U. S. Department of Commerce
Frederick B. Dent
Secretary

National Bureau of Standards
Richard W. Roberts, Director

National Bureau of Standards Certificate of Analysis

Standard Reference Material 916

Bilirubin

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 for bilirubin determination employed in clinical analysis and for routine critical evaluation of the daily working standards used in these procedures.

Purity (bilirubin content)	99.0 percent
Chloroform	0.8 percent
Insoluble (in chloroform)	less than 0.01 percent
Ash	0.01 percent

The value of the bilirubin content has a possible estimated inaccuracy of two percent. (See section on thin-layer chromatography).

The certification of this Standard Reference Material is based on the best current state-of-the-art knowledge. In spite of decades of work on bilirubin, it is still felt that much of its basic chemistry is incomplete.

The material in chloroform (ACS reagent containing ethanol) at 25.0 °C shows at 453 nm an absorptivity (±1 SD) of 104.6 ± 0.2 liter cm^{-1} g^{-1}. In methyl sulfoxide at 25.0 °C, the absorptivity (±1 SD) of the material at 453 nm is 107.7 ± 0.6 liter cm^{-1} g^{-1}.

It is recommended that bilirubin and its solutions be handled only under low-intensity incandescent light. The bilirubin should be stored under conditions that totally exclude light. Once removed from the ampoule, the vial should be replaced for storage in an air-tight container and stored at low temperature. The vial must be allowed to return to room temperature before opening.

The bilirubin used for this standard reference material was obtained from Pfanstiehl Laboratories, Inc., of Waukegan, Illinois. Analyses were performed by R. F. Brady, Jr., R. W. Burke, A. Cohen, B. Coxon, W. D. Dorko, D. Enagonio, A. J. Fatiadi, D. H. Freeman, J. M. Ives, B. A. Johnson, E. C. Kuehner, R. A. Paulson, T. C. Rains, R. Schaffer, W. P. Schmidt, J. T. Sterling, J. K. Taylor, B. F. West, W. J. Zielinski, Jr.

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

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.

Washington, D. C. 20234
March 10, 1971

J. Paul Cali, Chief
Office of Standard Reference Materials

(over)

The standard reference material was prepared from material that was isolated from hog bile and crystallized as the acid. It was purified further by treatment in chloroform solution with sodium sulfate according to Fog [1] and recrystallization from chloroform.

Ash was determined on 100-mg samples heated overnight at 250 °C and then for 3 hrs at 650 °C. Flame photometry and atomic absorption measurements on a solution of the ash obtained from 0.275 g of the material showed, in μg/g (ppm) of metal: <0.05 lithium, 7.3 sodium, 1.6 potassium, 2.1 calcium, and 2.3 magnesium.

Elemental composition found for the material was: C, 67.36; H, 6.24; N, 9.42; Cl, 0.73. Required for a bilirubin preparation containing 0.8 percent of chloroform: C, 67.25; H, 6.17; N, 9.50; Cl, 0.71. In these analyses, nitrogen was determined by the Kjeldahl technique; and chlorine was determined by Carius digestion followed by gravimetric determination as silver chloride. By the latter method and recalculated as chloroform, the average (±1 SD) of ten 25-mg samples, was 0.83 ± 0.04 percent. The chloroform content was also obtained by heating at 250 °C at 0.5 torr for 4 hrs; the average (± 1 SD) of six 100-mg samples was 0.79 ± 0.02 percent. (Only about one-half or one-tenth of the chloroform was removed at 180 °C and at 110 °C, respectively.) During gradual heating of the material in a quadrupole mass spectrometer, only ion-fragments characteristic of chloroform were observed until the sample was heated above 300 °C, whereupon water and carbon dioxide were detected, and at a somewhat higher temperature, the mass spectrum typical of bilirubin was obtained. Gas chromatography and nuclear magnetic resonance spectroscopy with the material dissolved in methyl sulfoxide confirmed the presence of chloroform. Because the chloroform is so firmly retained and homogeneously dispersed, it is recommended that the material be used as supplied.

Thermogravimetric analysis with the sample heated in a dry nitrogen atmosphere at 5 °C/min showed the initiation of the loss of a large proportion of sample weight at temperatures between 319 and 323 °C; determined in air, however, comparable weight-loss began between 288 and 292 °C.

For determining the absorptivity of the standard material, absorbances were recorded with a Cary 16 spectrophotometer on solutions using a capped 1.000 ± 0.001-cm path-length cuvette in a cell-holder thermostated at 25.0 ± 0.1 °C. Solution preparation and handling were performed under low intensity, incandescent light; at other times, solutions were totally protected from light. To preclude any change in concentration of chloroform solutions due to evaporation of the solvent during solution transfer, nitrogen gas was used to force the solution from the flask through Teflon tubing into the cuvette and, to effect rinsing of the cuvette, from the latter to an overflow. Solutions in methyl sulfoxide were transferred to the cuvette by pipet.

