



**CERTIFICATE OF ANALYSIS  
FOR CERTIFIED REFERENCE MATERIAL  
NMIA MX005**

**Certificate I: Steroid Metabolites in Freeze-Dried Human Urine**

**Batch No.: 2006.12**

**(i) Description of the material**

This reference material consists of freeze-dried human urine contained in a sealed glass bottle with a crimped rubber septum. In addition to natural levels of steroid metabolites, the material was fortified with the glucuronide conjugates of testosterone, androsterone and etiocholanolone. The material must be reconstituted with 20 mL of water as described in this certificate prior to use.

**(ii) Certified property values**

The certified property values for selected steroids in the urine reference material after reconstitution with water as specified in Section (v) are given in Table 1. The measurands are defined as the mass fractions, mass ratios or mass concentrations of all glucuronide conjugates ( $\beta$ -D-glucopyranosiduronic acid derivatives) of the stated steroid (expressed as equivalents of the free steroid) in the reconstituted material at the expiry date of this certificate. The masses of any corresponding endogenous free steroids present in insignificant amounts in the urine material are included in each of the certified property values except for those of testosterone and epitestosterone. The masses of any other possible conjugates of the steroids are not included in the measurands. Mass concentrations apply to the reconstituted material at 20 °C.

**Table 1: Certified property values for steroids present as glucuronide conjugates in the reconstituted human urine CRM NMIA MX005 expressed as equivalents of the free steroid. Uncertainties are expanded to provide a level of confidence of 95%.**

Steroid	CAS Registry Number	Mass fraction (ng/g)	Mass concentration (ng/mL)
5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol	1852-53-5	7.20 $\pm$ 0.40	7.27 $\pm$ 0.41
5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol	1851-23-6	20.9 $\pm$ 1.2	21.1 $\pm$ 1.2
androsterone	53-41-8	1176 $\pm$ 35	1187 $\pm$ 35
etiocholanolone	53-42-9	1281 $\pm$ 40	1293 $\pm$ 41
testosterone (T)	58-22-0	39.9 $\pm$ 1.7	40.3 $\pm$ 1.8
epitestosterone (E)	481-30-1	10.66 $\pm$ 0.59	10.76 $\pm$ 0.59
T/E mass ratio	-	3.74 $\pm$ 0.24	

**(iii) Expiration of certification**

The certified values are valid for sealed bottles of the material until **22 October 2017** provided that they are handled and stored in accordance with the instructions given on this certificate.

#### (iv) Intended use

The reference material is intended to be used to validate analytical methods for the measurement of testosterone, epitestosterone,  $5\alpha$ -androstane- $3\alpha$ - $17\beta$ -diol,  $5\beta$ -androstane- $3\alpha$ - $17\beta$ -diol, androsterone and etiocholanolone in human urine for doping, clinical or forensic analysis. It may also be used to calibrate secondary reference materials of similar composition or as a matrix calibration standard.

Note that the certified values are expressed in terms of equivalents of the free steroid. In order to calculate mass fractions or mass concentrations in terms of the glucuronide forms an appropriate correction factor must be applied.

#### (v) Instructions for use

##### HAZARD WARNING

CRM NMIA MX005 IS INTENDED FOR IN-VITRO DIAGNOSTIC ANALYSIS ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL POTENTIALLY CAPABLE OF TRANSMITTING DISEASE.

The recommended minimum sample size for analysis is 5 g of the reconstituted urine. The material must be reconstituted exactly as described below. The reconstituted material is stable for four weeks when sealed and stored at 4 °C.

##### Reconstitution protocol

1. Remove the freeze-dried urine bottle from cold storage and equilibrate to room temperature.
2. Remove the aluminium crimp cap, but leave the rubber septum in place.
3. Weigh the bottle with septum.
4. Fill a clean Pasteur pipette or a 10 mL syringe with purified water<sup>†</sup>, lift the septum to expose the slot in the septum and add 20.00 g<sup>#</sup> water into the bottle through the slot. If necessary, using a clean lint-free tissue, wipe off any drops of water on the exterior of the bottle, for example on the lip of the bottle, and then push the septum down.
5. Reweigh the bottle, septum and water to determine the mass of water added.
6. Seal the bottle with a crimp cap. Invert gently to dissolve all solid material and to rinse the sides of the bottle and the rubber stopper.
7. Heat the bottle at 40 °C for 30 minutes then equilibrate to room temperature.
8. Take subsamples of the reconstituted material. The remainder may be stored in the refrigerator for up to four weeks.

<sup>†</sup> Note if the material is to be stored in its reconstituted form the water and syringe used should be sterile.

<sup>#</sup> The certified values for this material correspond to the addition of 20.00 g water. If the mass of water added is not 20.00 g then a correction must be applied to take this into account. The reconstitution process has been tested with added water of approximately  $20.0 \pm 0.1$  g and thus masses within this range should be used.

