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Measurement of serum bilirubin and its mono- and diconjugates: application to patients with hepatobiliary disease*

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SUMMARY A technique has recently been described by Blanckaert and his colleagues that specifically and accurately quantifies unconjugated bilirubin, diconjugated bilirubin, and the C-8 and C-12 isomers of monoconjugated bilirubin. This technique has now been used to determine the distribution pattern of bilirubin and its ester conjugates in 91 sera from 65 patients with hepatobiliary disease, and the results were compared with two conventional diazo assays. Both diazo assays yielded values for total bilirubin concentration that were markedly and unpredictably higher than those obtained by the new technique, and the direct-reacting fraction by diazo assay showed little or no agreement with the fraction of total ester conjugates determined by the new method. Previous studies using the new method had shown that bilirubin conjugates are undetectable in sera from healthy adults or individuals with Gilbert's syndrome, but they were found in 89 of the 91 present patient sera. The fraction of total serum bilirubin represented by C-8 monoconjugates, C-12 monoconjugates, diconjugates, and total ester conjugates was higher in patients with biliary obstruction than in those with parenchymal liver disease, but extensive overlap between groups prevented determination of these conjugated species from being diagnostically useful. Overall, bilirubin ester conjugates in serum consisted of 30% C-8 monoconjugates, 37% C-12 monoconjugates, and 33% diconjugates, while urine contained predominantly diconjugates.

Bilirubin IXα is formed by cleavage of the heme ring at the α-methene bridge. It is conjugated in the liver predominantly with glucuronic acid and is excreted into bile. In patients with hepatobiliary disorders, bilirubin concentration in serum may be abnormally raised. Diazo assays based upon the method of Van den Bergh and Muller[1] are generally used to measure bilirubin in serum. In these assays, bilirubin reacts with aromatic diazonium salts and splits into two azodipyrroles that are quantified spectrophotometrically. Conjugated bilirubin is usually measured by the diazo reaction which occurs in the absence of an accelerator (direct-reacting fraction). Unconjugated bilirubin is measured as the difference between the total and direct-reacting pigments (indirect-reacting fraction). However, a variable portion of unconjugated bilirubin reacts 'directly' and the diazo reaction has been noted to be unreliable in quantifying unconjugated and conjugated bilirubin in serum,[2–4] even when used in conjunction with methods employing solvent partition.[5–9]

A technique (AMHPLC) has recently been developed for quantitative conversion of bilirubin mono- and diester diconjugates to their corresponding mono- and dimethyl ester derivatives by alkaline methanolysis.[10 11] These ester derivatives as well as underivatised unconjugated bilirubin, all of which

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are poorly soluble in water, are separated from serum by solvent extraction and quantified by high performance liquid chromatography using an internal standard.[11] This approach permits measurement of unconjugated, monoconjugated, and diconjugated bilirubin IXα in serum.[11] Moreover, bilirubin monoconjugates in which the conjugating moiety is attached to the propionic acid side chain on the C-8 carbon of the tetrapyrrole skeleton (C-8 monoconjugates) can be quantified independently from monoconjugates in which the conjugating moiety is attached to the propionic acid side chain on the C-12 carbon (C-12 monoconjugates). The purpose of this study was to assess the distribution pattern of unconjugated, monoconjugated, and diconjugated bilirubin in the serum of patients with a spectrum of hepatobiliary diseases using the AMHPLC technique. These results were also compared with direct-reacting and total bilirubin determined by conventional automated and manual diazo methods.

