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Production and Certification of a Reference Material for the Measurement of 19-Norandrosterone in Human Urine

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Abstract

A freeze-dried human urine reference material, fortified with 19-norandrosterone glucuronide, was produced following the International Organization for Standardization (ISO) Guides 34 and 35 and certified using a high-accuracy exact-matching isotope dilution mass spectrometry (IDMS) method for the concentration of total 19-norandrosterone. All measurements using the reference method were made as ng/g and then the final certified value was converted to ng/mL using the density of the reconstituted certified reference material (CRM). Very good homogeneity was demonstrated and the concentration of 19-norandrosterone in the freeze dried material was stable at the recommended storage temperature of -20°C 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 the level of the analyte only at 40°C; at this elevated temperature the level had dropped by 25% after 12 months storage. Stability testing of the reconstituted urine, kept in its liquid form at 4°C for 4 weeks, showed no change in the analyte level. The uncertainty of the assigned value was thoroughly assessed with all analytical biases investigated and factors covering sample homogeneity and stability incorporated. The use of high-accuracy IDMS determination of concentration ensured that the value assigned to the CRM was traceable to the SI with a well-defined uncertainty. The overall expanded relative uncertainty at the 95% confidence level was estimated to be 8%, which meets the needs of the World Anti-Doping Agency (WADA)-accredited user community. The uncertainty of the reference method used to assign the value to the CRM is 4%, the rest is largely due to the inclusion of stability factors to cover any potential change during the certification period. The certified level of 19-norandrosterone (as the sum of the free and glucuronide forms of the steroid) in the CRM was 2.15 ng/mL.

Introduction

In 2004, the WADA statistics showed that nandrolone was the second most commonly abused steroid amongst those detected. A urine matrix CRM (NMIA MX002) has thus been produced in conjunction with WADA for the major nandrolone metabolite, 19-norandrosterone. The reference material was prepared at the allowed cut-off level for 19-norandrosterone of 2 ng/mL. The exact measurand for the CRM was defined in accordance with the requirements of the WADA technical document TD2004 NA "Reporting Norandrosterone Findings" [1]. The measurand was thus defined as the total of the free and glucuronide forms of 19-norandrosterone. The certified reference material was certified using a high-accuracy isotope dilution mass spectrometry method [2] and by following the best practice guidelines outlined in ISO Guides 34 and 35 [3,4]. These guides provide full details for the appropriate preparation, instrumental analysis, homogeneity testing, stability testing and statistical analysis of reference materials.

Experimental

Production of the CRM: Urine samples were collected from donors and screened for the level of total 19-norandrosterone (19-NA), pH and specific gravity. The selected urines were treated with NaN_3 (13.5 g for 27 L of urine) to produce a 0.05% solution. The urine was clarified by filtering through a 0.65 µm filter (Sartopure PP2 Capsule, Sartorius) and then a 0.2 µm filter (Sartobran P sterile filter, Sartorius) using a peristaltic pump into a 50 L HDPE plastic drum. The 25 L of filtered urine was then fortified with a 19-norandrosterone glucuronide (19-NAG) solution (7.92 g of a 6.57 µg/g methanol solution, expressed as the free steroid) to give a level equivalent to 2.1 ng/mL of the free steroid. Portions (20 mL) of the urine were dispensed into 1206, 30 mL serum bottles, freeze-dried, stoppered and stored at -20°C.

Homogeneity testing: Thirty bottles for homogeneity testing were selected at a regular interval of every thirty-eighth bottle produced. The mass of urine dispensed and the mass fraction of total 19-NA in the reconstituted urine was determined for these bottles. A further eight bottles were reserved for moisture determination after freeze-drying.

Stability testing: Seventy-two of the freeze-dried bottles were stored at four different temperatures for accelerated and reference stability testing. The reference and recommended

storage temperature was -20°C and has been monitored at 1, 2, 3, 6 and 12 months to date, and hereafter will be monitored annually. Accelerated studies were conducted at 4°C, 22°C (room temperature) and 40°C with determinations at 1, 2, 3, 6 and 12 month time points. Seven bottles were reconstituted with water and stored refrigerated and tested over a 1 month period to examine the stability of the CRM in its liquid form.

Certification analyses: The high-accuracy gas chromatography high resolution mass spectrometry (GC/HRMS) IDMS method used to determine the mass fraction of total 19-NA in these samples is detailed separately in these proceedings. Briefly, the freeze-dried material was reconstituted with water (20 g) and a portion (4 g) was hydrolysed with β-glucuronidase, extracted with hexane, fractionated by analytical high performance liquid chromagraphy (HPLC) and the dried fraction silylated and analysed by GC/HRMS. This method produced a highly accurate value for the mass fraction of the sum of free and glucuronide forms of 19-NA. This mass fraction value in ng/g was converted to a certified mg/mL value using the density measured for the reconstituted CRM.

