Estimation of Unconjugated, Conjugated, and "Delta" Bilirubin Fractions in Serum by Use of Two Coated Thin Films

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We used two coated thin films to measure the concentrations of unconjugated, conjugated, and total bilirubin as well as bilirubin covalently bound to albumin ("delta" bilirubin) in more than 400 serum samples. We measured the unconjugated and conjugated species by determining their reflection densities at two wavelengths (400 and 460 nm) on a coating designed for the enhanced spectral measurement of bilirubin but which does not register the delta form. Total bilirubin was measured by use of a diazo-based thin film (Clin Chem 29: 37–41, 1983). We estimated the concentration of delta bilirubin by subtracting the sum of unconjugated and conjugated bilirubin from the concentration of total bilirubin. All measurements agree well with those by comparative methods, as shown by linear regression. Slopes ranged from 0.92 to 1.02, correlation coefficients from 0.935 and 0.998. Linear combinations of these values can also be used to compute other results; e.g., the sum of conjugated and delta bilirubin can be considered to be an estimate of "direct"-reacting bilirubin.

Additional Keyphrases: multilayer film analysis · reflection densitometry · effect of sample freezing and storage · changes in relative concentration of subcomponents during jaundice

Two methods have been reported from the Kodak Research Laboratories for measuring serum bilirubin concentrations in coated thin films designed for use with the Kodak Ektachem analyzer (1, 2). In the first, a mordant-enhanced spectral assay (the Kodak Ektachem NBIL slide), the concentration of bilirubin is determined from a measurement of reflectance at 400 nm for bilirubin bound by a cationic polymer (mordant) in the coating (1). In the second, the products of the reaction of bilirubin and a prefered diazonium salt are measured (2). Elsewhere (3) we have described the adaptation of the mordant-based slide to the simultaneous measurement of unconjugated (B,) and conjugated bilirubin (Bu)—the "B, Bu" slide.3

Concurrent with developing thin-film assays for bilirubin, work was underway to devise an independent method for the separate measurement of all subcomponents of bilirubin. During that work, in which a new liquid chromatographic (LC) method for bilirubin was developed (4), it became apparent that four, rather than three, forms of bilirubin are increased in the serum of adults with liver disorders. One of those forms, in which bilirubin is apparently covalently bound to albumin, was first reported by Kuenzle et al. (5), who designated it "delta" because it eluted fourth in their system. This form of bilirubin, B,, represents a significant fraction of the total serum bilirubin in some individuals (1, 6–11).

Owing to the design of the B, Bu slide, in which an optical screening layer minimizes interference from hemoglobin, B, is not detected (3). The diazo-based assay, on the other hand, measures total bilirubin in serum, regardless of subcomponent composition, in good agreement with a well-controlled Jendrassik–Gröf assay (2).

We describe here assays for bilirubin components in which two separate film coatings are used. B, and B, are measured by a two-wavelength determination of the reflectance from the B, Bu slide (3), and total bilirubin with use of the diazo-based coating (2); the concentration of B, is then computed by subtraction of the sum of B, + B, from the total bilirubin concentration.

We compared results for total bilirubin from the thin-film assays with those from the Jendrassik–Gröf assay. The concentrations of the bilirubin subcomponents were compared with derived values computed from both Jendrassik–Gröf and LC assays (3).

Materials and Methods

Materials

Equipment: A modified Kodak Ektachem four-chemistry analyzer was used for the thin-film assays. Raw data from the analyzer were directed to an HP-85 desktop computer (Hewlett-Packard Co., Corvallis, OR 97330), which calibrated, reported concentration results, and stored the data on magnetic tape for reanalysis. The Jendrassik–Gröf assay was performed with a Rotochem II-A centrifugal analyzer (Travenol Laboratories, Instrument Division, Silver Springs, MD 20910). The LC analysis has been described in detail elsewhere (4, 11).

Serum samples: Samples were obtained from a wide variety of inpatients and outpatients at Yale-New Haven Hospital, including patients with Gilbert's disease, Dubin-Johnson's syndrome, alcoholic liver disease, sepsis, obstruction, hemolysis, and infiltrative processes.

Sample handling: Thin-film assays were performed generally within 2 h of sample collection. After assaying them with the thin films, we froze the samples at −50 °C and shipped them to Rochester, NY, where they were reanalyzed by the Jendrassik–Gröf and the LC methods. The samples were stored in the dark and handled in subdued or yellow light whenever possible. To ascertain the extent of sample degradation during storage and shipping, we analyzed 54 samples by LC at Yale before freezing and again in Rochester after shipment.

Procedures

Assay methods: Structures and performance of the thin films for bilirubin analysis have been described (1, 2). The only modifications necessary for subcomponent analyses...
were to measure the reflectance from the $B_{B_1}$ slide at an additional wavelength (460 nm), to perform assays on both films sequentially, and to modify the algorithms for calibration and concentration computation.

