Quantitative Liquid-Chromatographic Estimation of Bilirubin Species in Pathological Serum

John J. Lauff, Mary E. Kasper, and Robert T. Ambrose

Earlier we described a "high-performance" liquid-chromatographic procedure for separating the four distinct fractions of bilirubin (un conjugated, mono conjugated, dic conjugated, and protein-bound) in pathological human serum (J. Chromatogr. 226: 391–402, 1981). We have modified the prechromatography precipitation of the serum globulins required in that method and have measured the bilirubin content of the precipitate spectrophotometrically. On average, the precipitate contained less than 10% of the total bilirubin in the serum samples. Adding the value obtained for the precipitate to that obtained by chromatography for the individual bilirubin fractions gave an estimate of the concentration of the total bilirubin in the sample. For 357 samples from 132 patients, this total value correlated well with that obtained by the Jendrassik–Gröf diazo procedure (slope = 1.00; r = 0.995, linear least-squares fit). The CV for the total and fractional bilirubin measurements was, on average, ±5% for pathological sera. Sample collected at different times from the same patient showed significant changes in the distribution of bilirubin among the four fractions. Only two of the published liquid-chromatographic methods for separating the bilirubin species in serum (1–9) have been demonstrated to separate, on the column, the protein-bound bilirubin species (β) as well as the more familiar diconjugated bilirubin (γ), mono conjugated bilirubin (β), and unconjugated bilirubin (α). The open-column chromatographic procedure of Kuenzle et al. (8, 9) required a large volume of serum and destruction of the packed column after every analysis—obviously a tedious procedure. In the "high-performance" liquid-chromatographic (HPLC) procedure we described earlier (1) the column was relatively long-lived and gave reproducible separations for samples with a concentration of total bilirubin ≤130 mg/L.

We have modified the earlier procedure by adding two steps, which allow one to quantify the total and fractional bilirubin in serum. First, before interfering globulins are precipitated prior to chromatography by added sodium sulfate, the serum is incubated at 37 °C with an equal volume of a solution of ascorbate-stabilized, phosphate-buffered, human serum albumin. Especially for samples with high concentrations of mono- and diconjugated bilirubin (β + γ), this treatment improves the analytical recovery of the bilirubin species for the subsequent liquid-chromatographic separation. Second, the protein fraction, removed by precipitation and filtration, is resolubilized in a solution of urea, mercaptoethanol, and Tris buffer. The concentration of bilirubin species that precipitates with the protein is then estimated by comparing the absorbance of the redisolved precipitate with that of unconjugated bilirubin–albumin standards dissolved in the same matrix. Likewise, the concentration of the fractions separated during chromatography is estimated by comparing their peak areas with those obtained when unconjugated bilirubin–albumin standards, diluted in the same matrix, are chromatographed.

Materials and Methods

All bilirubin-containing samples were handled under yellow-filtered light. Ascorbic acid, 2-methoxyethanol, 2-mercaptoethanol, phosphoric acid, sodium phosphate (mono- and dibasic), tris-(hydroxymethyl)aminomethane (Tris), and urea were of the highest purity available (Kodak Laboratory Chemicals, Rochester, NY 14650). Anhydrous sodium sulfate was purchased only in the granular form (MCB Reagents, Cincinnati, OH 45212). Unconjugated bilirubin and human serum albumin (Cohn Fraction V) were obtained from Sigma Chemical Co., St. Louis, MO 63178. "HPLC-grade" 2-propanol was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Chromatography columns packed with Lichrosorb RP-8 were obtained from either Brownlee Labs Inc., Santa Clara, CA 95050, or Chrompack Inc., Bridgewater, NJ 08807. Disk membrane filters, 0.45-μm pore size and 25-mm diameter (Type HA), were purchased from Millipore Corp., Bedford, MA 01730. Semimicro ultraviolet cuvettes, 1.0-mL nominal capacity, were obtained from Fisher Scientific Co., Pittsburgh, PA 15219; scintillation vials (cat. no. 986561) from Wheaton Scientific, Millville, NJ 08332; and Vacutainer Tubes (no. 6510) for collection of sera from Becton-Dickinson, Rutherford, NJ 07070.

Reagents

Ascorbic acid solution: 100 g/L in 0.5 mol/L phosphate buffer (sodium salt); final pH 5.8 ± 0.05. Prepare fresh daily.

Sodium sulfate solution: 277 g/L of anhydrous, granular sodium sulfate adjusted to pH 7.3 ± 0.1 and kept at 38 °C.

