Australian Government
Department of Industry, Science, Energy and Resources

National Measurement Institute

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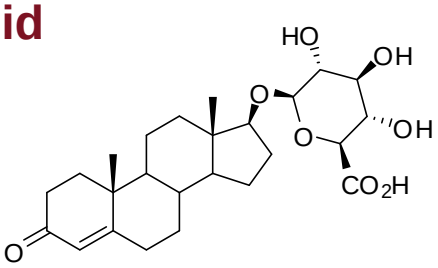
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA D507d: Testosterone glucuronic acid

Report ID: D507d.2021.01 (Ampouled 200625)

Chemical Formula: $C_{25}H_{36}O_8$

Molecular Weight: 464.6 g/mol



Certified value

Batch No.	CAS No.	Mass per ampoule
16-S-02	**1180-25-2**	**944 ± 31 μg**

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

IUPAC name: (17β)-3-Oxoandrost-4-en-17-yl β-D-glucopyranosiduronic acid.

Expiration of certification: The property values are valid till 27 April 2024, 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 ampoules that have been opened. In such cases it is recommended that the end-user conduct their own in-house stability trials.

Description: The compound is supplied as a dried aliquot in a sealed ampoule under an atmosphere of argon. The CRM is intended for a single use to prepare a standard solution containing D507d. This material was prepared by synthesis, and certified for identity and purity by NMIA.

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

Instructions for use: Open the ampoule and carefully rinse the interior at least three times with a suitable organic solvent (e.g. acetonitrile). This will transfer 944 ± 31 μg of anhydrous testosterone glucuronic acid. The mass of analyte in each ampoule is calculated from the assigned purity of the bulk and the concentration of bulk material in a stock solution used to prepare the ampoules.

Recommended storage: When not in use, this material should be stored at or below 4 ºC 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: In the absence of long term stability data the measurement uncertainty at the 95% coverage interval has been expanded to accommodate any potential change in the property value. The stability component has been estimated from stability trials conducted on similar materials by NMI Australia over the last ten years. 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 seven randomly selected ampoules 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 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.
13 May 2021

This report supersedes any issued prior to 13 May 2021

Characterisation Report:

HPLC:	Instrument:	Shimadzu Binary pump LC-20AB, SIL-20 A HT autosamplerThermo Scientific UltiMate 3000
	Column:	Alltima C-18, 5 □m (4.6 mm x 150 mm)
	Column oven:	40 °C
	Mobile Phase:	MilliQ with 0.1% formic acid/methanol (46:54)
	Flow rate:	1.0 mL/min
	Detector:	Shimadzu SPD-M20A PDA operating at 245 nm
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 99.4%, s = 0.05% (7 ampoules in duplicate, July 2020)
	Re analysis:	Mean = 99.3%, s = 0.01% (5 ampoules in duplicate, April 2021)

The following analytical data was obtained on the bulk material subsequently used in the preparation of the ampoules.

The identity was confirmed by a range of spectroscopic techniques, NMR, IR and MS. The certified purity value was obtained from a combination of traditional analytical techniques and quantitative nuclear magnetic resonance (qNMR). The techniques used in the mass balance approach include HPLC with UV detection thermogravimetric analysis, Karl Fischer analysis and ^{1}H NMR spectroscopy. The purity value is calculated as per Equation 1.

Purity = (100 % - I_{ORG}) x (100 % - I_{VOL} – I_{NVR}) Equation 1
I_{ORG} = Organic impurities of related structure, I_{VOL} = volatile impurities, I_{NVR} = non-volatile residue

The purity value by qNMR was obtained using the one-proton singlet at 5.9 ppm measured against a certified internal standard of potassium hydrogen maleate.Supporting evidence is provided by qualitative headspace GC-MS analysis of occluded solvents and elemental microanalysis.