**Comparison assays:** The Jendrassik–Gröf total and direct bilirubin assays were performed as described (12, 13). We estimated the concentrations of bilirubin subcomponents in two steps. After estimating the relative amount of each subcomponent by the LC procedure of Lauff et al. (1,1), we computed the concentration of each subcomponent by multiplying the total bilirubin concentration (as determined by the Jendrassik–Gröf procedure: J-G TBIL) by the proportion observed for that subcomponent in the LC separation ($LC \times J-G \ TBIL$) (3). For the LC method we monitored absorbance at 456 nm. Molar absorptivities of the components in the eluting solvent were assumed to be equal, as discussed by Lauff et al. (11). To avoid confusion associated with the diverse molecular masses of the bilirubin subcomponents, we express all measurements in micromoles per liter (1 mg of unconjugated bilirubin = 1.71 μmol). For comparison with results by the thin-film assay for $B_0$, we summed the mono- and diconjugated bilirubin concentrations as determined by $LC \times J-G \ TBIL$ (3).

**Calibration**

**Calibrators:** Reference materials are not available for most of the bilirubin subcomponents. Only unconjugated bilirubin can be obtained in quantity and relative purity. $B_4$ has been isolated from other bilirubin species but is always contaminated with excess albumin (6). Diconjugated bilirubin has been purified and characterized (14) but is unstable and not suitable as a reference material for routine use. Monoconjugated bilirubin has been isolated only in small amounts (1) and is not fully characterized; it is also too unstable to use as a reference material. Enough of all these components have been obtained, however, to verify their elution position in the LC separation.

The ditaurine conjugate of bilirubin has spectral and physical properties similar to those of the glucuronide conjugates in the $B_{B_1}$ slide and is stable enough to serve as a reference material. We describe its use as a calibrator for conjugated bilirubin species elsewhere (3). Calibrators for the thin-film assay for bilirubin fractions were prepared from bovine serum pools to which various amounts of unconjugated (Sigma Chemical Co., St. Louis, MO 63178) and ditaurine-conjugated bilirubin (Porphyrin Products, Logan, UT 94321) were added.

**Procedure:** The calibration function for the diazo-based thin-film total bilirubin assay (TBIL) can be represented as (2):

$$TBIL = a_0 + a_1D_1$$  \hspace{1cm} (1)

where $D_1$ is transformed reflection density (15), and $a_0$ and $a_1$ are linear-regression coefficients relating concentration and transformed density.

The assays for $B_0$ and $B_4$ were calibrated similarly, except that we computed the transformed reflection density at two wavelengths and used a nonlinear regression to compute the concentrations of unconjugated ($B_0$) and the combined mono- and diconjugated bilirubins ($B_4$) (3):

$$B_0 = b_0D_1(400 \text{ nm}) + b_2D_1(460 \text{ nm})$$  \hspace{1cm} (2)

$$B_4 = c_0 + c_1D_1(400 \text{ nm}) + c_2D_1(460 \text{ nm})$$  \hspace{1cm} (3)

One complication was the unavailability of authentic reference materials for glucuronide-conjugated bilirubin. Although the taurine conjugates are available and stable, their reflectance spectra, although qualitatively similar, differ quantitatively from those of the glucuronide conjugates. Thus we had to assign "apparent" $B_u$, $B_c$, and TBIL concentrations to each calibrator.

We determined "apparent" concentrations of $B_u$, $B_c$, and TBIL in the calibrators by assaying serum samples containing a wide range of all the bilirubin components, using both film coatings and comparative methods (LC and J-G TBIL). The component concentrations as determined by $LC \times J-G \ TBIL$ and the transformed reflection densities from the film assays were then substituted into equations 1–3 to determine "reverse" calibration coefficients. Finally, we used the transformed reflection densities observed for the various calibrator solutions on the coatings, in conjunction with the "reverse" calibration coefficients, to compute the apparent analyte concentrations in each calibrator fluid. This approach is similar to that used to compute "supplemental analyte values" and also resembles a proposal by Miller et al. (16) to compensate for matrix differences among secondary standards. One of the goals of this study was to confirm the feasibility of such an approach and to test its utility.

As already stated, we computed the concentration of $B_4$ by subtracting the concentrations of $B_0$ and $B_4$ from TBIL. Similarly, we computed a value corresponding to a presumptive direct bilirubin (DBIL) by subtracting only the concentration of $B_4$ from TBIL. Each of the assays was calibrated weekly with calibrators with assigned "apparent" concentrations of TBIL, $B_u$, and $B_4$. Concentrations of $B_4$ and DBIL were calculated from the other three; thus we used no calibrators for those components.

