

National Bureau of Standards

Certificate of Analysis

Standard Reference Material 921

Cortisol (Hydrocortisone)

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This Standard Reference Material is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of procedures employed in clinical analysis and for routine critical evaluation of the daily working standards used in these procedures.

Constituent	Percent
Cortisol.	98.9
21-Dehydrocortisol.	0.6
21-O-Acetylcortisol	0.2
21-Dehydrocortisone	0.1
Cortisone	0.1
Total Steroids.	99.9
Ash	0.002
Insoluble matter.	0.001
Loss on drying.	0.08

The cortisol assay has an estimated inaccuracy of 0.2 percent.

The cortisol used for this Standard Reference Material was obtained from the Upjohn Company, Kalamazoo, Michigan. Analyses were performed by R. F. Brady, Jr., A. Cohen, B. Coxon, M. Darr, W. D. Dorko, O. P. Enagonio, T. E. Gills, E. E. Hughes, W. P. Schmidt, and S. A. Wicks of the Analytical Chemistry Division.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Identification and quantitation of the four steroid impurities were accomplished by Fourier-transform proton-magnetic-resonance (pmr) spectroscopy and thin-layer chromatography (tlc) performed on fractions from the liquid chromatography of 100 mg of the material in ethanol on a column (91 x 0.8 cm) of poly(vinylpyridine) crosslinked with 8 percent of divinylbenzene. Fractions were eluted with ethanol at a pressure of 4.7 kg·cm⁻². Tlc of aliquots of the fractions was performed on silica gel GF₂₅₄ using 9:1 (v/v) chloroform-methanol. Equivalent sensitivity of detection was obtained by fluorescence quenching and by spraying with aqueous 20 percent sulfuric acid and heating at 120 °C. All the steroid impurities, except 21-dehydrocortisone, showed similar sensitivity. 4-Androsten-11β-ol-3,17-dione was found in liquid chromatography fractions, but could not be demonstrated directly by tlc of up to 1 mg of the bulk material. Because this compound was readily resolved from mixtures prepared with it and the bulk material and detected in proportion to amounts used for reference, the compound was adjudged an artifact produced during liquid chromatography. On the other hand, the major impurity, 21-dehydrocortisol, did not arise by the known copper-catalyzed oxidation of cortisol, as shown by its unaltered proportion by tlc of the bulk material even after EDTA-treatment of the system to remove copper.

The quantitative proportion of each steroid present in the sample was estimated from the dry weight (W) of the residue of each liquid chromatographic fraction and the measured intensity of the Fourier-transform generated methyl signals characteristic for each steroid, using the expression:

$$m_n M_n = \frac{W h_n M_n}{h_1 M_1 + h_2 M_2 \dots + h_m M_m}$$

where m_n and M_n are the number of moles present and the molecular weight, respectively, of the n^{th} component in a mixture of m components, h_n is the methyl signal intensity of the n^{th} component, and $h_1 \dots h_m$ are the corresponding intensities of components 1 \dots m , obtained by measurement of the methyl peaks above the methylene envelope of the steroids.

For proof of homogeneity, nine samples were withdrawn from the bulk Standard Reference Material according to a statistical plan. They were analyzed in a commercial carbon-hydrogen-nitrogen microanalyzer and were found to be homogeneous with respect to carbon and hydrogen content within the limits of precision of the method. Solutions

of the samples in 95 percent ethanol at 25 °C showed an absorbance maximum at 242 nm with a molar extinction coefficient of $16.1 \times 10^3 \text{ liter}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$.

Elemental macroanalysis of the material showed carbon, 69.49 ± 0.10 percent (2 SD of the mean) and hydrogen, 8.39 ± 0.05 percent (2 SD of the mean); the calculated values for cortisol are 69.58 percent and 8.34 percent, respectively.

The Standard Reference Material melted at 219.0–220.5 °C (corrected) when heated in an open capillary tube at $0.5 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$. The resulting pale yellow melt did not solidify on cooling. After sealing in a capillary tube under vacuum, the material melted at 220.5–221.5 °C without yellowing, but did not resolidify.

Thermogravimetric analysis of samples heated under dry nitrogen at $2 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ showed the initiation of loss of a large proportion of sample weight at 221 °C (uncorrected). However, for samples heated in air, loss of weight began at 204 °C. The attempted application of differential scanning calorimetry to samples heated under nitrogen gave thermograms that were very dependent on the rate and time of heating, and that were not reproducible.

The mass spectrum of the Standard Reference Material obtained by electron induced ionization at 70eV and a probe temperature of 220 °C showed strong ion currents at m/e 362 [M^+], 344 [$M-18(H_2O)$], 332 [$M-30(CH_2O)$], 303 [$M-59(COCH_2OH)$], 302 [$M-60(CH_2=C=O \text{ and } H_2O)$], 285 [$M-59-18$], 42 ($CH_2=C=O$), and 31 ($CH_2=O-H$)⁺. The peaks at m/e 302 and 163 correspond to the molecular ion and key fragment, respectively, of the thermal degradation product of cortisol, namely 4-Androsten-11 β -ol-3,17-dione.

Optical rotations of solutions of the Standard Reference Material in ethanol (c 1.00) were measured at 20 °C by means of automatic and high-precision, manual polarimeters and are as follows:

λ (nm)	$[\alpha]_{\lambda}^{20}$ (+2 SD of the mean)	
	degrees	radians
589	168.9 ± 0.1	2.948 ± 0.002
578	177.3 ± 0.1	3.094 ± 0.002
546	205.6 ± 0.1	3.588 ± 0.002
436	387.9 ± 0.4	6.770 ± 0.007
365	571.7 ± 0.6	9.978 ± 0.010

Insoluble matter was determined by filtration of solutions of the Standard Reference Material (1.8–2.0 g) in methyl sulfoxide (9–20 ml) through Millipore filters (1.5 μ m).

The loss of weight on drying was determined after heating three samples (2.8 g, each) of the material at 110 °C and 0.1 torr for 24 hours. No further weight loss occurred during heating of the samples for an additional 48 hours.

Ash was determined by heating of samples (10 g) of the Standard Reference Material to 700 °C. Spectrometric analysis of the ash indicated calcium as a major constituent, and sodium, magnesium, and silicon as minor constituents.

Destructive neutron activation analysis using SRM 1577, Bovine Liver, as a standard indicated that the Cortisol Standard Reference Material contains 0.035 ± 0.005 ppm (± 2 SD of the mean) of copper.