



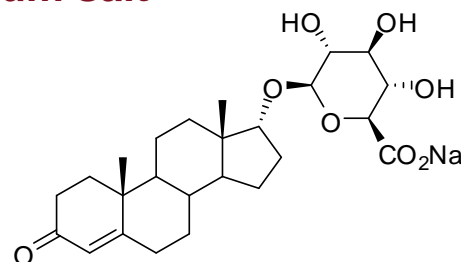
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA S035: Epitestosterone glucuronide sodium salt

Report ID: S035.2020.01

Chemical Formula: C₂₅H₃₅O₈Na

Molecular Weight: 486.5 g/mol



Certified value

Batch No.	CAS No.	Purity (mass fraction)
15-S-10	16996-33-1 (free acid)	88.8 ± 2.6%

The uncertainty has been calculated according to ISO Guide 35 and is stated at the 95% confidence limit ($k = 2$).

IUPAC name: Sodium (17 β)-3-oxoandrost-4-en-17-yl (5 ξ)- β -L-xylo-hexopyranosiduronate.

Expiration of certification: The property values are valid till 13 May 2023, i.e. three years from the date of re-certification provided the **unopened** material is handled and stored in accordance with the recommendations below. The material as issued in the unopened container and stored as recommended below should be suitable for use beyond this date, subject to confirmation of batch stability from the issuing body. The expiry date/shelf life does not apply to sample bottles that have been opened. In such cases it is recommended that the end-user conduct their own in-house stability trials. The material will be re-tested on an annual basis to ensure that the property values are still valid. In the event a product fails the stability trial, notification will be sent to all impacted customers.

Description: White solid was sourced from an external supplier, and certified for identity and purity by NMIA. Packaged in amber glass bottles with a septum and crimped aluminium cap or screw top cap.

Intended use: This certified reference material is suitable for use as a primary calibrator.

Instructions for use: Equilibrate the bottled material to room temperature before opening.

Recommended storage: When not in use this material should be stored at or below 4 °C in a closed container in a dry, dark area.

Metrological traceability: The certified purity value is traceable to the SI unit for mass (kg) through Australian national standards via balance calibration. In the mass balance approach all impurities are quantified as a mass fraction and subtracted from 100%. Quantitative NMR provides an independent direct measure of the mass fraction of the analyte of interest, calibrated with an internal standard certified for purity (mass fraction).

Stability: This material has demonstrated stability over a minimum period of 3 years. The measurement uncertainty at the 95% confidence interval includes a stability component which has been estimated from annual stability trials. The long-term stability of the compound in solution has not been examined.

Homogeneity assessment: The homogeneity of the material was assessed using purity assay by HPLC with UV detection on ten randomly selected 1-2 mg sub samples of the material. The material was judged to be sufficiently homogeneous at this level of sampling as the variation in analysis results between samples was not significantly different at a 95% confidence level from that observed on repeat analysis of the same sample.

Safety: Treat as a hazardous substance. Use appropriate work practices when handling to avoid skin or eye contact, ingestion or inhalation of dust. Refer to the provided safety data sheet.

S. R. Davies

Dr Stephen R. Davies,
Team Leader,
Chemical Reference Materials, NMI.
14 May 2020

This report supersedes any issued prior to 14 May 2020.

NATA logo notice: Accredited for compliance with ISO 17034. Accreditation No. 198 / Corporate Site No. 20844. The results of the tests, calibrations and/or measurements included in this document are traceable to Australian/national standards.

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

Characterisation Report:

The identity was confirmed by a range of spectroscopic techniques, NMR, IR and MS. The certified purity value by qNMR was obtained using the one-proton doublet at 5.88 ppm measured against a certified internal standard of potassium hydrogen maleate.

Supporting evidence is provided by HPLC with UV detection at 245 nm, thermogravimetric analysis, Karl Fischer analysis, ¹H NMR spectroscopy, headspace GC-MS analysis of occluded solvents and elemental microanalysis.

HPLC: Instrument: Waters Model 1525 Binary pump, 717 plus auto sampler
Column: Alltima C18, 5 µm (4.6 mm × 150 mm)
Column oven: 40 °C
Mobile Phase: MilliQ water with 0.1% formic acid/methanol (45:55)
Flow rate: 1.0 mL/min
Detector: Waters 2998 PDA operating at 245 nm
Relative mass fraction of the main component:
Initial analysis: Mean = 98.9%, s = 0.05% (10 sub samples in duplicate, September 2015)
Re-analysis: Mean = 98.9%, s = 0.02% (5 sub samples in duplicate, November 2016)
Re-analysis: Mean = 99.0%, s = 0.04% (5 sub samples in duplicate, April 2018)
Re-analysis: Mean = 98.9%, s = 0.02% (5 sub samples in duplicate, April 2019)
Re-analysis: Mean = 99.1%, s = 0.05% (5 sub samples in duplicate, May 2020)

