U. S. Department of Commerce
Malcolm Baldrige
Secretary

National Bureau of Standards
Ernest Ambler, Director

National Bureau of Standards

Certificate of Analysis

Standard Reference Material 998

Angiotensin I (Human)

This Standard Reference Material (SRM) is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of the renin assay and as a reference peptide for amino acid analysis and high performance liquid chromatography (HPLC).

Angiotensin I	94.1 ± 0.9 weight percent
Acetate (counter ion)	(6.3 ± 0.2) weight percent

The certified angiotensin I purity was assessed by HPLC and confirmed indirectly within the reported uncertainty by nuclear magnetic resonance (NMR).

In the HPLC procedure, the peptide content was measured by the method of standard additions using phenylalanine dissolved in 1 mol/L KOH as the added standard. The solution of the SRM and added standard was hydrolyzed in 6 mol/L HCl and the amino acid composition was determined. The phenylalanine concentration was determined by absolute weight and by measuring the absorbance of the standard solution and calculating the concentration using an apparent specific molar absorbance of $209 \pm 0.2\ L \cdot g^{-1} \cdot cm^{-1}$. The ratio of the concentration of phenylalanine to each of four amino acids (valine, leucine, histidine, and arginine) was used to calculate the amount of total peptide in each sample.

All reported uncertainties are stated as plus or minus one standard error of the listed value. The acetate content, in parentheses, was determined by a single method and is therefore NOT a certified value.

NOTICE AND WARNINGS TO USERS

This Standard Reference Material is intened for "in vitro" diagnostic use only.

Storage: SRM 998 should be stored in the sealed glass ampoule at −20 °C. It should be allowed to warm up to room temperature before opening. Under proper storage this material should be stable for 5 years. Samples of this SRM will be monitored by NBS. If the purity of the material degrades beyond the limits certified, purchasers will be notified by NBS. This material is not certified for use after 5 years from date of purchase.

The angiotensin I used for this SRM was obtained from Beckman Instruments, Inc., Bioproducts Operations, Palo Alto, Calif. Analytical and physical determinations were performed at NBS in the Organic Analytical Research Division by B. Coxon, S. Margolis and R. Weker.

The statistical analysis of the data was made by R. Paule, NBS National Measurement Laboratory.

The overall direction and coordination of the technical measurements leading to the certification were under the chairmanship of S. Margolis.

The technical and support aspects involved in the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by R. Alvarez.

Washington, D.C. 20234
January 11, 1983

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George A. Uriano, Chief
Office of Standard Reference Materials

Liquid chromatography was performed on dissolved 25-μg samples of this SRM using a 25 x 0.4 cm octadecylsilane column operated at a pressure of 5000 kPa. The samples had been dissolved in a solution of 81 parts by volume 0.1 mol/L triethylamine phosphate buffer at pH 3.5 and 19 parts by volume acetonitrile. Two eluent solutions were used: one, a solution of the same composition used to dissolve the SRM; the other, a solution of 75 parts by volume of the buffer to 25 parts by volume acetonitrile. Elution was monitored by measurement of absorbance at 215 and 280 nm. At an eluent ratio of 81:19, a major peak and minor peak with retention times of 16.6 and 12.2 minutes respectively, were detected at 215 nm and only the major peak was detected at 280 nm. The material corresponding to each peak was collected, hydrolyzed in 6 mol/L HCl and analyzed for amino acid composition. The amino acid content of the major peak indicated that it was angiotensin I. The minor peak ($<$ 1% of the absorbance at 215 nm) contained no detectable peptide material. Under these HPLC conditions, acetate was eluted in the solvent front and was not quantifiable. At an eluent ratio of 75:25, no additional peaks were detected at either wavelength indicating that non-peptide, uv absorbing materials were absent.

The acetate content of six samples of the SRM was determined by use of the 400 MHz NMR spectrometer in the pulse, Fourier transform mode. Solutions for this analysis were prepared by dissolution of the entire sample (approximately 0.5 mg) in 0.5 mL of deuterium oxide (100 atom % D). A small quantity of sodium 4,4-dimethyl-4-silapentanoate 2,2,3,3-d_4 was added to each solution to serve as an internal reference. Because the acetate methyl proton signal at δ 1.922 was found to overlap the proton signals of the angiotensin I, an acetate-free sample of angiotensin I was initially prepared to determine an accurate proton count for the signals underlying the acetate peak. Removal of the acetate from this sample was achieved by two lyophilizations of the material with 0.02 mol/L HCl. Analysis of this sample by proton NMR indicated that it was free of acetate and that the proton count in the spectral region of interest was three. Thereafter, the samples of the SRM were analyzed without lyophilizations, and the appropriate integral value for three protons was subtracted from the total integral of the acetate methyl peak and its underlying signals to obtain the integral of the acetate peak. The appropriate value for the integral of three protons was determined by averaging the integrals of a total of 29 proton signals in the high field-region, including many of those that originated from the methyl, methylene, and methine protons of angiotensin I. Digital integrals of the proton signals were plotted on chart paper and were measured manually. The following instrumental parameters were used: size of data set, 16384 points; 4 μs (30° flip angle); spectral width, 4 kHz; number of scans, 2000; relaxation delay, 1.95 s.