Results and Discussion

The production of a certified reference material such as this requires several key factors:

- appropriate control over the production processes
- an appropriate analysis method providing adequate accuracy and precision
- adequate homogeneity of the packaged material
- adequate stability of the material under various conditions
- an appropriate estimate of the uncertainty in the certified value of the material

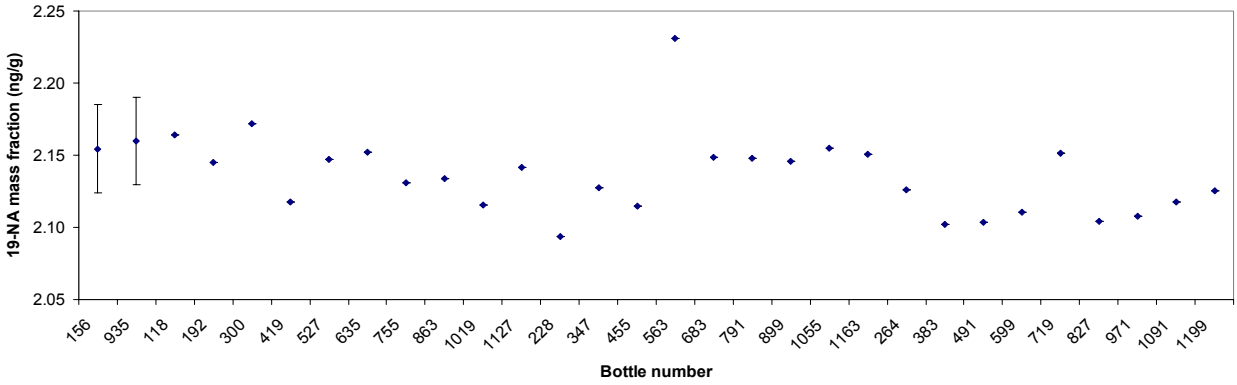
Monitoring production parameters of the CRM

The production of 1200 units from the bulk 25 L of urine involved the dispensing and freeze-drying into individual aliquots. The mass of urine dispensed was measured in 44 bottles selected at regular intervals over the entire production. The average mass dispensed was 20.1312 g with a standard deviation of 0.0104 g (0.05%). The average mass of the freeze dried urine powder was 0.4768 g with a standard deviation of 0.0008 g (0.17%). These two results demonstrate excellent control over these crucial production parameters.

Homogeneity testing

Results from homogeneity determinations both within bottles and between bottles are presented in Figure 1. All homogeneity testing was carried out by an IDMS GC/HRMS method developed for this certification project. Homogeneity within bottles was estimated by determining the 19-NA mass fraction in five subsamples from each of two bottles. The average mass fraction was 2.157 ng/g with a standard deviation of 0.015 ng/g (0.68%). Homogeneity between bottles was estimated by determining the 19-NA mass fraction in a subsample from each of 30 bottles. The average mass fraction was 2.128 ng/g with a standard deviation of 0.025 ng/g (1.16%).

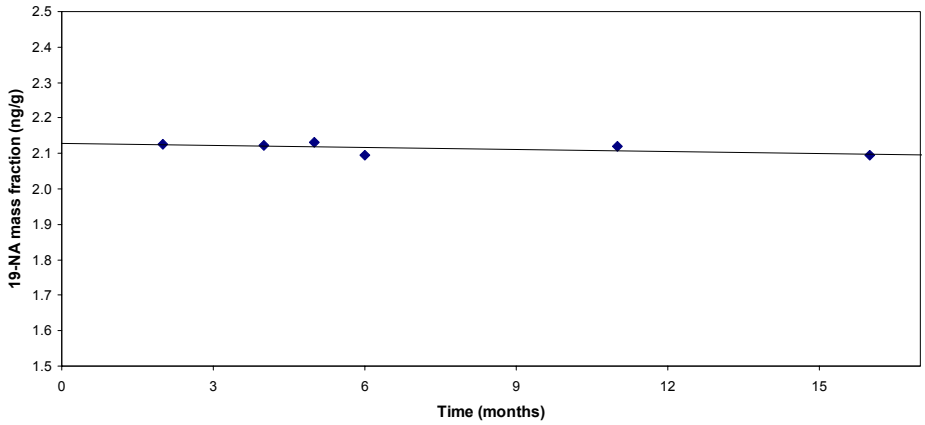
Figure 1: Homogeneity determination results. The first two bottles were subsampled five times to estimate within bottle homogeneity. Error bars represent 2 x the standard deviation of the five determinations. A single subsample was analysed for the remaining 28 bottles.