#### (vi) Production of the reference material

The material was prepared in December 2006 using urine collected from healthy volunteers. All donors had been vaccinated against Hepatitis A and B and have a very low risk of HIV. All specimens were screened for pH, specific gravity and for the level of testosterone/epitestosterone before use. Selected urine specimens were centrifuged and combined such that the pooled urine contained an approximate epitestosterone concentration of 10 ng/mL. After treatment with sodium azide (0.5 mg/g), thirty litres (30 L) of the pooled material was filtered through 0.65  $\mu$ m and 0.2  $\mu$ m filters. Twenty seven litres (27 L) of the filtered urine was fortified with testosterone glucuronide to a level equivalent to 40 ng/mL of the free steroid. A mixed solution containing 35 mg each of androsterone glucuronide and etiocholanolone glucuronide in 5 mL of methanol was also added. After stirring overnight the bulk material was dispensed in 20 mL aliquots into 50 mL clear glass bottles. The bottled urine was freeze-dried and then stored at -20 °C. The pH of the reconstituted

material was 6.8 measured at 22 °C using a calibrated pH meter. The residual water content of the freeze-dried material was measured by Karl Fischer titration as approximately 2.9%. The average mass of freeze-dried material in each bottle is 0.49 g.

NMIA MX005 was first certified on 4 May 2009 for the mass fractions, mass concentrations and mass ratio of testosterone and epitestosterone and its continued stability was verified in August 2011. The mass fractions and mass concentrations of androsterone and etiocholanolone in NMIA MX005 were determined on 8 June 2010 and those of 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol on 2 November 2010. The stability of the property values for these analytes was investigated in an isochronous stability trial in 2011. The continued stability of all property values was verified in April 2014 permitting an extension of the certificate expiry date and a reduction of the expanded uncertainty associated with the reference values for epitestosterone, androsterone, etiocholanolone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol.

## (vii) Analytical procedures

The mass fractions of testosterone, epitestosterone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, androsterone and etiocholanolone were measured by isotope dilution mass spectrometry [1]. Deuterated free steroid analogues of 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol and etiocholanolone and the isotopically-labeled analogues of testosterone glucuronide, epitestosterone glucuronide and androsterone glucuronide were added to the urine prior to sample preparation. The freeze-dried material was reconstituted with water according to the protocol in (v). Separate analytical methods were used for testosterone and epitestosterone, for 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, and for androsterone and etiocholanolone. Testosterone and epitestosterone were analysed using 5 g of the reconstituted urine combined with a mixed solution of  $^2\text{H}_3$ -testosterone glucuronide and  $^2\text{H}_3$ -epitestosterone glucuronide. A subsample of 10 g of reconstituted urine was analysed for 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol after being combined with a mixed solution of  $^2\text{H}_3$ -5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and  $^2\text{H}_5$ -5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol. A separate subsample of 10 g reconstituted urine was analysed for androsterone and etiocholanolone after being combined with a mixed solution of  $^2\text{H}_4$ -androsterone glucuronide and  $^2\text{H}_5$ -etiocholanolone. The urine samples were hydrolysed with  $\beta$ -glucuronidase enzyme to convert the steroid glucuronide metabolites to their equivalent free steroid forms and these were extracted with hexane. The hexane extracts were evaporated and the residues reconstituted in methanol/water (50:50) prior to clean-up by HPLC. The separate fractions containing testosterone and epitestosterone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, and androsterone and etiocholanolone were evaporated and derivatised to trimethylsilyl derivatives (di-TMS forms). Analysis was performed by GC-HRMS.

The conversion from mass fraction units of ng/g to mass concentration units of ng/mL at 20 °C was achieved by measuring the density of the reconstituted material. The density was measured as  $1009.24 \pm 0.03 \text{ kg/m}^3$  at 20 °C ( $\pm 0.05 \text{ °C}$ ).

## (viii) Homogeneity assessment

Assessment of the inter-bottle homogeneity of the human urine CRM NMIA MX005 was conducted in accordance with ISO Guide 35 [2]. Seventeen bottles of freeze-dried urine material were selected from the batch using a stratified sampling plan. Duplicate sub-samples were analysed from 10 of these bottles and single sub-samples were analysed from the remaining 7 bottles. Homogeneity testing was carried out using the analytical procedures described in Section (vii). Reconstituted urine samples of five gram were used for analysis and determination of the within-bottle and between-bottle variances for testosterone and epitestosterone. Ten gram samples were used for the remaining analytes. The uncertainties in the certified values incorporate these variances.