Methods

PATIENTS AND COLLECTION OF SAMPLES

Hospitalised or clinic patients with hepatobiliary disease under treatment at the University of California, San Francisco, between September 1978 and September 1980 were investigated. An effort was made to select patients with a wide range of serum bilirubin concentrations and with widely differing diagnoses. A total of 91 serum samples was collected from 65 patients as follows: 11 samples from nine patients with surgically confirmed biliary tract obstruction, 15 samples from 10 patients with acute viral hepatitis, 13 samples from eight patients with primary or secondary hepatic neoplasms, seven samples from six patients with postoperative cholestasis, five samples from two patients with drug-induced liver injury, four samples from two patients with fulminant hepatic failure, three samples from two patients with biopsy-proven cirrhosis, four samples from four patients with biopsy-proven chronic active hepatitis, three samples from three patients with alcoholic hepatitis, two samples from one patient with hepatic trauma, one sample each from patients with haemochromatosis and sickle cell disease with cholestasis, and 22 samples from 16 patients with hepatobiliary disease of uncertain aetiology. Five urine samples were collected from four icteric patients (two biliary obstruction, one acute viral hepatitis, and one undiagnosed) within 24 hours of drawing the serum sample. All sera were frozen shortly after collection, stored in the dark at −20°C, and analysed within three weeks. Unconjugated, monoconjugated, and diconjugated bilirubin have previously been shown to be stable under these conditions.[11] Second-voided urine samples were collected in plastic containers containing small amounts of ascorbate and EDTA, were stored frozen at −20°C, and were analysed within three weeks. All studies were approved by the University of California, San Francisco, Committee on Human Research.

ANALYTICAL TECHNIQUES

Specific determination of unconjugated bilirubin IXα and its mono- and diester conjugates in serum and urine was performed by alkaline methanolysis combined with high performance liquid chromatography (AMHPLC) using an internal standard as previously described.[11] This assay has a 6% coefficient of variation and is able to detect as little as 0·2 μM (0·012 mg/dl) of unconjugated, monoconjugated, or diconjugated bilirubin IXα in 0·6 ml of serum.[11] Direct-reacting and total diazo-positive bilirubin in serum was measured by two methods commonly used in clinical laboratories. The automated procedure marketed by Dupont Instruments for use on the Dupont automatic clinical analyser (ACA method, 4% coefficient of variation) was used to measure total bilirubin in 80 samples and direct-reacting bilirubin in 49 samples. The manually performed reference diazo method of Michaelsson, Nosslin, and Sjolin[12] (Michaelsson method, 4% coefficient of variation), calibrated with standard solutions of bilirubin in normal human serum as described by Billing *et al*,[3] was used to measure total and direct-reacting bilirubin concentration in 40 samples. The samples analysed for total and/or direct-reacting bilirubin by these diazo techniques did not differ from the entire group of 91 samples with respect to either the proportion of patients with biliary obstruction or the mean total bilirubin concentration by AMHPLC.

Results

Total bilirubin concentration by AMHPLC in the 91 patient sera ranged from 5 to 750 μM. Both diazo techniques yielded values for total and indirect bilirubin that were substantially and erratically higher than those by AMHPLC for total and unconjugated bilirubin in serum (Fig. 1), particularly at low bilirubin concentrations where values were often several-fold higher than those with AMHPLC. Bilirubin conjugates (C-8 and C-12 monoconjugates and diconjugates) were detectable in 89 of the 91 sera and averaged 62±25% (mean ±SD) of total bilirubin. The direct-reacting fraction by diazo assay correlated poorly (Michaelsson, r=0·54) or not at all (ACA, r=0·02) with the