Stability monitoring at -20 ℃

Results of stability monitoring determinations by GC/HRMS are presented in Figure 2. These results confirmed that the total mass fraction of 19-NA in the freeze-dried material was stable at -20ºC over the period of testing; the slope of the regression line was not significantly different from zero. The regression line was extrapolated to determine the maximum possible change in the mass fraction of 19-NA over a 36-month period up to April 2008 (the current certification period of the CRM). This value was used to estimate a long-term stability factor for the uncertainty in the certified value. The relative standard uncertainty in this stability factor was 2% over this time period.

Figure 2: Stability monitoring of freeze-dried CRM bottles stored at -20ºC.

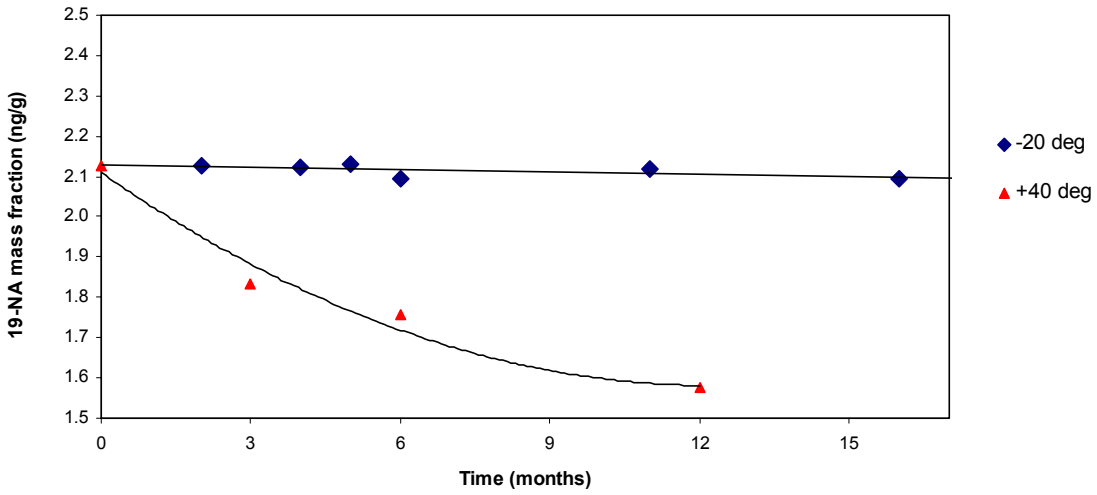


Accelerated stability testing

Results from accelerated stability testing are summarised in Figure 3 where the results for -20°C and 40°C are shown. The mass fraction of 19-NA did not change significantly at -20°C, 4°C or 22°C over the 12-month period but there was a 26% decrease in the 19-NA level in bottles stored at 40ºC over the same period.

The 19-NA mass fraction results at 40ºC were used to determine the short-term stability of the material at potentially elevated temperatures during transportation. Extrapolation of the regression equation fitted to the 40ºC results was used to estimate the potential decrease in the analyte level over 0.25 months (eg during a week of transport at 40ºC). This decrease was 0.6% of the certified value and was thought to be adequately incorporated in the long-term stability factor of 2% described above. Hence an additional short-term stability factor was not included in the CRM uncertainty.

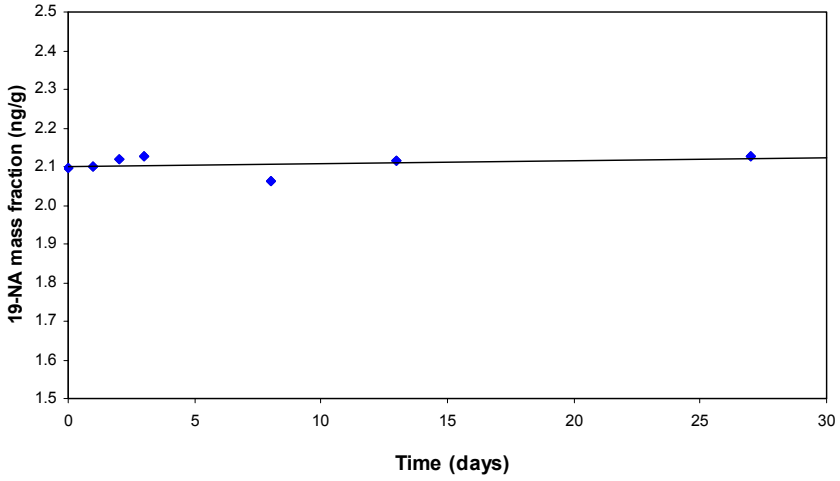
Figure 3. Accelerated stability trial results of the freeze-dried material.



