Conjugated Bilirubin: A Better Indicator of Impaired Hepatobiliary Excretion Than Direct Bilirubin*

DEAN ARVAN, M.D.† and TERRY L. SHIREY, Ph.D.‡§

†Department of Pathology and Laboratory Medicine, University of Rochester, Rochester, NY 14642 and ‡Eastman Kodak Company Rochester, NY 14650

ABSTRACT

A prototype KODAK EKTACHEM Clinical Chemistry Slide (BuBc) provided a measurement of serum conjugated bilirubin which was at least as sensitive to developing conjugated hyperbilirubinemia as values provided by a direct (diazo) bilirubin assay. Under conditions where the impairment of hepatobiliary excretion was relieved in patients being treated for various hepatobiliary diseases, conjugated bilirubin was cleared from serum more rapidly than alkaline phosphatase, the delta bilirubin fraction, and bilirubin measured by the direct and total bilirubin assays. It is concluded that the conjugated bilirubin measurement provided by the BuBc Slide appears to be an earlier indicator of relief from hepatobiliary cholestasis, or conversely of residual impairment, than direct bilirubin, total bilirubin, or alkaline phosphatase.

Introduction

In 1982, Pelligrini and his colleagues12 reported findings from a five-year prospective study in which total bilirubin and alkaline phosphatase were determined for patients having treatable cholestatic disorders prior to surgical intervention and postoperatively. They concluded that in patients in whom surgery was successful in relieving biliary obstruction, total bilirubin returned to normal at a predictable rate, whereas alkaline phosphatase remained abnormal for prolonged periods. They further concluded that total bilirubin was a better indicator than alkaline phosphatase in identifying those patients with residual biliary obstruction.

Measurement of direct bilirubin has been reported as being a more sensitive indicator than total bilirubin of compromises in the excretory function of the hepatobiliary system. Based on a literature review, Rosati14 suggested that as many as one-third of patients with liver or hepatobiliary disease have slightly elevated direct levels with normal total values; thus, measurement of total bilirubin alone may miss about 30 percent of patients with occult liver disease. In spite of its greater sensitivity, however, the direct bilirubin assay has recently
been shown to suffer from lack of accuracy for conjugated bilirubin, the fraction believed to signal the presence of disease. For example, Lo and Wu\(^9\) found that the direct assay they were using detected only 65 to 75 percent of gravimetrically determined bilirubin diglucuronide, while Lauff et al\(^8\) demonstrated that their direct assay measured 76 to 89 percent of any delta bilirubin present, the latter thought to represent the bilirubin fraction covalently bound to protein.

The ability to resolve total bilirubin into its fractions and to quantitate them by chromatography\(^5,7\) or by a multilayered slide system\(^15,19\) has been recently demonstrated. In studies reported in this paper, the question was addressed of whether or not the information obtained by the measurement of the bilirubin fractions improved the specificity and sensitivity of assessing cholestasis as compared to the traditional tests. Using the novel technology of a multilayer slide system, sequential serum samples from patients with various hepatobiliary disorders were assayed for the bilirubin fractions along with being tested for total and direct bilirubin and alkaline phosphatase.

**Materials and Methods**

**Bilirubin Units**

Bilirubin concentrations are reported in micromoles per liter to avoid confusion from the different fractions having different molecular weights. The conversion of weight per volume to molar equivalents per volume is one mg unconjugated bilirubin equivalent per dL = 17.1 \(\mu\)moles per L.