For the determination of chloroform-insoluble matter, sample (0.1 g) and solvent (200 ml), protected from light, were agitated in an ultrasonic bath, heated to 60 °C briefly, and then cooled and filtered, using pressure, (with 50 ml of the solvent for washing) through a tared 0.4 μm-porosity membrane.

Extraction by Bicarbonate. Yellow extracts were obtained by shaking a solution of the standard reference material (0.010 g) in chloroform (40 ml) with 10-ml volumes of 0.6 molar aqueous sodium bicarbonate. Their absorption spectra, with maxima in the range 415--430 nm, showed absorbance values that ranged from 0.035 to 0.030 in successive extracts using 1-cm cells. The qualitative and quantitative compositions of such extracts were examined using the filtered chloroform solutions containing 0.1-g quantities of the standard reference material that were prepared in the tests for chloroform insolubles: Extractions twice with 50-ml volumes of the bicarbonate solution and with water, and neutralization of the combined aqueous extracts followed by extraction into chloroform, yielded pigment (dry weight approximately 0.2 mg) that was shown by its spectrum and specific absorptivity to be typical of bilirubin in chloroform. Thus, bicarbonate extraction provided no evidence of impurities more acidic than bilirubin.

Extraction by Carbonate. Extraction of a 200-ml chloroform solution containing 0.1 g of standard with 100-ml volumes of 0.1 molar sodium carbonate reduced the 1-cm absorbance value of the chloroform layer to approximately 0.22 after the second extraction, and to 0.08 and then 0.01 with the third and fourth extractions, respectively. On washing with water and then evaporating the extracted chloroform layer to dryness, residue weights were typically about 0.01 mg. Hence, this extraction process showed a negligible proportion of nonacidic impurities.

Thin-layer Chromatography. An impurity in the standard material may be detected by thin-layer chromatography with polyamide as the absorbent and 3:1 (v/v) methanol--aqueous ammonium hydroxide (3.3 percent) as the developer [2]. Thus, 0.05 mg of the material (spotted from a chloroform solution) provides, on development, an elongated, yellow-orange streak preceded (and usually separate) by a faintly-visible, yellow spot. Under 366-nm irradiation, a pink fluorescence develops very quickly at the location of this spot. The material responsible for the pink fluorescence and its precursor have not been characterized. A means for obtaining a reliable estimate of this contribution to overall impurity has not been ascertained, but is under study. Repurification of the standard material does not affect this behavior.

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

This material is for use as a standard in clinical chemistry. A standard solution for the development of a calibration curve may be obtained as follows. Weigh out exactly 20.2 mg of SRM 916, transfer to a 100-ml volumetric flask, and dissolve in 2 ml of 0.1M sodium carbonate and 1.5 ml of 0.1N sodium hydroxide. The solution should be clear and red in color. Dilute this solution to 100 ml with "acceptable" [3], pooled serum and mix well. Since bilirubin is light sensitive, the flask should be wrapped in aluminum foil and kept as dark as possible. It is further recommended that the solution preparation and handling be carried out under low-intensity incandescent light [3,4].

Standard solutions of lower concentration may be prepared by dilution of appropriate aliquots of the above solution with "acceptable" pooled serum.

Standard Reference Material 916 is supplied in a vial, which is sealed in an ampoule under argon. The sample must be protected from light. After opening, the material should be kept in the tightly-closed vial in a desiccator, protected from light by a black plastic bag. Refrigeration at 4 °C is suggested. Under proper storage, experience at NBS indicates this material to be stable for at least 3 years. If the material degrades beyond the limits certified, purchasers will be notified by NBS. It is recommended that this material not be used after 3 years from the date of purchase.

Solutions of bilirubin, prepared as described above, may be preserved for about a week under refrigeration at −20 °C.

References

[1] Fog, J., Scand. J. Clin. Lab. Invest., 16, 49 (1964).

[2] Petryka, Z. J., and Watson, C. J., J. Chromatog., 37, 76 (1964).

[3] Recommendation on a uniform bilirubin standard, Clin. Chem. 8, 405-407 (1962).

[4] Tietz, N. W., Fundamentals of Clinical Chemistry, W. B. Saunders Co., Philadelphia, Pa., 1970, pp. 755-762.

This Standard Reference Material has been measured and certified at the laboratories of the National Bureau of Standards, Gaithersburg, Maryland. All inquiries should be addressed to:

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The date of issuance and certification of this Standard Reference Material was March 10, 1971.