Australian Government
Department of Industry, Science and Resources

National Measurement Institute

NATA
WORLD RECOGNISED ACCREDITATION

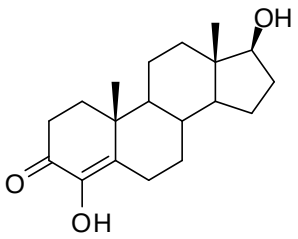
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA D851b: 4-Hydroxytestosterone

Report ID: D851b.2024.01 (Ampouled 130821)

Chemical Formula: $C_{19}H_{28}O_3$

Molecular Weight: 304.4 g/mol



Certified value

Batch No.	CAS No.	Mass per ampoule
11-S-02	2141-17-5	959 ± 9 μg

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

IUPAC name: (17β)-4,17-Dihydroxyandrost-4-en-3-one.

Expiration of certification: The property values are valid till 11 December 2034, ten 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 D851b. This material was prepared by synthesis and certified for identity and purity by NMI Australia.

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. toluene). This will transfer 959 ± 9 μg of anhydrous 4-hydroxytestosterone. 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 all impurities are quantified as a mass fraction and subtracted from 100%.

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 GC-FID 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.
12 December 2024

This report supersedes any issued prior to 12 December 2024.

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

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

Characterisation Report:

GC-FID:	Instrument:	Varian CP-3800
	Column:	HP-1 or DB-17, 30 m × 0.32 mm I.D. × 0.25 μm
	Program:	180 °C (1 min), 30 °C/min to 250 °C (10 min), 30 °C/min to 280 °C (5 min)
	Injector:	200 °C
	Detector Temp:	320 °C
	Carrier:	Helium
	Split ratio:	20/1
	Relative mass fraction of the main component as the TMS derivative:	
	Initial analysis:	Mean = 98.2%, s = 0.08% (7 ampoules in duplicate, August 2013)
	Re-analysis:	Mean = 98.3%, s = 0.04% (5 ampoules in duplicate, August 2014)
	Re-analysis:	Mean = 98.3%, s = 0.03% (5 ampoules in duplicate, May 2017)
	Re-analysis:	Mean = 98.1%, s = 0.14% (5 ampoules in duplicate, May 2020)
	Re-analysis:	Mean = 98.3%, s = 0.04% (5 ampoules in duplicate, December 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 mass balance from a combination of traditional analytical techniques, including GC-FID, 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.

Supporting evidence is provided by qualitative headspace GC-MS analysis of occluded solvents andelemental microanalysis.

GC-FID:	Instrument:	Varian CP-3800
	Column:	HP-1, 30 m × 0.32 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:	200 °C
	Detector Temp:	320 °C
	Carrier:	Helium
	Split ratio:	20/1
	Relative mass fraction of the main component as the TMS derivative:	
	Initial analysis:	Mean = 98.4%, s = 0.1% (10 sub samples in duplicate, July 2013)
GC-FID:	Instrument:	Varian CP-3800
	Column:	TG-17MS, 30 m × 0.32 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 °C
	Detector Temp:	320 °C
	Carrier:	Helium
	Split ratio:	20/1
	Relative mass fraction of the main component as the TMS derivative:	
	Initial analysis:	Mean = 98.4%, s = 0.1% (10 sub samples in duplicate, July 2013)
HPLC:	Instrument:	Waters Model 1525 Binary pump, 717 plus
	Column:	Grace Alltima C-18, 5 μm (4.6 mm x 150 mm)
	Mobile Phase:	A = MilliQ water B = Acetonitrile
		Gradient, 45% B (0-16 min), 60% B (25 min), 45% B (35 min)
	Flow rate:	1 mL/min
	Column oven:	40 °C
	Detector:	Waters PDA 2998 operating at Max plot 235-400 nm
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 98.6%, s = 0.04% (10 sub samples in duplicate, April 2013)
Karl Fischer analysis:		Moisture content 0.1% mass fraction (May 2013)
Thermogravimetric analysis:		Volatile content 0.2% and non volatile residue 0.8% mass fraction (May 2013)