Initial eluent: One volume of 2 mol/L phosphate solution buffer (sodium salt, pH 6.7), two volumes of 2-methoxyethanol, and 37 volumes of water. Adjust the pH to 2.0 with phosphoric acid and de-gas by bubbling with helium.

Final eluent: One volume of phosphoric acid, two volumes of 2-methoxyethanol, and 38 volumes of 2-propanol. De-gas with helium.

Human serum albumin diluent: 100 g/L with 0.25 mL of ascorbic acid solution (above) added for every 10 mL of solution; final pH adjusted to 7.8 ± 0.1.

Urea/mercaptoethanol solution: per liter, 480 g of urea, 6 g of Tris, and 14 mL of 2-mercaptoethanol. Final pH 9.1. Filter to remove turbidity.

Instrumentation

Samples were filtered in a stirred, multicell filtration unit (Type MMC; Amicon Corp., Lexington, MA 02173). As many as eight samples could be processed at one time.

For chromatographic separations we used either of two nearly identical chromatographic systems. The first consisted of two Model M6000A pumps, a Model 720 system controller, and a Wisp 710B autoinjector (all from Waters Research Laboratories, Eastman Kodak Co., Rochester, NY 14650.

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Associates, Milford, MA 01757), an oven from a Model 830 liquid chromatograph maintained at 41 °C (Du Pont Instruments, Wilmington, DE 19888), a variable-wavelength spectrophotometer monitoring absorbance at 436 nm (Model LC-55; Perkin-Elmer Corp, Norwalk, CT 06856), a second spectrophotometer monitoring absorbance at 280 nm (Model Holochrome; Gilson Medical Electronics, Middleton, WI 53562), a dual-pen recorder (Model SR-206; Heath/Schumberger, Benton Harbor, MI 49022), and a recording integrator (Model 3390A; Hewlett-Packard, Avondale Div., Avondale, PA 19311), both of which were used to record the data. The integrator measured the area of peaks monitored at 436 nm. To ensure full measurement of all the bilirubin species separated in a sample, a flat baseline was forced by appropriate selection of the integration parameters from just before the elution of the δ-bilirubin peak to just after elution of the β-bilirubin peak.

The second instrument was similar but had a column heater set at 41 °C (Jones Chromatography Inc., Columbus, OH 43214), a Waters Model 440 absorbance detector equipped with 436- and 280-nm filters in separate detector compartments, and a Waters data module to record and integrate the peaks. Again, a flat baseline was used, as above, for integration of the peaks corresponding to δ, γ, and β bilirubin.

Sample Collection and Storage

Samples were obtained by venipuncture and collected into Vacutainer Tubes coated with a red dye to minimize photolysis. The serum was separated by centrifugation and as soon as practical, the specimens were stored at ~70 °C and then shipped on solid CO2 to our laboratory. Aliquots (0.25 mL) of each sample were transferred to scintillation vials, for subsequent liquid-chromatographic analysis, and to small screw-cap vials, for Jendrassik–Gröf diazo analysis for bilirubin. Both sets were stored frozen until analysis.

Prechromatographic Treatment

After 0.25 mL of HSA diluent was added to each sample vial (usually we treated seven samples at one time), the vials were sealed, gently shaken to ensure mixing, and then placed in a 37–39 °C water bath 1–2 cm deep. After 20 min all vials were removed from the bath and treated two at a time with 7 mL each of sodium sulfate solution to precipitate the serum globulins. After rescaling and shaking the vials thoroughly, we replaced them in the water bath for 1.5–2.0 min. The turbid contents were removed with a 10-mL plastic syringe and transferred to a chamber in the Amicon filtration unit, assembled with a single 0.45-μm pore-size Millipore filter in place. We did not use the Gelman prefilters that we used in our earlier work (1) because the prefilters increased the filtration rate but it also made it difficult to determine, while filtering samples, whether a filter had broken.

The entire unit was tightened together as tightly as possible with hand pressure only, and filtration proceeded under pressure (346 kPa; 50 psi) with helium. Each chamber was connected with plastic tubing to a 25-mL volumetric flask. Each receiving flask contained 1.5 mL of the 100 g/L ascorbic acid solution.

Immediately after sample addition, the filter chamber was stirred and then pressurized to force filtration in a reasonable time (~1–20 min/sample; average ~10 min). Samples with high concentrations of total bilirubin generally took longer to filter. We then added a second 7-mL aliquot of sodium sulfate to each of the vials that had contained the serum samples, which were then sealed and reheated.