Other substances, except angiotensin I and acetate, were not detected by NMR. Therefore the angiotensin I concentration, by difference, is 93.7 weight percent.

Reproducibility of filling ampoules was assessed by randomly selecting 10 ampoules from the entire lot of angiotensin I. The contents of each ampoule were dissolved in 2 mL of the HPLC eluent described previously (ratio 81:19) and a 100 μL aliquot of this solution was chromatographed on an octadecylsilane column. Duplicate HPLC measurements were made for angiotensin I using the 215 nm peak. The calculated coefficient of variation between ampoules was 2.9%.

The total peptide in each ampoule was determined, in duplicate, using five randomly selected ampoules. The contents of each ampoule was dissolved in a weighed amount of 0.1 mol/L HCl. An aliquot of this solution was transferred to a weighed vial and the weight of the aliquot was determined. An aliquot of a phenylalanine solution (6.2 mmol/L) was then added and weighed. The resulting mixture was hydrolyzed in 6 mol/L HCl and the amino acid composition was determined.

The amount of peptide was calculated from the equation:

$$\frac{X}{X + M} = \frac{L_s \times P_{st}}{L_{st} \times P_s}$$

where X = μmol of peptide in the ampoule, M = μmol of phenylalanine added, L_s = height of leucine peak in the spiked sample, P_{st} = height of phenylalanine peak in the standard amino acid mixture, L_{st} = height of leucine peak in the standard amino acid mixture, and P_s = height of phenylalanine peak in the spiked sample. The samples had a mean weight of 526 μg and a standard error of 10 μg. The calculated coefficient of variation between ampoules was 4.0%. Similar results were obtained when the peak height of arginine was substituted for that of leucine. These results, in conjunction with the ampoule-filling study, indicate that users wishing to obtain very highly accurate measurements must weigh the angiotensin I before use.

The amino acid composition of nine randomly selected samples was determined by the chromatographic method of Benson and Hare (Proc. Natl. Acad. Sci., U.S. 72: 139, 1975). The molar ratios of the amino acids were: aspartic acid (asp) 1.01, arginine (arg) 0.95, valine (val) 1.05, tyrosine (tyr) 0.97, isoleucine (ile) 1.01, phenylalanine (phe) 1.05, leucine (leu) 1.04, and histidine (his) 1.94. This method did not permit the determination of imino acids.

However, the determination of the amino acid composition of 6 randomly selected samples by integration of selected identified resonances from the proton NMR spectrum of Angiotensin I enabled quantification of proline (pro) in addition to the other amino acids. The molar ratios of the constituent amino acids determined by this method were: his 9, 0.99 ± 0.03; his 6, 0.99 ± 0.05; phe, 1.00 ± 0.03; tyr, 1.03 ± 0.03; leu, 0.98 ± 0.04; pro, 1.00 ± 0.04; asp, 1.04 ± 0.04; ile, 0.98 ± 0.04; leu + arg, 1.98 ± 0.04; and ile + val 2.10 ± 0.04. These results independently confirm the results obtained by the method of Benson & Hare.

The D-amino acid composition of the acidic and neutral amino acids of ten randomly selected samples was determined by the method of Engel and Hare (Carnegie Inst. Yearbook, 1981). The percent D isomer content was: val 0.66 ± 0.30, ile 0.54 ± 0.18, pro 1.11 ± 0.40, leu 0.35 ± 0.14, asp 2.17 ± 1.00, phe 2.17 ± 0.41, and tyr < 1.0. These levels of the D isomers are consistent with the generation of D-amino acids during the hydrolysis and derivatization procedure. The D-amino acid content of the basic amino acids could not be determined by this method. However, the lack of other peptide peaks in the HPLC chromatograms, a method that resolves D-amino acid containing peptides (NBSIR 79-1947, Development of a Standard Reference Material for Angiotensin I) suggests that the levels of D isomers of the basic amino acids are comparable to those of the other D-amino acids.

Five angiotensin I samples were examined for non-peptide impurities by low resolution mass spectrometry. The measurements were made on a high resolution, double focussing, mass spectrometer operated at an ionizing energy of 70 eV and a source temperature of 250 °C. The following impurities were tentatively identified: acetic acid, low levels of alkyl groups containing up to at least 8 carbon atoms, very low levels of phthalate esters in three of five samples and extremely low levels of dimethylsilicone polymers.