best precision in the peak areas by ideally ensuring that any background effects were maintained as constant as possible. The final optimised conditions are outlined in the Experimental section.

Potential measurement biases

The development of high-accuracy reference methods requires considerable effort to be put into examining any potential biases in the method. This is necessary so that appropriate uncertainties can be assigned to the various factors involved and to ensure traceability to the SI within the claimed uncertainty. The major factors that were investigated are summarised in Table 1.

Table 1: Potential method bias factors investigated during validation of the method

Factor	Means of investigation
Calibration standards	• Use of the free and glucuronide forms of the calibration standards • Preparation of multiple standard solutions by different operators and in different solvents
Hydrolysis effects	• Measurement of the hydrolysis efficiency from standard addition experiments • Comparison of results using the free steroid and its glucuronide forms of the internal standard
Interference effects	• Use of different GC columns • Use of different MS resolutions
Matrix effects	• Standard addition experiments • Matrix-matched calibration standards using blank urine

The investigation of some of these key factors is described below. These individual experiments were combined with the development of a completely independent LC/MS/MS method.

Measurement of the hydrolysis efficiency

When d_4-19-NA was used as the internal standard, the hydrolysis step was not controlled and any reduction in enzymatic hydrolysis yield would directly affect the accuracy of the result. Hence, two methods were used to measure the efficiency of the hydrolysis step:

- Determination of the recovery of 19-NA and 19-NAG reference standards added gravimetrically to the urine CRM using d_4-19-NA internal standard (standard addition), and
- Determination of total 19-NA in the urine CRM using each of the internal standards d_4-19-NA and d_4-19-NAG, thus controlling the hydrolysis step

The recovery of the 19-NAG standard measured against the free steroid was 99.2%, i.e. the different forms of the standard agreed very well. The recoveries measured for the standard addition experiments are shown in Table 2. Within the method precision there was no observable difference between the recoveries determined from the addition of the free steroid or the glucuronide form and thus there appears to be very efficient hydrolysis of the glucuronide to its free form.

Table 2: Results for addition of free or glucuronide forms of 19-norandrosterone to the urine CRM

Standard addition experiment	Recovery
Urine CRM with added free steroid	99.4%
Urine CRM with added glucuronide	100.9%

At the end of the certification project for NMIA X002 a small quantity of d_4-19-NAG became available (provided by the Cologne laboratory). This material was tested by the GC/HRMS method and the mean results for each of the internal standards are given in Table 3. The results show excellent agreement between the two internal standards with a relative percent difference of 0.8% indicating that there was no significant bias involved in the hydrolysis stage.

Table 3: Results for the urine CRM using d_4-19-norandrosterone glucuronide internal standard

Internal standard	Calibration standard	Mean result (ng/g)	Number of replicate analyses
GC/HRMS result with d_4-19-norandrosterone glucuronide internal standard	19-norandrosterone	2.112	3 bottles, each sample injected 5 times
GC/HRMS result with d_4-19-norandrosterone internal standard	19-norandrosterone	2.128	30 bottles, each sample injected 5 times

Confirmatory results using different GC columns

Potential chromatographic interferences were assessed by comparing the difference in the results obtained using two GC columns of very different polarity (5% phenyl methyl polysiloxane versus 50% cyanopropylphenyl-dimethylpolysiloxane) resulting in a 100°C difference in the analyte elution temperature. The results obtained are summarised in Table 4. The difference in the 19-NA mass fractions determined between the two columns was 1.5% indicating that the presence of significant interferences was unlikely.

Table 4: Results for the urine CRM using two different GC columns by GC/HRMS. The values below are the average 19-NA mass fractions determined using the same extracts from nine bottles.

GC Column	19-NA (ng/g)	Number of replicate analyses
DB 5ms	2.119	9 bottles, each sample injected 5 times
DB 225ms	2.152	9 bottles, each sample injected 5 times
Relative percent difference	1.6%	

LC/MS/MS determination of 19-norandrosterone glucuronide in urine CRM

A LC/MS/MS method was developed to confirm the results from the GC/HRMS method. Initially, d_4-androsterone glucuronide was used as an internal standard, but when a small amount of d_4-19-norandrosterone glucuronide became available this was used as the internal standard. The method involves no enzyme hydrolysis and gives a direct measurement of the level of glucuronide present. Since a 19-NAG standard was used in calibration blends a completely independent assessment of the mass fraction was obtained. LC/MS/MS results are summarised in Table 5.

Table 5: Results for the urine CRM using the LC/MS/MS method with two internal standards

Confirmatory method	Calibration standard	Mean result (ng/g)	Number of replicate analyses
LC/MS/MS result with d_4-androsterone glucuronide internal standard	19-norandrosterone glucuronide	2.156	3 bottles, each sample injected in triplicate
LC/MS/MS result with d_4-19-norandrosterone glucuronide internal standard	19-norandrosterone glucuronide	2.158	3 bottles, each sample injected in triplicate

The results show excellent agreement between both internal standards and with the GC/HRMS certified value of 2.128 ng/g. The relative percent difference in the mean of the two LC/MS/MS results to the GC/HRMS certified value is 1.3%. This reflects excellent agreement between the two methods with each method measuring a subtly different measurand. As the LC/MS/MS method measures the glucuronide form only, while the GC/HRMS method measures the sum of the free and glucuronide forms, this suggests that 19-NA exists predominantly in its glucuronide form in this material.