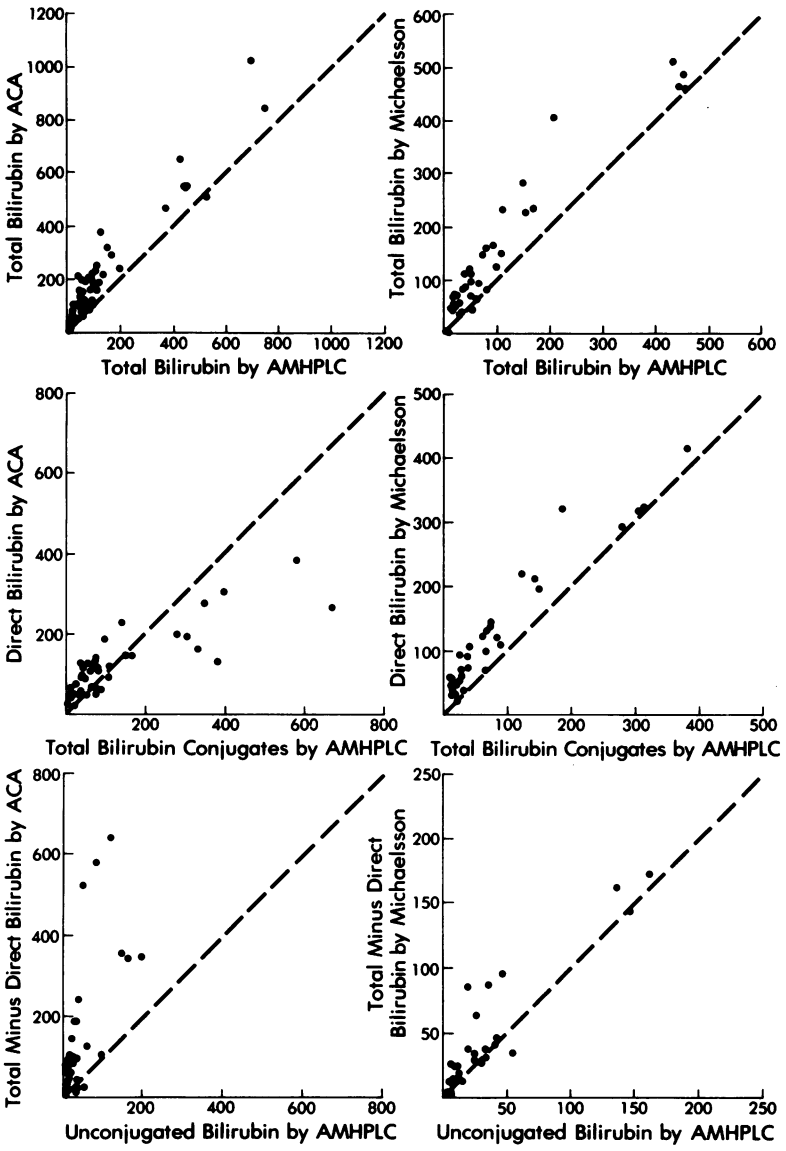

Fig. 1 *Comparison of two diazo assays with the AMHPLC technique for measuring the concentration of bilirubin IXα (in μM) and its ester conjugates in serum. - - - line of identity.*

fraction of ester conjugates by AMHPLC (Fig. 2). At low concentrations of total bilirubin, the direct-reacting fraction by both diazo techniques was higher than the fraction of ester conjugates measured with AMHPLC. At high bilirubin concentrations the direct-reacting fraction by the ACA but not by the Michaelsson technique was lower than the fraction of ester conjugates by AMHPLC.

Total bilirubin ester conjugates by AMHPLC increased in direct proportion to total bilirubin concentration (Fig. 3). Similarly, C-8 monoconjugates, C-12 monoconjugates, and diconjugates of bilirubin increased in direct proportion to total conjugates and comprised 30±11%, 37±12%, and 33±16%, respectively, of total ester conjugates in the entire patient group (Fig. 3). The proportion of total ester conjugates represented by C-8 monoconjugates (26±5%), C-12 monoconjugates (36±7%), or diconjugates (38±10%) was not significantly altered in patients with biliary obstruction. The percentage of total serum bilirubin present as ester conjugates in patients with biliary obstruc-

