



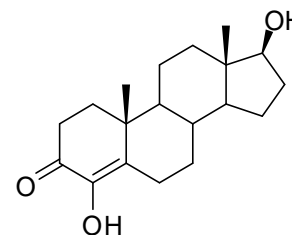
# CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

## NMIA D851b: 4-Hydroxytestosterone

Report ID: D851b.2020.01 (Ampouled 130821)

Chemical Formula: C<sub>19</sub>H<sub>28</sub>O<sub>3</sub>

Molecular Weight: 304.4 g/mol



### Certified value

Batch No.	CAS No.	Mass per ampoule
11-S-02	2141-17-5	959 ± 19 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 12 May 2025, i.e. five 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 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. toluene). This will transfer 959 ± 19 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 °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%.

**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.  
18 May 2020

This report supersedes any issued prior to 18 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.

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## Characterisation Report:

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 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)

### 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 <sup>1</sup>H NMR spectroscopy. The purity value is calculated as per Equation 1.

$$\text{Purity} = (100 \% - I_{\text{ORG}}) \times (100 \% - I_{\text{VOL}} - I_{\text{NVR}}) \quad \text{Equation 1}$$

$I_{\text{ORG}}$  = Organic impurities of related structure,  $I_{\text{VOL}}$  = volatile impurities,  $I_{\text{NVR}}$  = non-volatile residue.

Supporting evidence is provided by qualitative headspace GC-MS analysis of occluded solvents and elemental 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
	Carrier:	Helium, 1.0 mL/min
		Transfer line temp: 280 °C
		Split ratio: 20/1
	Program (parent compound):	180 °C (1 min), 10 °C/min to 300 °C (3 min)
	Program ( <i>bis</i> -TMS deriv):	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 <i>tris</i> -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 <sup>+</sup> , 89), 289 (19), 262 (21), 147 (100), 140 (60), 133 (22), 125 (22), 113 (43), 91 (45), 79 (43), 67 (37), 55 (37) <i>m/z</i>
	<i>Tris</i> -TMS (14.7 min):	520 (M <sup>+</sup> , 100), 505 (5), 433 (4), 431 (4), 147 (23), 73 (54) <i>m/z</i>
LC-MS:	Instrument:	Waters 2695 (HPLC)/Micromass Quatro
	Column:	X-Bridge C-18, 100 mm x 2.1 mm I.D. x 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 °C
		Desolvation gas temperature: 350 °C
	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 <sup>+</sup> ) <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 µm
	Program:	50 °C (5 min), 7 °C/min to 120 °C, 15 °C/min to 220 °C (8.3 min)
	Injector:	150 °C
	Carrier:	Helium, 1.2 mL/min
		Transfer line temp: 280 °C
		Split ratio: 50/1
	Solvents detected:	Acetone
TLC:	Conditions:	Kieselgel 60F <sub>254</sub> . Chloroform/ethyl acetate (4/1) Single spot observed, R <sub>f</sub> = 0.40. Visualisation with vanillin
IR:	Instrument:	Biorad FTS3000MX FT-IR
	Range:	4000-400 cm <sup>-1</sup> , KBr powder
	Peaks:	3523, 3431, 2949, 2911, 1664, 1624, 1388, 1249, 1167, 1077, 872, 610 cm <sup>-1</sup>
<sup>1</sup> H NMR:	Instrument:	Bruker Avance-400
	Field strength:	400 MHz
		Solvent: CDCl <sub>3</sub> (7.26 ppm)
	Spectral data:	δ 0.78 (3H, s), 0.89-1.00 (3H, m), 1.08 (1H, dt, <i>J</i> = 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, <i>J</i> = 2.3, 4.1, 14.9 Hz), 3.64 (1H, t, <i>J</i> = 8.6 Hz) 6.09 (1H, s) ppm
<sup>13</sup> C NMR:	Instrument:	Bruker Avance-400
	Field strength:	100 MHz
		Solvent: CDCl <sub>3</sub> (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 <sub>19</sub> H <sub>28</sub> O <sub>3</sub> )