



## CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

### NMIA MX017: Steroid Metabolites in Freeze-Dried Human Urine

#### Certified values

Steroid #	CAS Registry Number	Mass fraction (ng/g)	Mass concentration (ng/mL)	Coverage Factor (k)
5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol	1852-53-5	41.2 $\pm$ 1.8	41.6 $\pm$ 1.8	2.10
5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol	1851-23-6	66.0 $\pm$ 2.9	66.8 $\pm$ 2.9	2.11
androsterone	53-41-8	1652 $\pm$ 29	1670 $\pm$ 29	1.99
etiocholanolone	53-42-9	1359 $\pm$ 34	1374 $\pm$ 35	2.00
testosterone (T)	58-22-0	88.1 $\pm$ 4.2	89.1 $\pm$ 4.2	2.01
epitestosterone (E)	481-30-1	21.9 $\pm$ 1.0	22.1 $\pm$ 1.0	2.09
T/E mass ratio	-	4.03 $\pm$ 0.26		2.00

*#The measurands are the mass fractions, mass ratios or mass concentrations of the free steroids (if present) and 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 other conjugates of the steroids are not included. Mass concentrations apply to the reconstituted material at 20 °C. Uncertainties are expanded to provide a level of confidence of 95%.*

**Expiry:** 30 September 2020

**Batch No.:** 2016.12

**Description:** 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 and 19-norandrosterone. The material must be reconstituted with 20 mL of water as described in this certificate prior to use.

**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.

**Instructions for use:** The recommended minimum sample size for analysis is 1 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 syringe with purified water<sup>†</sup>, lift the septum to expose the slot in the septum and add 20.00 g water<sup>#</sup> 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 around the sides of the bottle and the rubber stopper.
7. Heat the bottle at 40 °C for 30 minutes and equilibrate to room temperature before taking subsamples of the reconstituted material. The remaining material may be stored at 4 °C 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 so masses within this range should be used.

**Storage:** Store at -20 °C out of direct light in the closed container as issued. Exposure of the material to elevated temperatures should be avoided

**Metrological traceability:** The certified values for mass fraction 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, epitestosterone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol, androsterone 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 provide traceability 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 were converted to mass concentrations using an SI-traceable density measurement.

**Stability:** The stability of the material under the recommended storage conditions has been verified via a protocol compliant with ISO Guide 35:2017<sup>1</sup> and will continue to be monitored. Long term stability was assessed at -20 °C. Transport stability was assessed at 23 °C and the material has been shown to be sufficiently stable for three weeks.

**Homogeneity:** Fourteen bottles were selected using a stratified sampling plan. Duplicate sub-samples were analysed from 8 of these bottles and single sub-samples were analysed from the remaining 6 bottles. Reconstituted urine samples of one gram were used for analysis and determination of the within-bottle and between-bottle variances for androsterone and etiocholanolone. Nine gram samples were used for the remaining steroids. The uncertainties in the certified values incorporate these variances.

**Safety:** CRM NMIA MX017 is intended for in-vitro diagnostic analysis only. Handle product as a biohazardous material potentially capable of transmitting disease.

**Production:** Preparation of the material was completed in December 2016 using urine collected from healthy volunteers. Specimens from individual donors were screened for potential analytical interferences before use. Selected urine specimens were centrifuged and combined such that the pooled urine contained an approximate epitestosterone concentration of 23 ng/mL. After treatment with sodium azide (0.5 mg/g), the pooled material was filtered through 0.65  $\mu$ m and 0.2  $\mu$ m filters. The filtered urine was fortified with testosterone glucuronide to provide a T/E ratio of 4 and with 19-norandrosterone glucuronide to give an approximate concentration of 8 ng/g. After stirring overnight the bulk material was accurately 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 is 6.9 measured at 20 °C using a calibrated pH meter. The average mass of freeze-dried material in each bottle is 0.60 g.

**Analytical method:** The certified 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 including an investigation of the measurement system factor<sup>2</sup>. Isotopically-labelled analogues of the individual steroid glucuronides were added to the urine prior to sample preparation. The freeze-dried material was reconstituted with water according to the specified protocol. Two separate analytical methods were used. Testosterone, epitestosterone, 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol were analysed using 9 g of the reconstituted urine combined with a mixed solution containing glucuronides of <sup>2</sup>H<sub>3</sub>-testosterone, <sup>2</sup>H<sub>4</sub>-epitestosterone, <sup>2</sup>H<sub>4</sub>-5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol and <sup>2</sup>H<sub>3</sub>-5 $\beta$ -androstane-3 $\alpha$ -17 $\beta$ -diol. A separate 1 g subsample of the reconstituted urine was analysed for androsterone and etiocholanolone after being combined with a mixed solution containing glucuronides of <sup>2</sup>H<sub>4</sub>-androsterone and <sup>2</sup>H<sub>5</sub>-etiocholanolone. The urine samples were hydrolysed with  $\beta$ -glucuronidase enzyme from E.coli to convert the steroid glucuronide metabolites to their equivalent free steroid forms and extracted with hexane. The hexane extracts were evaporated and the residues reconstituted in methanol prior to clean-up by HPLC. The HPLC fractions were dried and then derivatised to trimethylsilyl derivatives (di-TMS forms) prior to analysis by GC-MS/MS and GC-HRMS. The density was measured as 1.0112 g/mL with a standard uncertainty of 0.0002 g/mL at a temperature of 20 °C using an oscillating loop densitometer.

**Measurement uncertainty:** Standard uncertainties were estimated and combined as described in the JCGM Guide to the Expression of Uncertainty in Measurement<sup>3</sup>. 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 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.

*P Armishaw*

Paul Armishaw  
Manager Chemical Reference Values  
30 January 2019  
Report ID: MX017.2019.01

## References:

1. Reference Materials – Guidance for characterisation and assessment of homogeneity and stability. ISO Guide 35:2017
2. Complete Equation for the Measurement of Organic Molecules Using Stable Isotope Labeled Internal Standards, Exact Matching, and Mass Spectrometry. Burke, Daniel G and Mackay, Lindsey G. 13, 2009, Analytical Chemistry, Vol. 80, pp. 5071-5078.
3. Joint Committee for Guides in Metrology; Evaluation of measurement data — Guide to the Expression of Uncertainty in Measurement; JCGM 100:2008.

**CIPM MRA Notice:** This certificate is consistent with the capabilities that are included in Appendix C of the CIPM MRA drawn up by the CIPM. Under the CIPM MRA, all participating institutes recognize the validity of each other's calibration and measurement certificates for the quantities, ranges and measurement uncertainties specified in Appendix C. The "CIPM MRA Logo" and this statement attest only to the measurement(s) applied for determining the certified values on the certificate (for details see <http://www.bipm.org>).

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