After the initial filtration of all samples was completed, the pressure was released from each filtration chamber, and another 0.25 mL of HSA and 0.25 mL of water were added. The unpressurized chambers were stirred for ≤1 min. The time was kept to a minimum because the HSA solution can redissolve some of the precipitated globulins, which, if allowed to pass through the filter into the receiving flask, would degrade the chromatographic column. After the rapid HSA wash, the sodium sulfate aliquot, which had been reheated in the original sample vial, was added to the appropriate filtration chamber. The samples were stirred rapidly for ~1 min to reprecipitate completely any redissolved globulins, then pressure was applied to force the solution through the precipitate. The wash was collected in the same volumetric flask as the first filtrate. The samples were diluted to volume with distilled water.

HPLC Separation

The HPLC separation of bilirubin species was the same as that described earlier (1) except that 800-μL samples were injected. Two injections of each sample were made in a mirror-image sequence (i.e., order of injection: std. 1, sample 1, sample 2, std. 2, sample 2, sample 1, std. 3) during each overnight run, to minimize the effects of hydrolysis while the samples awaited injection and to check whether an incorrect sample volume had been injected.

Recovery of Globulins

After the filtrates were collected and diluted to volume, 1 mL of the urea/mercaptoethanol reagent was added to each filtration chamber and stirred for 2 min; the filtration chambers were represurized and the redissolved globulins were collected in 5-mL screw-cap vials. The chambers were then rinsed with 0.5 mL of urea/mercaptoethanol reagent, which was collected in the same vials. The recovered precipitates were transferred to semimicro cuvettes and scanned from 650 to 350 nm vs urea/mercaptoethanol.

Calculations

To stabilize standard bilirubin–albumin solutions prepared in human serum albumin solution (50 g/L), we added 0.25 mL of ascorbic acid solution (100 g/L) per 100 mL of albumin solution and adjusted the pH to 7.3–7.4 before dilution to volume. To ensure complete dissolution of the unconjugated bilirubin, we used dimethyl sulfoxide and sodium carbonate (0.1 mol/L), as described by Routh (10), and thorough ultrasonic mixing (2–5 min, no specks visible) before diluting with the HSA solution. One stock bilirubin–albumin solution containing ~200 mg of unconjugated bilirubin per liter was diluted serially with the HSA solution (50 g/L) to make calibrator solutions with concentrations as low as 5–8 mg/L. Each of these solutions was split into ~0.5-mL aliquots and stored until needed in separate sealed 5-mL screw-top vials in a freezer. These calibrator solutions (at least seven different concentrations) were used for about one month. As demonstrated by Doumas (10), these standards may have decomposed by as much as 2% during one month. In contrast, standards for the diazo procedure were stored at liquid-nitrogen temperature, where no decomposition would be expected.

For each day of HPLC analysis, we thawed a fresh series of the calibrator bilirubin–albumin solutions and transferred 0.25 mL of each to individual 25-mL volumetric flasks that contained 0.5 mL of the 100 g/L HSA diluent. We then added to each flask 1.5 mL of ascorbate solution (100 g/L) and 14 mL of sodium sulfate solution (277 g/L). Before we injected these standards onto the HPLC column, we diluted them to volume with distilled water. Using the resulting
peak areas for unconjugated bilirubin in each standard and the concentration of bilirubin in the calibrator solutions, we calculated a linear least-squares fit (Hewlett-Packard 11C calculator).

Because the standards contained albumin, a peak corresponding to the δ-fraction (protein-bound bilirubin) position in the chromatograms was observed at 436 nm, even though no δ bilirubin was present. This occurs because the absorption spectrum of albumin that we used to make up the concentrated standards, as well as for the HSA diluent, extends beyond 436 nm. Since albumin and δ bilirubin coelute, correction must be made for the contribution of albumin to the δ peak in serum samples. The area of this "pseudo-δ peak" was averaged for all standards run in one day and usually was equivalent to 8 mg of unconjugated bilirubin per liter (SD ~1 mg/L). Because the same concentration of albumin was added to the samples as to the standards, we subtracted the average value obtained for the standards from the value observed for δ bilirubin in each serum injection.

After correcting the δ-bilirubin areas for each sample injection for the albumin blank, we summed the areas of all the bilirubin peaks for each sample injection, then calculated the fractional amount of each bilirubin peak by dividing each by the total area. The values for both injections from one sample were then averaged. Using the averaged total area for a sample and the slope and intercept obtained from the linear least-squares fit of the standards, we estimated the concentration of the total bilirubin species that remained soluble in the samples.