Stability of reconstituted urine

Seven bottles of freeze-dried material were reconstituted with water, combined and re-bottled to ensure that the contents of each bottle were identical. Each bottle was crimp sealed and refrigerated. Duplicate portions (4 g) were taken from the bottles immediately after reconstitution then at 2 h and 4 h after reconstitution, and then at 1, 2, 3, 8, 13 and 27 days after reconstitution. Bottles were resealed after portions were taken and returned to refrigerator for subsequent sampling to mimic use in a routine laboratory. The mean of the duplicate results at each time point is plotted in Figure 4. There was no significant change in the 19-NA mass fraction over the 27 day period when tested by regression analysis. The average mass fraction of all the subsamples taken from the seven bottles was 2.104 ng/g with a standard deviation of 0.023 ng/g (1.1%).

Figure 4: Mass fraction of total 19-NA in samples of reconstituted CRM stored in the refrigerator for 27 days.

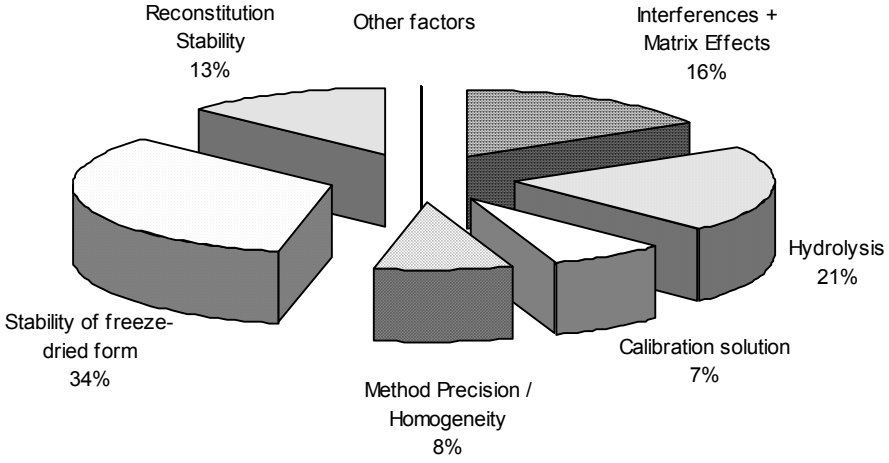


Uncertainty estimation

The total uncertainty in the certified value of the freeze-dried urine CRM was estimated according to the ISO Guide to the Expression of Uncertainty in Measurement (GUM) [5] and as outlined in ISO Guides 34 and 35 [3,4]. The uncertainty was estimated by incorporating additional factors into the reference method uncertainty defined previously in these proceedings. The additional factors were long-term stability of the freeze-dried material, stability of the reconstituted material and a density correction factor. An additional factor for homogeneity was not necessary as this was incorporated within the method precision factor which was calculated as the standard deviation of the results for the 30 bottles. The major contributors to the uncertainty are summarised in Figure 5.

The total relative expanded uncertainty calculated by combining these factors was 8% of the CRM's certified value (at the 95% level of confidence), certified for a certification period until April 2008. The uncertainty of the reference method used to assign the value to the CRM is 4%, the rest is largely due to the inclusion of stability factors to cover any potential change during the certification period. The uncertainty is certainly fit for purpose for the user laboratories of this material.

Figure 5: Major contributors to the total uncertainty for the CRM



Certified value

The certified value for the CRM was calculated from the homogeneity determination data from the GC/HRMS results for 30 bottles averaged against four different calibration solutions. The average mass fraction of the total of 19-NA and 19-NAG expressed as the free steroid was 2.13 ng/g. The IDMS data obtained in mass fraction units (ng/g) were converted to concentration units (ng/mL) using the measured density of the reconstituted material. The density of the reconstituted material (specific gravity) measured in the NMI mass laboratory was 1.00875 ± 0.00006 g/mL at 20°C. The pH of the reconstituted material was 6.7 at 22°C, measured using a routine laboratory pH meter.

The certified levels (as mass fractions (ng/g) and mass concentrations (ng/mL)) assigned to the urine material NMIA MX002 are summarised below in Table 1.

Table 1: Summary of certified values

Material	Certified value	Relative expanded uncertainty
19-norandrosterone urine CRM NMIA MX002	2.13 ± 0.17 ng/g	8.1%
	2.15 ± 0.17 ng/mL	8.1%

Conclusions

A urine matrix reference material was successfully produced for 19-norandrosterone at a level close to the WADA cut-off of 2 ng/mL and certified using a high-accuracy IDMS method. The material showed no evidence of any significant between-bottle inhomogeneity and displayed very good stability, except at elevated temperatures. It has a current expiry date of 30 April 2008 when stored at -20 ºC. The material is certified such that it may also be stored refrigerated in its reconstituted form for up to four weeks.

The certified value for the CRM was assigned using a rigorous reference method and is traceable to the SI. The uncertainty in the certified value was estimated by incorporating all appropriate components.

Acknowledgements

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