### (ix) Storage stability assessment

The freeze-dried material should be stored at -20 °C out of direct light in the closed container as issued. The stability of the material in the freeze-dried form, when stored under these conditions, was demonstrated by an isochronous stability trial conducted at -20 °C. No instability was observed in accelerated stability trials at 4 °C but degradation of testosterone and epitestosterone glucuronides occurred after prolonged storage at 22 °C and significant degradation occurred at 40 °C. Exposure of the material to elevated temperatures should therefore be avoided.

The stability of the material after reconstitution with sterile pure water was also investigated at 4 °C. The stability study demonstrated that the reconstituted urine material may be kept refrigerated for up to four weeks in a sealed container.

The uncertainties in the property values were expanded to incorporate the long-term storage stability component extrapolated to cover the period of certification of the property values [2].

### (x) Measurement uncertainty

Measurement uncertainties were estimated according to international standards [2, 3] using the standard operating procedures of the National Measurement Institute. All factors that could reasonably be expected to affect the measurement result were identified and the standard uncertainty of each estimated from experimental data. The standard uncertainties of the various components were combined as described in the Guide to the Expression of Uncertainty in Measurement [3]. The combined standard uncertainties were expanded to a level of confidence of 95% using a coverage factor calculated from the effective degrees of freedom obtained from the Welch-Satterthwaite equation.

The individual components contributing to the measurement uncertainty estimates were the mass fractions assigned to calibration standards, gravimetric mass measurements, precision of the analytical method, batch homogeneity, long-term storage stability of the material at -20 °C, stability of the material during transportation of the freeze-dried material and potential sources of bias in the reference analytical procedure. The major contributing factors to the measurement uncertainties were the mass fractions assigned to calibration standard solutions and the long-term storage stability component extrapolated to cover the period of certification. The coverage factors (k) used and the effective degrees of freedom ( $v_{\text{eff}}$ ) associated with the measurement uncertainties in the certified values are summarised in Table 2.

**Table 2: Coverage factors and the effective degrees of freedom for the measurement uncertainties associated with the mass fractions of steroids in NMIA MX005**

	<b>k</b>	<b><math>v_{\text{eff}}</math></b>
<b>5<math>\alpha</math>-androstane-3<math>\alpha</math>-17<math>\beta</math>-diol</b>	2.13	15
<b>5<math>\beta</math>-androstane-3<math>\alpha</math>-17<math>\beta</math>-diol</b>	2.23	11
<b>androsterone</b>	2.01	52
<b>etiocholanolone</b>	2.00	60
<b>testosterone</b>	2.05	28
<b>epitestosterone</b>	2.03	33
<b>T/E mass ratio</b>	2.05	28

The certified values for testosterone, epitestosterone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, androsterone and etiocholanolone apply to any correctly stored and un-opened bottle of Batch 2006.12 of NMIA MX005 until the expiration date specified on this certificate. The corresponding expanded uncertainties represent the 95% confidence intervals for the property values certified until the date of expiry. The validity of the certification of the material

may be extended by the National Measurement Institute as additional information about the long-term storage stability of the material becomes available.

### (xi) Metrological traceability of property values

The certified values are traceable to the SI unit for mass, the kilogram (kg) through the Australian national standards for mass. The primary ratio method of isotope dilution mass spectrometry (IDMS) was used and the quantities used in the calculation of the certified values are traceable to the SI units. The pure substance reference materials of testosterone glucuronide, epitestosterone glucuronide, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, androsterone glucuronide and etiocholanolone used for the calibration of the IDMS method were traceable to the SI units through certification by NMIA. Balances used in the preparation of samples and calibration standards were all appropriately calibrated and traceable to the SI unit of the kilogram.

The certified mass concentrations are traceable to the SI units for mass (kg) and length (m) through the relevant Australian national standards. The steroid mass fractions obtained by IDMS analysis were converted to mass concentrations using an SI-traceable density measurement performed at the Mass Laboratory of the National Measurement Institute.

### (xii) Legal notice

Neither the National Measurement Institute as a representative of the Commonwealth of Australia, nor any person acting on behalf of the National Measurement Institute, assumes any liability with respect to the use of, or for damages resulting from the use of this reference material or the information contained in this certificate.

#### References:

1. L.G. Mackay, C.P. Taylor, R.B. Myers, R. Hearn and B. King; *Accred. Qual. Assur.*, **8**, 2003, 191-194.
2. *Reference Materials – General and statistical principles for certification*. ISO Guide 35, Third Edition (2006), Annex B5.
3. Joint Committee for Guides in Metrology; *Evaluation of measurement data — Guide to the Expression of Uncertainty in Measurement*; JCGM 100, First edition (2008).

Authorised by:



Dr. E. John Murby  
Team Leader, Chemical Reference Methods  
National Measurement Institute  
Dated: 28 April 2014

The property values specified in this report supersede any issued prior to 28 April 2014.