To estimate the bilirubin remaining with the precipitated proteins, we scanned the absorbance from 650 to 350 nm of 0.25 mL of a series of bilirubin–albumin calibrators added to 1.5 mL of the urea/mercaptopethanol solution. From the maximum absorbance monitored in the range 420–450 nm and the concentration of bilirubin added, a linear least-squares fit of the data was calculated. Alternatively, we also constructed a linear standard curve by standard addition of bilirubin–albumin calibrators to proteins precipitated from serum samples that had already been redissolved in urea/mercaptopethanol. Nearly the same slope (3% difference) and intercept were obtained by either method. By applying a correction for volume differences between samples and these standards, we used the slope and intercepts to calculate the concentration of bilirubin removed with the redissolved proteins from serum samples. We made this calibration twice and used it for all the samples in the study.

For the redissolved globulins from serum samples in urea/mercaptopethanol solution, we used the maximum absorbance in the range 405–450 nm in the calculations. This wide range was used because the precipitates, depending on the relative concentration of fractions in the samples, may include any of the bilirubin fractions, the maximum absorbances of which vary within these limits. For very turbid samples (as noted visually or determined from scans of the urea/mercaptopethanol solution), the bilirubin value for the precipitate could not be estimated. For samples that were not turbid (>97%), we estimated the total bilirubin content of the samples by adding the concentration of bilirubin estimated to have been lost with the precipitated proteins to that obtained by summing the peaks from the chromatographic separation.

We estimated the concentrations of each of the bilirubin fractions by multiplying the fractional amounts determined by the chromatographic separation by the concentration of total bilirubin that remained soluble for the chromatographic separation (i.e., the value for the precipitate was not included).

Reference Bilirubin Values by the Jendrassik–Gróf Method

Jendrassik–Gróf diazo values for bilirubin content in serum were obtained in a separate laboratory by a procedure similar to that of Doumas et al. (11) but including a direct-reaction modification (12). The actual analyses were performed with an automated centrifugal analyzer (Rotochem; American Instrument Co., Division of Travenol Laboratories, Silver Spring, MD 20910). The diazo analyses were carried out either on the same day as the chromatographic analysis or within two weeks, during which time the samples were stored in a freezer.

Results

Chromatographic Separation

Figure 1 shows typical chromatograms of a pathological serum sample. The inverted upper trace monitored the absorbance of the column eluant at 280 nm, and the lower at 436 nm. The traces are offset by the amount indicated. When the offset is taken into account, the δ peak on the 436-nm trace eluted at the same time as albumin on the 280-nm trace. The δ fraction is identified in chromatograms by its co-elution with human serum albumin. The retention of unconjugated bilirubin (α) is determined from standard samples. The assignment of monoconjugate (β) and diconjugate (γ) peaks was made by comparison with purified samples of bilirubin diglucuronide and samples of dog bile, the bilirubin content of which has been characterized by others (13).

The chromatogram in Figure 1, obtained from separation of processed serum on a fresh column with very good resolution, shows that in many samples minor peaks can be resolved from the four major peaks. We have observed that on hydrolysis of dog bile at alkaline pH in albumin solution a small peak eluted at retention 16.33 min, most probably from an isomer of the monoconjugate (β). Thus, in calculating the individual fractions, we added the minor peaks to the large main peaks, as indicated in the Figure. With a column that had poorer resolution, the minor peaks would, in fact, often merge with the larger ones.

![Fig. 1. HPLC separation of bilirubin species in pathological serum](image-url)

Numbers indicate elution time (in min) for each peak. Upper curve, absorbance at 280 nm; lower curve, absorbance at 436 nm. Abs., absorbance; A, absorbance unit.
Table 1. Summary of Bilirubin (Total and Fractions), as Determined by HPLC, In Patients' Sera

<table>
<thead>
<tr>
<th>Concentration, mg/L</th>
<th>Percentage of Total Bilirubin</th>
<th>Total Soluble Bilirubin</th>
<th>Bilirubin in ppt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δγ</td>
<td>β</td>
<td>α</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>31</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>SD</td>
<td>30</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>Range</td>
<td>37</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>% of total bilirubin</td>
<td>21</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

*For 357 serum samples from 132 subjects.