Measurement uncertainty

An ISO GUM [5] approach to the uncertainty estimation for the GC/HRMS reference method was used whereby a full measurement equation was defined and a standard uncertainty for each parameter in the measurement equation was estimated. The basic IDMS equation (equation 1) was expanded to include appropriate contributing factors from sample preparation. The expanded equation is given in equation 2.

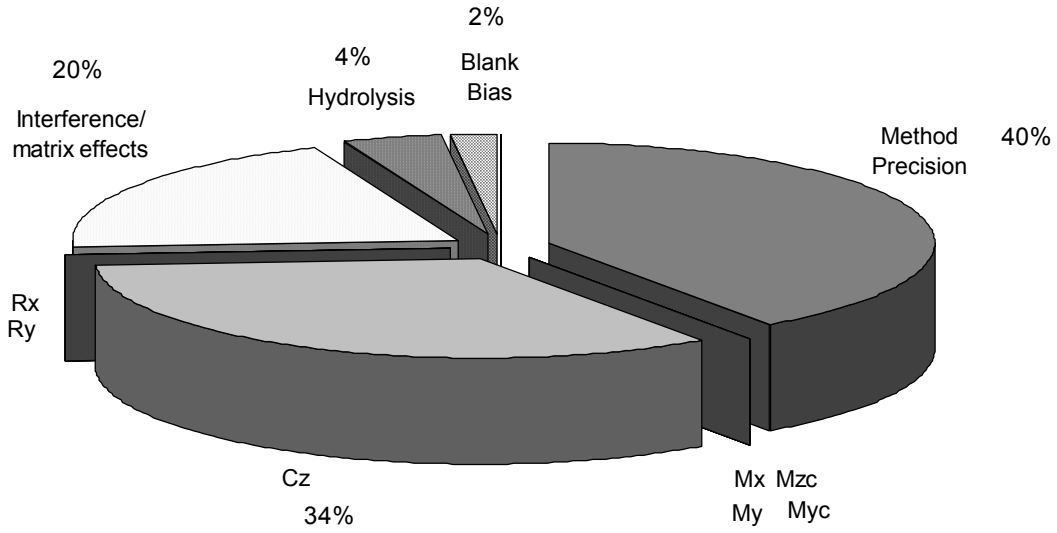
$$C_X = P_M.F_1.F_2.F_3.C_Z \cdot \frac{M_Y \cdot M_{ZC}}{M_X \cdot M_{YC}} \cdot \frac{R_Y' - R_B'}{R_B' - R_X'} \cdot \frac{R_{BC}' - R_X'}{R_Y' - R_{BC}'} \qquad (2)$$

P_M = overall method precision factor
F_1 = interference/matrix effects factor
F_2 = hydrolysis effects factor
F_3 = blank factor

Standard uncertainties were estimated for the mass fraction of the calibration solution, the masses used to prepare the sample and calibration blends and the isotope ratios. A method precision factor was used to capture all precision effects resulting from multiple measurements. In this case, factors having only a precision component could be omitted in the uncertainty budget. The main factors this applies to are the peak area ratios in the sample and calibration blends, R'_B and R'_{Bc} as the effects of any biases on these ratios were minimised by the exact-matching approach employed here.

Standard uncertainties for the hydrolysis and interferences parameters in equation 2 were estimated using the data available from the confirmatory experiments which were carried out. For each of the resulting standard uncertainties, their degrees of freedom were determined [5]. The standard uncertainties were combined using appropriate sensitivity coefficients for each input factor. Sensitivity coefficients mathematically describe the dependence of the result C_X on each specific input parameter. The combined uncertainty is then converted to an expanded uncertainty at the 95% level of confidence using the appropriate k factor using the total effective degrees of freedom (in this case k = 2.00). The contribution of each of the major factors to the total expanded uncertainty is given in Figure 2.

Figure 2: Contribution to the overall expanded uncertainty of the GC/HRMS reference method as applied to the measurement of the urine CRM.



The overall relative expanded uncertainty of the reference method was 3.7% at the 95% level of confidence. This very low uncertainty is a result of considerable optimisation of the method specifically for the urine matrix of the CRM it was developed to analyse.

Conclusions

A high-accuracy exact-matching IDMS GC/HRMS method for the analysis of 19-NA in urine was developed. The developed GC/HRMS method achieved the desired uncertainty of 3-4% as outlined in the original WADA funding proposal. This target was set in order to achieve an appropriately low uncertainty for the value assigned to the resulting certified reference material being produced at NMI (NMIA X002). This low uncertainty was achieved by considerable optimisation of the method. It should also be noted that all potential biases in the method were rigorously investigated and included in the uncertainty budget. This method can be considered a primary method of measurement providing traceability to the SI [2].

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