**Reference Total and Direct Bilirubin Analyses**

A modified Jendrassik-Grof procedure described by Nosslin\(^11\) was used to measure total bilirubin. Direct bilirubin was assayed according to the method of Gambino.\(^4\)

**Alkaline Phosphatase Analyses**

The activity of alkaline phosphatase was measured using a dinitrophenyl phosphate procedure on an SMA-12.*

**Bilirubin Fractions by Chromatography**

High performance liquid chromatography (HPLC) was performed at the Health Sciences and Human Factors Laboratory at Eastman Kodak Company using the procedure of Lauff et al.\(^6,7\) Globulins were removed from serum by sodium sulfate precipitation prior to HPLC resolution of the bilirubin fractions. Typically, four peaks were noted in patients having hepatobiliary disease: an alpha peak (from unconjugated bilirubin), beta and gamma peaks (from bilirubin mono- and diglucuronides, respectively), and the delta peak (from tightly protein-bound bilirubin). The areas of the peaks were summed, and each peak was divided by the total area to indicate the percent of the total bilirubin represented by that fraction. These percentages were multiplied by the reference Jendrassik-Grof total bilirubin value for the sample to obtain fractional values. The beta and gamma peaks were combined to give the conjugated bilirubin value.\(^19\)

**Bilirubin Fractions by Multilayered Slides**

KODAK EKTACHEM Clinical Chemistry Slides (NBIL), read at both 400 and 460 nm, served as prototype KODAK

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* Technicon Instruments Corp., Tarrytown, NY 10591.
EK TACHEM Clinical Chemistry Slides (BuBc) with their ability to quantitate the unconjugated and conjugated bilirubin fractions simultaneously. In addition, an experimental diazo slide for total bilirubin, the KODAK EKTACHEM Clinical Chemistry Slide (TBIL), was used to assay all samples. As reported by Sundberg and colleagues, the difference in bilirubin detected by the TBIL slide and the NBIL slide is the delta bilirubin fraction. Also, by subtracting the unconjugated bilirubin value obtained with the NBIL slide from the total bilirubin value measured on the TBIL slide, direct bilirubin may be estimated. This value should be comparable to the direct-reacting fraction derived from the conventional Jendrassik-Grof method.

All slides were analyzed on a KODAK EKTACHEM ½ Analyzer modified to read NBIL slides at the two required wavelengths.

**Samples**

Five hundred and two serum samples were collected from in-house patients at Strong Memorial Hospital. Following centrifugation, each serum was split; part was assayed by the multilayered slides and by conventional Jendrassik-Grof total and direct bilirubin analyses within one-half to six hours following collection, and part was frozen and sent to the Health and Environment Laboratory at the Eastman Kodak Company for HPLC analyses. Sequential samples collected from 36 patients with hyperbilirubinemia owing to various causes provided the data from which certain trends were retrospectively noted. Diagnoses, pertinent history, and therapy were obtained from these patients' charts.

**Results**

In figure 1 bilirubin measurements obtained from multilayered slides are compared with those given by HPLC and by reference Jendrassik-Grof solution assays on samples from a patient who was hospitalized for cholangiocarcinoma. Comparable data indicating similar agreement between the methodologies were obtained for all patients studied. It should be noted that the proportion of each bilirubin fraction varied over the course of the disease. Also, as has been reported by Weiss and Powers, the delta fraction increased as a proportion of total bilirubin as obstruction was relieved.

To address the question whether a bilirubin fraction(s) might provide more clinically useful information in patients suffering from either intra- or extrahepatic obstruction than the direct or total bilirubin assays or alkaline phosphatase, sera from 13 patients recovering from various hepatobiliary diseases, including pancreatic masses, common duct neoplasms, and biliary stones, were assayed periodically over the course of each patient's hospital stay. In figure 2 are illustrated measurements obtained from a patient who was surgically treated for cholelithiasis whereas figure 3 illustrates a patient presenting with alcoholic hepatitis. These figures describe a feature that was apparent in virtually all such patients; conjugated bilirubin was cleared from serum more rapidly than alkaline phosphatase, delta bilirubin, or bilirubin detected by the total or direct bilirubin assays. In figure 4 are illustrated results from these patients in a format similar to that used by Pelligrini. This figure reinforces the observation that conjugated bilirubin appears to be an earlier indicator of excretory restoration than the other bilirubin measurements. Within the first day of decreasing hyperbilirubinemia signaling obstructive relief, the conjugated fraction had decreased to an average of 51 percent (ranging from 10 to 97 percent) of its pre-relief value for the six patients from
CONJUGATED VERSUS DIRECT BILIRUBIN