Using this procedure, we have analyzed 357 serum samples from 132 individuals, most with high concentrations of bilirubin. Table 1 is a statistical summary of our data for each fraction, total bilirubin, and the recovered precipitate. On the average the Δ fraction made up the greatest percentage of total bilirubins and the γ fraction the least. In fact, in no sample of human serum have we found γ, the diconjugate fraction, to be the major component.

Analytical Variables

Accuracy. Over a five-month period the 357 serum samples summarized in Table 1 were quantified by the chromatographic technique and the Jendrassik–Gröf diazo procedure to assess the accuracy of the chromatographic procedure. Figure 2 shows the correlation observed for the total bilirubin value by HPLC, including that which remained with the precipitate, γ, and for the total found by the diazo procedure, x. A linear least-squares fit of the data yields the equation y = x + 1.4 mg/L (n = 357, r = 0.9995, SEslope = 0.0053, SEintercept = 0.65, Sxx = 8.8).

Figure 3 shows the correlation observed for the summation of the δ, γ, and β bilirubin fractions (Δ) with the direct-reacting Jendrassik–Gröf diazo value (x). For this calculation, we summed the HPLC value for soluble bilirubin species and that amount which remained with the precipitate and multiplied this total by the fractional sum of δ, γ, and β bilirubins determined from the chromatographic separation (this assumes that the precipitate contained bilirubin species in the same proportion as observed in the chromatographic separation of soluble bilirubin species). The linear least-squares equation was y = 1.28x - 5 mg/L (r = 0.992, n = 354). Excluding the δ fraction from the HPLC value lessened the correlation (r = 0.967) and decreased the slope to 0.85. Thus, the direct diazo value appeared to measure the δ fraction as well as the mono- and diconjugated species. That the slope is greater than 1, however, suggests that the direct diazo value does not measure all of this material.

Precision. Table 2 summarizes precision studies for the chromatographic procedure. For this study six sera, varying in total bilirubin content from 26 to 344 mg/L, were each divided into three aliquots. The average CV for the total bilirubin values was 3.0% and for the individual fractions averaged from ±2.4 to ±6.2%. Measurement of the precipitate fraction was less precise (CV = 22–37%). This result is expected because of the simple procedure used to measure the bilirubin content of the precipitate, which may be a complex mixture of all the bilirubin fractions, albeit at

Table 2. Precision Studies

<table>
<thead>
<tr>
<th>Serum</th>
<th>Within-day (n = 3)</th>
<th>Between-day (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ</td>
<td>γ</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>256G Meanα</td>
<td>22.4</td>
</tr>
<tr>
<td>C.V.</td>
<td>279D Meanβ</td>
<td>20.2</td>
</tr>
<tr>
<td>C.V.</td>
<td>258G Meanγ</td>
<td>73.0</td>
</tr>
<tr>
<td>C.V.</td>
<td>287A Meanδ</td>
<td>58.6</td>
</tr>
<tr>
<td>C.V.</td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>C.V.</td>
<td>239G Meanε</td>
<td>85.4</td>
</tr>
<tr>
<td>C.V.</td>
<td>5.5</td>
<td>2.8</td>
</tr>
<tr>
<td>C.V.</td>
<td>5.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Values in mg/L. +n = 2. For values >2.0 mg/L. +n = 4.
relatively low concentrations. The variability in the precipitation step and the variation in the turbidity present after dissolution in urea/mercaptoethanol are reflected in the poor precision for measuring this fraction.

Day-to-day precision values in Table 2 were obtained from five different sera ranging in total bilirubin content from 33 to 292 mg/L and analyzed on each of five successive days. Two samples were lost during the study because of sample mishandling. The average CV for total bilirubin for this limited sample set was 3.8% and for the individual fractions varied between 3 and 7%.

Interferences. We investigated three potential spectral or chromatographic interferences: carotene, biliverdin, and hemoglobin. Carotene, because of its hydrophobicity, eluted, if at all with this chromatographic system, only as a broad peak at the end of the gradient run. Although biliverdin did elute early between the γ and β peaks, at 436 nm its molar absorptivity is much less than that of the bilirubin species, so that for a biliverdin peak to appear in the chromatogram as large as a bilirubin peak, the sample would appear distinctly green. Obviously such abused samples are not suitable for this method. Finally, hemoglobin added to serum samples at −1 g/L led to the elution of minor peaks between the β peak and the α peaks. Because these peaks are well resolved from those for the bilirubin species, they present no problem during the chromatographic separation. However, we could not scan the precipitate from badly hemolysed samples accurately because of spectral interference from hemoglobin.

