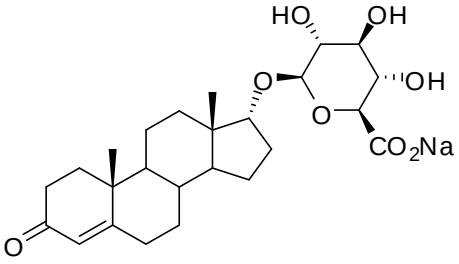
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

Report ID: S035.2018.01

Compound Name: **Epitestosterone glucuronide sodium salt**
Collection Number: S035
Chemical Formula: $C_{25}H_{35}O_8Na$
CAS Number: 16996-33-1 (free acid)
Structure:

Description: White solid
Batch Number: 15-S-10
Molecular Weight: 486.5
Release date: 6th December 2016



Synonyms: Epitestosterone glucuronoside sodium salt
Epitestosterone glucosiduronate sodium salt
(17α)-3-Oxoandrost-4-en-17-yl glucopyranosiduronic acid sodium salt

Purity (mass fraction): 87.9 ± 2.6% (95% coverage interval)

The purity value was obtained by quantitative nuclear magnetic resonance (qNMR). The one proton singlet at 5.88 ppm was 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, ^{1}H NMR spectroscopy, headspace GC-MS analysis of occluded solvents and elemental microanalysis.

HPLC:	Instrument:	Waters Model 1525 Binary pump, 717 plus autosampler
	Column:	Alltima C18, 5 μm (4.6 mm × 150 mm)
	Column oven:	40 °C
	Mobile Phase:	MilliQ water with 0.1% formic/methanol (45:55)
	Flow Rate:	1.0 mL/min
	Detector:	PDA (245.4 nm)
	Relative mass fraction of 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)
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.
Karl Fischer analysis:		Moisture content 6.8% mass fraction. (October 2016) Moisture content 8.7% mass fraction. (April 2018)
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 (5.88 ppm) = 89.8%, s = 0.1% (5 sub samples, November 2016)

Spectroscopic and other characterisation data

GC-MS: *Bis*-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 × 0.25 mm I.D. × 0.25 μm	
Program:	180 °C (1 min), 30 °C /min to 250 °C (10 min), 30 °C /min 300 °C (3 min)	
Injector:	250 ºC	Transfer line temp: 280 ºC
Carrier:	Helium, 1.0 mL/min	Split ratio: 20/1

The retention time of the *bis*-TMS derivative of epitestosterone 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.

Bis-TMS (10.1 min): 432 (100), 417 (16), 327 (12), 209 (17), 73 (62) *m/z*

ESI-MS:

Instrument:	Waters Acquity TQ API mass spectrometer
Operation:	Negative ion mode, infusion at 5 μL/min
Ionisation:	ESI spray voltage at 3.5 kV negative ion
EM voltage:	650 V
Cone voltage:	20 V
Peak:	463.4 ($M-H^+$) *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:	Propan-2-ol and diethyl ether	

TLC:

Conditions:	Kieselgel $60F_{254}$. Chloroform/Methanol/Water (70/30/2)
	Single spot observed, R_f = 0.20. Visualisation with UV at 254 nm

IR:

Instrument:	Bruker Alpha FT-IR
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: CD_3OD (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, *J* = 7.9, 8.9 Hz), 3.38 (1H, t, *J* = 8.8 Hz), 3.44 (1H, t, *J* = 9.6 Hz), 3.50 (1H, d, *J* = 9.7 Hz), 3.98 (1H, d, *J* = 5.7 Hz), 4.24 (1H, d, *J* = 7.7 Hz), 5.71 (1H, s) ppm	
	2-Propanol (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: CD_3OD (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 (decomp.)

Microanalysis: Found: C = 59.6 %; H = 7.4 %

Calc: 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)

Expiration of certification
The property values are valid till 17th April 2021, 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 long-term stability of the compound in solution has not been examined.

This material has been given a shelf life of three years from the date of re-certification. 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.

In the absence of 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 10 years.

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.

Metrological traceability
The certified purity value is traceable to the SI unit for mass (kg) through Australian national standards via balance calibration. The purity was derived by subtraction of the mass of impurities from the mass of the reference material. Organic purity is traceable to the SI-derived coherent unit one through chromatographic separation and response factor determination of individual components. Volatile and non-volatile residue content is directly traceable to mass through use of Karl Fischer and thermogravimetric analysis. 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).

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.

Intended use
For ***in vitro*** laboratory analysis only.

Caution
Treat as hazardous substance. Use appropriate work practices when handling to avoid skin or eye contact, ingestion or inhalation of dust.

Legal notice
Neither NMI nor any person acting on NMI's behalf 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.

Authorised by:

S. R. Davies

Dr Stephen R. Davies,
Team Leader,
Chemical Reference Materials, NMI.
Dated: 20 April, 2018.

Characterisation data and property values specified in this report supersede those in all reports issued prior to 20th April 2018.