**Figure 1.** Bilirubin measurements on sequential samples received from a patient with cholangiocarcinoma. Measurements from multilayered slides are compared with (1) solution total and direct, Jendrassik-Grof bilirubin assays (reference JG), and (2) the fractional values given by multiplying the percentage of bilirubin in HPLC peaks by the total bilirubin given by the Jendrassik-Grof procedure (HPLC-JG). Bilirubin measurements are designated TBIL for total, DBIL for direct, Bu for unconjugated, Be for conjugated and BS for delta. PTC stands for percutaneous transhepatic cholangiography.

*17.1 μM = 1 mg Bu equivalent/dL

**Figure 2.** Bilirubin (abbreviations in figure 1) and alkaline phosphatase (ALP) measurements on a patient with cholelithiasis. The patient was decompressed surgically on day 3. The bilirubin measurements on this and subsequent patients reported were obtained from the multilayered slides.
whom adequate data were available, while direct bilirubin had decreased to 68 percent (ranging from 35 to 98 percent) and total bilirubin to 67 percent (ranging from 33 to 92 percent). By the end of a week, the average conjugated bilirubin was less than 20 percent of its pre-relief value; direct and total bilirubin being typically greater than 20 percent of theirs at the end of two weeks.

Even though the conjugated fraction appears to be a more specific indicator of restored excretory function, it is important to know how sensitive it is to remaining or developing excretory impairment compared to the direct bilirubin assay. One way to address this question is to measure the increasing conjugated and direct bilirubin during an early period of increasing total bilirubin in patients with excretory impairment. Among the patients in our study were 12 who had total bilirubin increases ranging from 9 to 360 \( \mu \text{moles/L} \) in seven days or less. The combined increase in total bilirubin in all 12 patients was 1408 \( \mu \text{moles/L} \). The comparable figure for the conjugated fraction in these patients dur-
ing the same period was 990 \mu moles per L whereas for direct bilirubin, 996 \mu moles per L.

Thus, in the evolution of cholestatic jaundice, conjugated and direct bilirubin measurement gave comparable increases, each amounting to about 70 percent of the increase in total bilirubin. However, considering that the combined starting conjugated bilirubin concentration for all episodes of increasing total bilirubin for all 12 patients was 1316 \mu moles per L while that for direct bilirubin was 2064 \mu moles per L, the relative sensitivity of the conjugated fraction to developing cholestasis (990/1316 = 0.75) compared to that of direct bilirubin (996/2064 = 0.48) was greater. In figure 5 are illustrated how the two values increased comparably in a patient suffering from hepatic dysfunction secondary to hemorrhagic shock. Alkaline phosphatase appeared not to be as sensitive to the developing liver dysfunction as the total, direct, or conjugated bilirubin values in this patient.

Discussion

Multilayered Slides vs. Solution and HPLC Bilirubin Assays

The close agreement of the bilirubin measurements given by the multilayered slides with those obtained from the conventional solution Jendrassik-Grof total and direct assays and the fractional separations provided by HPLC supported our using the slide values in our evaluation of patient samples.¹

Conjugated Bilirubin—A More Specific Indicator of Impaired Hepatobiliary Excretion

Either or both the conjugated and delta bilirubin fractions are candidates for signaling impaired hepatobiliary excretion since they are both detected by the direct bilirubin assay, the assay traditionally used to monitor this dysfunction. The conjugated fraction appears to be present in negligible quantities in normal non-neonatal patients; the average concentration measured by radioisotope dilution² and by HPLC, as performed by Muraca and Blanckaert,¹⁰ being about 1.7 \mu moles per L. Furthermore, the delta fraction is not detectable in appreciable amounts in sera from normal adults or adults having disorders characterized by unconjugated hyperbilirubinemia.¹⁶ Delta bilirubin appears in serum only when hepatic excretion of conjugated bilirubin has been impaired. The presence of either conjugated or delta bilirubin in serum, therefore, suggests that an abnormality in excretion has occurred.¹⁶

