Australian Government
Department of Industry, Science and Resources

National Measurement Institute

NATA
WORLD RECOGNISED ACCREDITATION

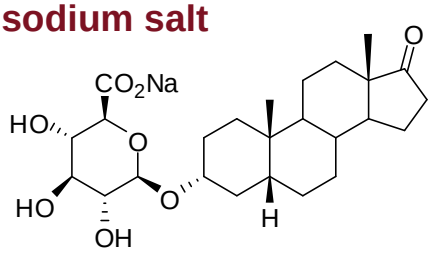
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA D607b: Etiocholanolone-3-O-β-glucuronide, sodium salt

Report ID: D607b.2024.01 (Ampouled 221027a)

Chemical Formula: $C_{25}H_{37}NaO_8$

Molecular Weight: 488.5 g/mol



Certified value

Batch No.	CAS No.	Mass per ampoule
15-S-14	3602-09-3 (free acid)	899 ± 20 μg

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

IUPAC name: Sodium (3α)-17-Oxo-5β-androstan-3-yl β-D-glucopyranosiduronic acid.

Expiration of certification: The property values are valid till 23 October 2027, 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: White solid prepared by synthesis and certified for identity and purity by NMI Australia The analyte is supplied as a dried aliquot in a sealed ampoule under an atmosphere of argon, intended for a single use to prepare a standard solution containing D607b.

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. methanol). This will transfer 899 ± 20 μg of anhydrous etiocholanolone-3-*O*-β-glucuronide, sodium salt. 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: This material has demonstrated stability over a minimum period of three 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 UHPLC with charged aerosol 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.
28 October 2024

This report supersedes any issued prior to 28 October 2024.

NATA Accreditation No. 198 / Corporate Site No. 14214.

Characterisation Report:

UHPLC:	Instrument:	Thermo Scientific Ultimate 3000 or Thermo Scientific Vanquish Flex UHPLC
	Column:	ACE Super C18, 5 μm (4.6 mm x 250 mm)
	Column oven:	40 °C
	Mobile Phase:	Milli Q water/methanol (27:73) Formic acid at 0.2% v/v was present in the aqueous phase.
	Flow rate:	1 mL/min
	Detector:	Dionex Charged Aerosol Detector or Vanquish Charged Aerosol Detector
	Power function:	1.0
	Nebuliser temp:	25 °C
	Relative peak area of the main component:	
	Initial analysis:	Mean = 99.07%, s = 0.06% (7 sub samples in duplicate, November 2022)
	Re-analysis:	Mean = 99.24%, s = 0.04% (5 sub samples in duplicate, December 2023)
	Re-analysis:	Mean = 99.13%, s = 0.04% (5 ampoules in duplicate, October 2024)

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 by quantitative nuclear magnetic resonance (QNMR) using the one proton doublet at 4.64 ppm against a certified internal standard of potassium hydrogen maleate.

Supporting evidence is provided HPLC with ELS detection, Karl Fischer analysis, ^{1}H NMR and elemental microanalysis.

HPLC:	Instrument:	Shimadzu Binary pump LC-20AB, SIL-20 A HT auto sampler
	Column:	X-Bridge, 5 μm (4.6 mm x 150 mm)
	Column oven:	40 °C
	Mobile Phase:	Milli Q water/methanol (40:60) Formic acid at 0.2% v/v was present in the aqueous phase
	Flow rate:	1 mL/min
	Detector:	Shimadzu ELSD-LT II or Waters ELSD 2424
	Relative peak area of the main component:	
	Initial analysis:	Mean = 100.0%, s = 0.01% (7 sub samples in duplicate, March 2016)
	Re-analysis:	Mean = 99.5%, s = 0.09% (5 sub samples in duplicate, February 2020)
Karl Fischer analysis:		Moisture content 10.1% mass fraction (November 2016) Moisture content 9.9% mass fraction (March 2020) Moisture content 10.4% mass fraction (March 2022)
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 (4.64 ppm) = 89.2%, s = 0.04% (3 sub samples, November 2016)

Spectroscopic and other characterisation data

GC-MS:	The free steroid was liberated upon treatment with β-glucuronidase enzyme (E. Coli K12) and derivatised with MSTFA.	
	Instrument:	Agilent 6890/5973
	Column:	HP-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 to 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 etiocholanolone 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 (8.5 min):	434 (M^+, 54), 419 (63), 329 (25), 239 (11), 208 (6), 169 (31), 73 (100) *m/z*
IR:	Instrument:	FT-IR, Biorad WIN FTS40
	Range:	4000-400 cm^{-1}, KBr pellet
	Peaks:	3617, 3450, 1731, 1615, 1410, 1166, 1072 cm^{-1} ppm
^{1}H NMR:	Instrument:	Bruker Avance III 500
	Field strength:	500 MHz
	Solvent:	MeOH-d_4 (3.31ppm)
	Spectral data:	δ 0.87 (3H, s), 0.98 (3H, s), 1.01 (1H, dt, *J* = 3.0, 14.0 Hz), 1.21-1.49 (7H, m), 1.50-1.71 (6H, m), 1.74-2.00 (6H, m), 2.08 (1H, dt, *J* = 19.0, 9.1 Hz), 2.43 (1H , dd, *J* = 8.6, 19.3 Hz), 3.18 (1H, t, *J* = 8.2 Hz), 3.39 (1H, t, *J* = 8.8 Hz), 3.43 (1H, t, *J* = 8.8 Hz), 3.54 (1H, d, *J* = 9.3 Hz), 3.82 (1H, m), 4.41 (1H, d, *J* = 7.8 Hz) ppm
		Methanol estimated at 0.02% mass fraction was observed in the ^{1}H NMR.
^{13}C NMR:	Instrument:	Bruker Avance III 500
	Field strength:	126 MHz
	Solvent:	MeOH-d_4 (49.0 ppm)
	Spectral data:	δ 14.2, 21.2, 22.8, 23.8, 26.5, 27.5, 28.0, 33.0, 35.0, 36.0, 36.3, 36.7, 36.8, 42.1, 43.6, 52.8, 73.8, 75.0, ,76.2, 77.9, 79.2, 101.8, 177.0, 224.2 ppm
Melting point:		> 230 °C
Microanalysis:	Found:	C = 55.7%; H = 8.1% (April 2016)
	Calculated:	C = 55.3%; H = 8.0% (Calculated for $C_{25}H_{37}O_8Na$ + 10.1% water)