A Stable "Bilirubin" Standard

P. K. Bilissis and R. J. Speer

An aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride has been successfully employed as a routine secondary "bilirubin" standard. It possesses a stability which is, unfortunately, not a characteristic of bilirubin itself.

The advent of automation for the daily determination of bilirubin in many serum samples has intensified our need for stable bilirubin standards for routine, repetitive calibration purposes. Crystalline bilirubin serves as a suitable primary standard, and when dissolved in chloroform, it yields reasonably stable stock solutions. However, when this is diluted with methanol to prepare a working standard, the resulting solution lacks adequate stability for routine daily use. Similarly, when bilirubin is dissolved in dilute alkali and quickly diluted with albumin solutions, the working standards are not as stable as one might desire. Some authors have suggested the use of pooled serum specimens or of commercial quality control sera, but these are neither as convenient nor as economical as one might like. In seeking a solution to this prosaic but practical problem, we have sought a pure, stable, commercially available aromatic amine (or related material) which would couple with diazotized sulfanilic acid under the appropriate reaction conditions to yield a chromogen with spectral qualities similar to those of azobilirubin. Such a material, N-(1-naphthyl) ethylenediamine dihydrochloride, has been found, and its applicability as a simulated bilirubin standard has been proved during the past 18 months of daily routine use.

Experimental

Concentrated Stock Solution  Pre pared by dissolving 129 mg. of N-(1-naphthyl) ethylenediamine dihydrochloride in sufficient distilled

From the Department of Pathology, Baylor University Medical Center, Dallas, Tex.
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water to make 200 ml. This solution is stable for many months when stored in a brown bottle at 4°.

**Simulated "Bilirubin" Working Standards** Distilled water is employed to dilute the concentrated stock solution (5.0 ml. stock diluted to 100 ml. is approximately equivalent to 5 mg./100 ml. bilirubin; 10.0 ml. stock diluted to 100 ml., approximately 10 mg./100 ml. etc.)

These solutions should be stored in brown polyethylene dispenser bottles at 4° when not in actual use and are stable for at least 2 months. The apparent bilirubin concentration of each should be checked against a freshly prepared bilirubin standard with the specific analytical procedure and the particular spectrophotometer for which they are to be employed prior to their use as daily secondary standards.

**Fig. 1.** Pigments derived from coupling diazotized sulfanilic acid with A, crystalline bilirubin (in weak alkali diluted with albumin solution); B, N-(1-naphthyl)ethylenediamine dihydrochloride solution; C, Versatol Pediatric (Warner-Chileott); and D, icteric human serum.

**Spectral Characteristics**

Figure 1 illustrates the close correspondence between the \( \lambda \) max of authentic azobilirubin in serum and that of the azo dye derived from N-(1-naphthyl) ethylenediamine dihydrochloride. These characteristics have permitted the use of this simulated standard in the Beckman Model B, Beckman Model DK-2, Spinco Ultramicrophotometer,* and the Technicon Autoanalyzer† on a routine basis for the past 18 months.

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*Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
†Technicon Instrument Corp., Chauncey, N. Y.