HPLC:	Instrument:	Waters alliance 2650 or Waters Model 1525 Binary pump, 717 plus autosampler
	Column:	Alltima C-18, 5 μm (4.6 mm x 150 mm)
	Column oven:	40 °C
	Mobile Phase:	MilliQ with 0.1% formic acid/methanol (45:55 or 46:54)
	Flow rate:	1.0 mL/min
	Detector:	Waters 2998 PDA operating at 245nm
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 99.2%, s = 0.05% (10 sub samples in duplicate, February 2016)
	Re-analysis:	Mean = 99.3%, s = 0.01% (5 sub samples in duplicate, March 2017)
	Re-analysis:	Mean = 99.5%, s = 0.06% (5 sub samples in duplicate, April 2020)
Karl Fischer analysis:		Moisture content 4.3% mass fraction (February 2016 and March 2017) Moisture content 4.2% mass fraction (April 2020)
Thermogravimetric analysis:		Non-volatile residue < 0.2% mass fraction
QNMR:	Instrument:	Bruker Avance-III-500
	Field strength:	500 MHz
	Solvent:	AcOH-d_4 (2.07 ppm)
	Internal standard:	Potassium hydrogen maleate (99.6% mass fraction)
	Initial analysis:	Mean (5.9 ppm) = 94.6%, s = 0.05% (3 sub samples, September 2016)

Spectroscopic and other characterisation data

GC-MS:	Persilylated Derivative	
	Instrument:	Agilent 6890/5973
	Column:	HP Ultra 1, 17 m x 0.22 mm I.D. x 0.11 μm
	Program:	180 °C (1 min), 12 °C/min to 300 °C (5 min)
	Injector:	220 °C
	Split ratio:	15/1
	Transfer line temp:	300 °C
	Carrier:	Helium, 1.0 mL/min
	The retention time of the persilylated derivative is reported along with the major peaks in the mass spectrum. The latter are reported as mass/charge ratios and (in brackets) as a percentage relative to the base peak.	
	Per-Der (15.0 min):	502 (3), 388 (15), 375 (13), 343 (64), 305 (7), 257 (7), 247 (6), 233 (6), 217 (40), 204 (53), 169 (13), 147 (30), 73 (100) *m/z*
ESI-MS:	Instrument:	Micromass Quatro Micro
	Operation:	Negative ion mode, direct infusion at 5 μL/min
	Ionisation:	ESI spray voltage at 3.0 kV negative ion
	EM voltage:	650 V
	Cone voltage:	30 V
	Peak:	463.0 ($M\text{-}H^+$) *m/z*
HS-GC-MS:	Instrument:	Agilent 6890/5973/G1888
	Column:	DB-624, 30 m x 0.25 mm I.D. x 1.4 μm
	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:	None observed
TLC:	Conditions:	Kieselgel 60F_{254}. Chloroform/Methanol (1/1) Single spot observed, R_f = 0.36. Visualisation with UV at 254 nm
IR:	Instrument:	Biorad FTS300MX FT-IR
	Range:	4000-400cm^{-1}, KBr powder
	Peaks:	3450, 3300, 2926, 1665, 1655, 1434, 1362, 1235, 1176, 1092, 1057, 1035 cm^{-1}
^{1}H NMR:	Instrument:	Bruker DMX600
	Field strength:	600 MHz Solvent: MeOH-d_4 (3.31 ppm)
	Spectral data:	δ 0.89 (3H, s), 0.92-1.06 (3H, m), 1.20 (1H, m), 1.23 (3H, s), 1.27-1.35 (1H, m), 1.46-1.53 (1H, m), 1.56-1.73 (5H, m), 1.86-1.89 (1H, m),1.97-2.11 (3H, m), 2.25-2.32 (2H, m), 2.43-2.51 (2H, m), 3.21 (1H, dd, *J* = 7.9, 9.2 Hz), 3.35 (1H, t, *J* = 9.2 Hz), 3.50 (1H, t, *J* = 9.7 Hz), 3.68 (1H, t, *J* = 8.6 Hz), 3.73 (1H, d, *J* = 9.8 Hz), 4.37 (1H, d, *J* = 7.7 Hz), 5.70 (1H, s) ppm
	Methanol estimated at 0.01% mass fraction was observed in the ^{1}H NMR	
^{13}C NMR:	Instrument:	Bruker DMX600
	Field strength:	150 MHz Solvent: MeOH-d_4 (49.0 ppm)
	Spectral data:	δ 12.0, 17.7, 21.7, 24.2, 29.8, 32.8, 33.9, 34.7, 36.7, 36.8, 38.3, 40.0, 44.1, 51.6, 55.4, 73.2, 75.0, 76.6, 77.5, 90.2, 105.1, 124.1, 172.5, 175.2, 202.4 ppm
Melting point:		175-179 ºC
Microanalysis:	Found:	C = 62.0%; H = 8.0% (February 2016)
	Calculated:	C = 62.0%; H = 8.0% for $C_{25}H_{36}O_8$ with 1.1 mole equivalents H_2O (4.0% mass fraction)