**Results**

The effect of freezing and shipping on the relative concentrations of the bilirubin subcomponents (as determined by LC) was minimal. The percentages of the total bilirubin attributable to each subcomponent before freezing and shipping and after receipt in Rochester were compared for 54 samples; there was no appreciable change.

The agreement of the diazo-based thin film and J-G methods for total bilirubin is similar to that reported previously (2). The concentrations of $B_0$ (γ) and $B_4$ ($\gamma'$) estimated from two-wavelength measurements of the $B_{B_1}$ slide correlated well with the $LC \times J-G \ TBIL$ results ($r = 0.935$ and $0.996$, respectively $\gamma = (0.92 \pm 0.019)x + (11.0 \pm 0.7) \mu mol/L, S_{\gamma} = 8.7 \mu mol/L, n = 338$; and $\gamma' = (0.94 \pm 0.008)x + (2.3 \pm 1.0) \mu mol/L, S_{\gamma'} = 14.5 \mu mol/L, n = 337$).

The concentration of $B_4$ computed from the $B_u$, $B_c$, and TBIL results from both thin films is compared with the $LC \times J-G \ TBIL$ values in Figure 1. Although this comparison requires five independent estimates to be combined (three thin-film determinations, one LC, and one J-G TBIL), the scatter about the regression line ($S_{\gamma}$) is not appreciably greater than for the other subfractions. Even though this component represents the total difference between the two thin-film methods, the correlation and slope by linear regression on results by $LC \times J-G \ TBIL$ are also comparable to those of the other bilirubin fractions. Such good agreement between independent and quite different measurement techniques increases our confidence in the validity of both methods.

Figure 2 shows a comparison of the J-G direct bilirubin concentration and the thin-film value. Owing to uncertainties in calibration, existing assays for direct bilirubin may only roughly approximate the concentration of bilirubin species commonly assumed to be "direct reacting" (13). Such a comparison generally results in a slope larger than unity, probably because of the failure of the J-G direct bilirubin assay to detect all the conjugated and delta bilirubin (11, 13).
only one of which can measure $B_B$ ($d$). None is suitable for routine use, and all suffer from the lack of pure reference materials for all but unconjugated bilirubin.

Our work shows that the thin-film assays for bilirubin and its subcomponents, based on calibration with unconjugated and ditaurre conjugated bilirubin, yield results comparable with those obtained by use of LC in conjunction with the J-G total bilirubin assay. Apparent concentrations for $B_B$, $B_C$, and TBIL assigned to the calibrators resulted in assays that were calibratable and stable.

Although the thin-film method does not obviate the need for reference materials, it does provide a convenient and reproducible means for measuring bilirubin subcomponents and results by the thin-film method are in substantial agreement with those by a comparison method based on significantly different physical principles.

Figure 3 illustrates changes in the concentration of the bilirubin subcomponents with time in sequential serum samples from two individuals. As the total bilirubin concentration increased, the fraction contributed by unconjugated bilirubin decreased; few samples had more than 75 µmol/L $B_B$ per liter (45 mg/dL). Either $B_B$ or $B_C$ or more likely both were usually responsible for increases in jaundice in adults. As symptoms were relieved and the total bilirubin concentration diminished, the concentration of $B_B$, if increased at all, returned to normal, and the $B_B$ concentration dropped to insignificant values. The concentration of $B_C$, on the other hand, changed much more slowly and often remained above normal for several weeks, a phenomenon probably related to the lifetime of albumin in serum.

Owing in part to the previous difficulty in quantifying bilirubin components, little is known about the significance of changes in their relative concentrations in serum (10, 11, 17, 20). The limited work we have done, however, shows that the relative amounts of each subcomponent can change dramatically over the course of acute liver dysfunction and recovery. We hope that the availability of a convenient means to measure total bilirubin and its components will encourage more general awareness of the shifting pattern of bilirubin subcomponents, so that we can learn more about the significance of these changes.

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References
Fig. 3. (left) Bilirubin analyses of serum from a 62-year-old man with progressive renal failure undergoing dialysis treatment; (right) Bilirubin analyses of serum from a 58-year-old woman with a tumor obstructing the biliary tract.

(left) Sepsis was diagnosed on 4/10/82 and began to improve on 4/22, when we obtained our first sample. The broken line in a indicates the total bilirubin concentration reported by the hospital's routine assay. (right) External drainage was begun on 2/22, but partial obstruction returned on 3/1. Note in particular how sensitive B, is to the relief and return of obstruction. Results are presented three ways: (a) Concentration of total bilirubin as a function of time. (b) Concentration of unconjugated (•••), mono- and diconjugated (©©©), and delta bilirubin (¥¥¥¥) as a function of time. (c) Fraction of total bilirubin attributable to each subcomponent as a function of time; symbols as in b.