Spectroscopic and other characterisation data

GC-MS:

Instrument:	Agilent 6890/5973	
Column:	TG-1MS, 30 m x 0.25 mm I.D. x 0.25 μm	
Injector:	250 °C	Transfer line temp: 280 °C
Carrier:	Helium, 1.0 mL/min	Split ratio: 20/1

Program (parent compound): 180 °C (1 min), 10 °C/min to 300 °C (3 min)
Program (*bis*-TMS derivative): 180 °C (1 min), 30 °C/min to 250 °C (10 min), 30 °C/min to 300 °C (3 min)

The retention times of the parent compound and major *tris*-TMS derivative are 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.

Parent (11.2 min): 304 (M^+, 89), 289 (19), 262 (21), 147 (100), 140 (60), 133 (22), 125 (22), 113 (43), 91 (45), 79 (43), 67 (37), 55 (37) *m/z*

Tris-TMS (14.7 min): 520 (M^+, 100), 505 (5), 433 (4), 431 (4), 147 (23), 73 (54) *m/z*

LC-MS:

Instrument:	Waters 2695 (HPLC)/Micromass Quatro	
Column:	X-Bridge C-18, 100 mm × 2.1 mm I.D. × 3.5 μm	
Column temp:	20 °C	
Solvent system:	A: MilliQ water [45% v/v] B: Acetonitrile [55% v/v]	
Sample prep:	1000 ppm in MilliQ water/Acetonitrile (45:55)	
Flow rate:	0.2 mL/min	Injection volume: 10 μL
Ionisation mode:	Electrospray positive ion	
Capillary voltage:	3.0 kV	Cone voltage: 20 V
Source temp:	130 ºC	Desolvation gas temperature: 350 ºC
Cone gas flow rate:	28 L/hr	Desolvation gas flow rate: 763 L/hr

The retention time of 4-hydroxytestosterone is reported along with the major peak in the mass spectrum. The latter is reported as a mass/charge ratio.

4.2 min: 305.4 ($M+H^+$) *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:	Acetone	

TLC:

Conditions: Kieselgel 60F_{254}. Chloroform/ethyl acetate (4/1)
Single spot observed, R_f = 0.4. Visualisation with vanillin.

IR:

Instrument:	Biorad FTS3000MX FT-IR
Range:	4000-400 cm^{-1}, KBr powder
Peaks:	3523, 3431, 2949, 2911, 1664, 1624, 1388, 1249, 1167, 1077, 872, 610 cm^{-1}

^{1}H NMR:

Instrument:	Bruker Avance-400
Field strength:	400 MHz
Solvent:	$CDCl_3$ (7.26 ppm)
Spectral data:	δ 0.78 (3H, s), 0.89-1.00 (3H, m), 1.08 (1H, dt, *J* = 4.1, 12.8 Hz), 1.17 (3H, s), 1.25-1.70 (9H, m), 1.81-1.87 (2H, m), 1.92-2.12 (3H, m), 2.44-2.57 (2H, m), 3.00 (1H, ddd, *J* = 2.3, 4.1,14.9 Hz), 3.64 (1H, t, *J* = 8.6 Hz) 6.09 (1H, s) ppm

^{13}C NMR:

Instrument:	Bruker Avance-400
Field strength:	100 MHz
Solvent:	$CDCl_3$ (77.16 ppm)
Spectral data:	δ 11.2, 17.3, 20.7, 23.1, 23.5, 30.6, 30.7, 31.9, 34.8, 35.4, 36.6, 38.0, 42.9, 50.7, 54.5, 81.8, 140.2, 141.3, 193.7 ppm

Melting point: 221-223 °C

Microanalysis:

Found:	C = 74.2%; H = 8.7% (May 2013)
Calculated:	C = 75.0%; H = 9.3% (Calculated for $C_{19}H_{28}O_3$)