Column Lifetime

The useful lifetime of columns used in this study was shorter than reported earlier (1) and not always consistent from column to column. We suspect some batch-to-batch variation in the column manufacture. Although good resolution of the bilirubin species would remain for many more injections, columns usually were not suitable for quantitative work after 80–150 injections of serum.

There were several indicators of column degradation. First, the albumin peak height on the 260-nm recorder traces in standard samples would decrease daily from run to run as the column aged. When the cumulative loss of peak height amounted to ~25% of the height of albumin on the column when first used, losses in the δ-bilirubin peak also became evident during a single day. Reproducibility of the area of the δ-bilirubin peak in duplicate separations during a single day's run changed from a usual variation of a few percent to a systematic loss of 10% or more in the second injection. Thus, the summed area of all bilirubin species for the second injection of each sample was consistently less, rather than randomly lower or higher. Some components in the samples, even after removal of the globulins, apparently still caused some gradual column degradation, which affected primarily the protein-bound bilirubin (δ) and which varied with samples and columns.

Variation in the Distribution of Bilirubin Species in Pathological Serum with Time

During this study we obtained multiple samples of serum from the same patients at different times. Figure 4 shows the changes in the total and fractional bilirubin content of one patient's serum. Marked changes in these chromatographic patterns with time were noted for many patients.

Discussion

We chose this reversed-phase HPLC separation because it gave a good separation of the bilirubin species in pathological sera, including the δ-bilirubin fraction, which is tightly bound to protein. Other less acidic or less polar solvent systems did not cleanly separate this component. Unfortunately, not all serum proteins were compatible with this chromatographic system, and for a reproducible and relatively stable separation we first had to precipitate the globulins from serum samples with sodium sulfate (1).

At moderate bilirubin concentrations, precipitation of the globulins present in serum caused no specific losses of any one of the bilirubin species. But as the total bilirubin exceeded 130 mg/L, not only did the percentage losses become greater, but also the chromatographic patterns became distorted because more of the conjugates, particularly γ, were lost to the precipitate. The incubation with HSA minimized losses to the precipitate and hence minimized distortion in the observed chromatographic patterns.

The measurement of all the bilirubin fractions in our procedure with only unconjugated bilirubin used as a standard material is undoubtedly a source of inaccuracy and imprecision. Another source of error is the spectrophotometric measurement of bilirubin remaining with the precipitate, which may contain various amounts of all the bilirubin fractions, relative to standards containing only unconjugated bilirubin (see Table 2). Still, as Figures 2 and 3 demonstrate, these approximations yield good correlations with the more precise diazo values. Moreover, the relative sample-to-sample variation in these fractions in our procedure should be directly proportional to the true changes in concentration in the samples. Often it is the changes in the relative concentrations of the fractions that are of clinical interest (16–19, 20, 22–23).

Although other HPLC methods may quantify the conjugates (β + γ) more accurately, owing to the inclusion of reportedly stable standards for these species (2), none monitors the albumin-bound bilirubin (δ fraction). As the data in Table 1 show, overall the δ fraction represents a substantial

![Fig. 4. Change of bilirubin concentrations in patient's serum with time](image-url)
portion of the total bilirubin in pathological sera (sometimes >80%) and is observed in sera from almost all patients with high concentrations of the conjugates, not just from those patients with a specific disorder (14). In addition, as the correlation from Figure 3 demonstrates, the direct diazo value is affected by this fraction in addition to the β and γ fractions. Earlier methods, which did not measure the δ fraction, demonstrated poor correlations between direct diazo values and the conjugates (2, 15), presumably for this reason. Chromatographic methods that do not quantify this fraction are thus likely to incorporate large systematic errors for "real" serum samples.

Furthermore, as the time-course plots for the recovery of a patient demonstrate (Figure 4), the relative amounts of the fractions, and particularly the δ fraction, often vary systematically during recuperation. Thus the overall chromatographic pattern, including the fractional amount of the δ fraction, may be of some diagnostic significance, as has been reported earlier (16–21). More detailed studies of the patterns from patients included in this study will be presented elsewhere (22, 23).

We thank Dr. A. Gautam and Dr. J. Weiss at Yale–New Haven Hospital for the collection of serum samples; L. Evans and L. Mullaly of the Clinical Chemistry Technical Center, Eastman Kodak Co., for the Jendrassik–Grof bilirubin analyses; J. Widener and C. Little for technical assistance; and M. Sundberg for the use of a microcomputer to produce correlation plots.

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