Of interest is the fact that delta bilirubin increases in proportion to the other fractions during the time of clinical improvement.¹⁶ This could mean that the delta fraction continues to be synthesized as the conjugated fraction is cleared or that the delta fraction has a longer half-life in serum. In our experience, the concentration of the delta fraction had little or no tendency to increase as the concentration of the conjugated fraction decreased, supporting the conclusion of Weiss et al¹⁶ that the proportional increase in the delta fraction relative to the other fractions during remission is probably due to its longer half-life in serum. Measurement of the delta fraction, then, in the conventional direct bilirubin assay may, in at least some instances, obscure the fact that obstruction has been relieved. Measurement of the conjugated fraction, by itself, appears to give a more specific indication of excretion status.

The results from the patients diagnosed as having cholangiocarcinoma (figure 1), cholelithiasis (figure 2), and alcoholic hepatitis (figure 3) demonstrate that the rate of decrease of the conjugated fraction during restoration of excretion or improvement toward normality is dependent on the nature of the underlying
pathology and the choice of therapy. In figure 4 are presented a composite picture of slowly as well as rapidly resolving diseases with cholestatic components which reinforces the more general finding that conjugated bilirubin decreases more rapidly than the direct and total bilirubin values during remission.

Conjugated Bilirubin—A Sensitive Indicator of Cholestasis

In figure 5 is illustrated the more general finding that as cholestasis developed, the concentration of the conjugated fraction increased as rapidly as direct bilirubin. This was not entirely a predictable result considering that the conjugated fraction measured by the slide is not the same as the bilirubin detected by the direct solution assay. During a period of rapidly developing cholestasis, both conjugated and delta fractions are formed; the conjugated being the dominant fraction. Conjugated bilirubin, as measured by the BuBc slide, increases quantitatively as rapidly as the value obtained by the direct solution assay (the latter including estimates of both the conjugated and delta fractions). This finding can probably be explained by the fact that two opposing factors are at play in the direct solution measurement. On the one hand this measurement underestimates the conjugated fraction (see introduction); on the other hand, it includes the measurement of the delta fraction. The conjugated fraction measured by the slide is not subject to these opposing factors as has been confirmed by quantitative HPLC measurements.\(^1\)\(^{15}\)\(^{19}\) Should a patient in remission from a recent episode of impaired excretion experience recurring cholestasis, the likelihood that the direct assay would be larger than the value for the conjugated fraction at the onset of the new episode is high, considering that the delta fraction would be lingering from the previous episode. This would explain why the percentage increase in the conjugated fraction was greater than that for the direct assay during recurrence. Further studies are needed to confirm these findings and to verify the enhanced utility of the conjugated bilirubin fraction.

**Figure 5.** Bilirubin (abbreviations in figure 1) and alkaline phosphatase (ALP) measurements on a patient following hemorrhagic shock. Conjugated bilirubin increases as rapidly as direct bilirubin during a hyperbilirubinemic event. Day 6, where total bilirubin was at a minimum in the sequential samples obtained from the patient, represents the base value preceding increasing total bilirubin values interpreted as increasing impairment, as discussed in the text.
Clinical Significance

Pelligrini et al.\textsuperscript{12} reported that total bilirubin provided a better prognostic indicator than alkaline phosphatase in identifying patients with residual biliary obstruction, knowledge of which could spare costly studies in the early postoperative period to differentiate patients with patent biliary tracts from those with residual biliary obstruction. The finding that the conjugated fraction decreases more rapidly than direct or total bilirubin (figure 4) during relief from cholestasis suggests that it may be an even more timely indicator. In addition, because of its comparable sensitivity to developing impairment without its measuring the delta fraction, the conjugated fraction may confirm residual or renewed excretory abnormality in a shorter period of time than the conventional bilirubin measurements.

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