Karl Fischer analysis: Moisture content 6.8% mass fraction (October 2016)
Moisture content 8.7% mass fraction (April 2018)
Moisture content 10.8% mass fraction (April 2019)
Moisture content 7.8% mass fraction (May 2020)

Thermogravimetric analysis: Volatile content 2.9% mass fraction
Due to the material being the sodium salt, the non-volatile content could not be determined by thermogravimetric analysis.

qNMR: Instrument: Bruker Avance-III-500
Field strength: 500 MHz
Solvent: AcOH-d₄ (2.07 ppm)
Internal standard: Potassium hydrogen maleate (99.6% mass fraction)
Initial analysis: Mean (5.88 ppm) = 89.8%, s = 0.1% (5 sub samples, November 2016)

Spectroscopic and other characterisation data

GC-MS:	<i>Bis</i> -TMS derivative: The free steroid was liberated upon treatment with β -glucuronidase enzyme (E. Coli K12) and derivatised with MSTFA. Instrument: Agilent 6890/5973 Column: TG-1MS, 30 m x 0.25 mm I.D. x 0.25 μ m Program: 180 °C (1 min), 30 °C /min to 250 °C (10 min), 30 °C /min 300 °C (3 min) Injector: 250 °C Transfer line temp: 280 °C Carrier: Helium, 1.0 mL/min Split ratio: 20/1 The retention time of the <i>bis</i> -TMS derivative of epitestosterone is reported along with the major peaks in the mass spectra. The latter are reported as mass/charge ratios and (in brackets) as a percentage relative to the base peak. <i>Bis</i> -TMS (10.1 min): 432 (100), 417 (16), 327 (12), 209 (17), 73 (62) <i>m/z</i>
ESI-MS:	Instrument: Waters Acquity TQ API mass spectrometer Operation: Negative ion mode, infusion at 5 μ L/min Ionisation: ESI spray voltage at 3.5 kV negative ion EM voltage: 650 V Cone voltage: 20 V Peak: 463.4 (M-H ⁺) <i>m/z</i>
HS-GC-MS:	Instrument: Agilent 6890/5973/G1888 Column: DB-624, 30 m x 0.25 mm I.D. x 1.4 μ m Program: 50 °C (5 min), 7 °C/min to 120 °C, 15 °C/min to 220 °C (8.3 min) Injector: 150 °C Transfer line temp: 280 °C Carrier: Helium, 1.2 mL/min Split ratio: 50/1 Solvents detected: Propan-2-ol and diethyl ether
TLC:	Conditions: Kieselgel 60F ₂₅₄ . Chloroform/Methanol/Water (70/30/2) Single spot observed, R _f = 0.2. Visualisation with UV at 254 nm
IR:	Instrument: Bruker Alpha Platinum ATR Range: 4000-400 cm ⁻¹ , neat Peaks: 3394, 2932, 2870, 2847, 1663, 1611, 1408, 1231, 1159, 1113, 1066, 1043, 1020 cm ⁻¹
¹ H NMR:	Instrument: Bruker Avance III-500 Field strength: 500 MHz Solvent: MeOH- <i>d</i> ₄ (3.31 ppm) Spectral data: δ 0.78 (3H, s), 0.99 (1H, m), 1.11 (1H, m), 1.24 (3H, s), 1.27 (1H, m), 1.44-1.87 (9H, m), 1.93 (1H, m), 2.00 (1H, m), 2.11 (1H, m), 2.25-2.34 (2H, m), 2.43-2.54 (2H, m), 3.18 (1H, dd, <i>J</i> = 7.9, 8.9 Hz), 3.38 (1H, t, <i>J</i> = 8.8 Hz), 3.44 (1H, t, <i>J</i> = 9.6 Hz), 3.50 (1H, d, <i>J</i> = 9.7 Hz), 3.98 (1H, d, <i>J</i> = 5.7 Hz), 4.24 (1H, d, <i>J</i> = 7.7 Hz), 5.71 (1H, s) ppm Propan-2-ol (0.8%), methanol (0.05%) and diethyl ether (0.1%) were quantified by ¹ H NMR
¹³ C NMR:	Instrument: Bruker Avance III-500 Field strength: 126 MHz Solvent: MeOH- <i>d</i> ₄ (49.0 ppm) Spectral data: δ 17.3, 17.8, 21.7, 25.7, 29.8, 32.5, 33.7, 34.1, 34.7, 36.9, 37.2, 40.1, 45.9, 50.2, 55.3, 73.7, 74.9, 76.4, 78.0, 85.7, 101.6, 124.0, 175.6, 177.1, 202.5 ppm
Melting point:	> 250 °C (decomposition.)
Microanalysis:	Found: C = 59.6%; H = 7.4% (September 2015) Calculated: C = 55.9%; H = 7.5% (Calculated for C ₂₅ H ₃₅ NaO ₈ + 3.7% water + 1.2% propan-2-ol + 0.2% diethyl ether)