Australian Government
Department of Industry, Science, Energy and Resources

National Measurement Institute

CIPM MRA

NATA
ACCREDITED FOR TECHNICAL COMPETENCE

CERTIFIED REFERENCE MATERIAL
CERTIFICATE OF ANALYSIS

NMIA MX017: Steroid Metabolites in Freeze-Dried Human Urine

Certificate II: Carbon Isotope Delta Value

Certified values

Steroid	CAS No.	$\delta^{13}C_{VPDB}$ / ‰	Coverage Factor (k)	V_{eff}
19-Norandrosterone	**1225-01-0**	**–29.82 ± 0.41**	**2.0**	**35**

The measurand is defined as the carbon isotope ratio delta value of the stated free steroid in reconstituted CRM after hydrolysis of glucuronide conjugates. The certified value is reported as relative to the primary reference Vienna Pee Dee Belemnite (VPDB), normalised by consensus $\delta^{13}C_{VPDB}$ values of +1.95 ‰ (NBS 19) and -46.6 ‰ (LSVEC). [a] *The uncertainties are expanded to provide a level of confidence of 95%.*

Expiry: 30 September 2021 **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 19-norandrosterone glucuronide. 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 and act as quality control or matrix calibration standard for measurement of carbon isotope ratio of 19-norandrosterone in human urine in doping.[1,2] The concentration of the stated steroid is not certified and is only approximately 8 ng/mL after reconstitution. In-vitro use only.

Instructions for use: The recommended minimum sample size is 20 mL for carbon isotope ratio measurement of free 19-norandrosterone. The volume can be reduced if derivatisation is employed but the minimal volume to obtain optimum instrument response has to be verified by end-user.

Reconstitution protocol

1. Remove the freeze-dried urine bottle from cold storage and equilibrate to room temperature.
2. Remove the aluminium crimp cap and septum, add 20.0 mL purified water.
3. Replace septum and seal the bottle with a crimp cap. Invert gently to dissolve all solid material around the sides of the bottle and the rubber stopper.
4. Heat the bottle at 40 °C for 30 minutes and equilibrate to room temperature before taking subsamples of the reconstituted material.

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 carbon isotope delta value is traceable to the VPDB reference via the NMIA MX018 steroid carbon isotopic reference materials normalised by two secondary isotopic reference materials IAEA-CH-6 (sucrose) and IAEA-CH-7 (polyethylene)[3], in accordance with IUPAC recommendation.[4]

Stability: The stability of the material under the recommended storage conditions has been verified and will continue to be monitored. Transport stability was assessed at room temperature (23 ± 3 °C) over a period of 33 days with no evidence of change in certified value.

Homogeneity: Ten bottles were selected using randomized sampling and tested in a single batch under repeatability conditions using the GC-C-IRMS. No significant inhomogeneity was detected.

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 µm and 0.2 µm 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/mL. 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: Analysis was performed on 20 mL of reconstituted urine after hydrolysis with β-glucuronidase (E.coli). Free steroids were extracted into hexane and purified by two-dimensional HPLC. In-house quality control samples including water spikes, matrix spikes and solvent standards were included to monitor fractionation due to sample transformation procedures. The purified fractions were dried and reconstituted in cyclohexane/2-propanol (4:1) for analysis by GC-C-IRMS. The identical treatment principle was followed to ensure samples and calibration solutions were combusted and transferred the same way into the IRMS. Each sample was injected twice in a randomised order bracketed by calibration standards. Frequent bracketing of the test sample with calibration solutions allowed any drift in the instrument to be fully captured. An approximate δ-value of the internal working gas was used to calculate all raw δ-values for samples and standards. A multi-point isotopic bracketing calibration approach was adopted to normalise all the measured δ-values of samples using steroid isotopic CRM mixtures NMIA MX018-1 and MX018-3. A linear regression line fitted through the measured and the reference delta values from the eight steroid compounds, in the two calibration mixtures, allowed the measured δ-values of 19-norandrosterone in the MX017 to be normalised to the VPDB scale.

Measurement uncertainty: Standard uncertainties were estimated and combined as described in the JCGM Guide to the Expression of Uncertainty in Measurement[5]. The major contributions to the combined measurement standard uncertainty were between-unit homogeneity, measurement precision and reproducibility, potential instability due to transport conditions, potential bias from extraction and matrix effects, and the uncertainty from the calibration procedure. The combined standard uncertainties were expanded with coverage factors calculated from degrees of freedom obtained from the Welch-Satterthwaite equation.

Raluca Iavetz
Manager Chemical Reference Values
9 March 2020

Accreditation No.198

References:

1. WADA *Technical Document – TD2019NA: Harmonisation of analysis and reporting of 19-norsteroids related to nandrolone, Version 1.0*
2. WADA *Technical Document – TD2019IRMS: Detection of synthetic forms of endogenous anabolic androgenic Steroids, Version 1.0*
3. IUPAC *Technical Report: Assessment of international reference materials for isotope-ratio analysis,* Pure Appl Chem, 2014, 86, 425-467
4. IUPAC *Standard atomic weights of 14 chemical elements revised,* Chem Intl, 2018, 40(4), 23-24
5. JCGM *Evaluation of measurement data – Guide to the expression of uncertainty in measurement*. JCGM100:2008

[a] **IUPAC CIAWW Notice:** In 2017, IUPAC advised that LSVEC is no longer suitable for normalization of the VPDB scale but all carbon isotope delta measurements are to be normalized to VPDB using at least two appropriate international reference materials *(online: https://www.ciaaw.org/carbon-references.htm)*

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).

Legal Notice: Terms and Conditions associated with the provision of this reference material can be found on the NMIA website**.**

END

105 Delhi Road, North Ryde, NSW 2113 - GPO Box 2013, Canberra ACT 2601
ABN: 74 599 608 295