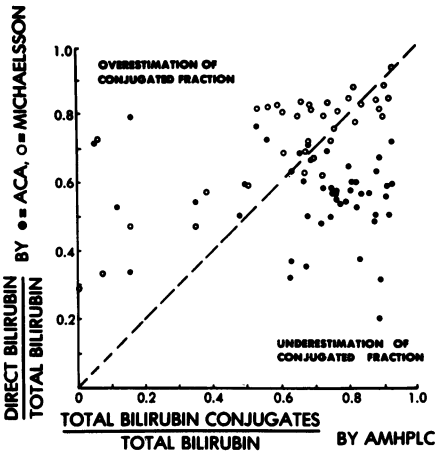

Fig. 2 *Comparison of the ACA (r=0·02) and Michaelsson (r=0·54) diazo assays with the AMHPLC technique for quantifying the conjugated bilirubin fraction in serum. - - - line of identity.*

tion (80±11%) exceeded ($p<0·01$) that of patients with hepatocellular dysfunction (55±26%). Considerable overlap, however, existed between these two groups, so that this was not a diagnostically useful determination (Fig. 4). The percentage of total serum bilirubin present as C-8 monoconjugates (21±6% versus 16±8%, $p>0·05$, Fig. 5), C-12 monoconjugates (28±4% versus 20±11%, $p<0·02$, Fig. 6), and diconjugates (31±12% versus 20±14%, $p<0·05$, Fig. 7) also tended to be greater in patients with biliary obstruction than in those with hepatocellular dysfunction. Again, however, considerable overlap between groups prevented determination of the individual conjugated species from being diagnostically useful.

The results of AMHPLC measurements of urinary bilirubin are summarised in the Table. In contrast with patient sera in which bilirubin monoconjugates predominated, diconjugates accounted for most (62±14%) of the bilirubin excreted in urine.

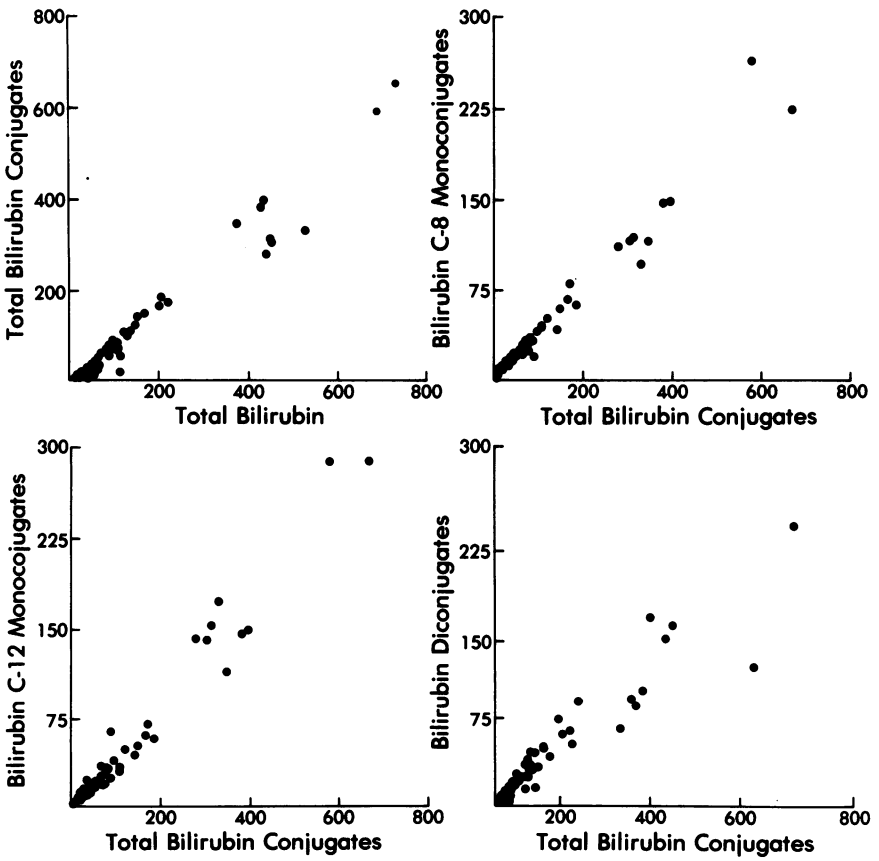

Fig. 3 *Bilirubin and bilirubin ester conjugates (in μM) in sera of patients with hepatobiliary disease measured by the AMHPLC technique.*

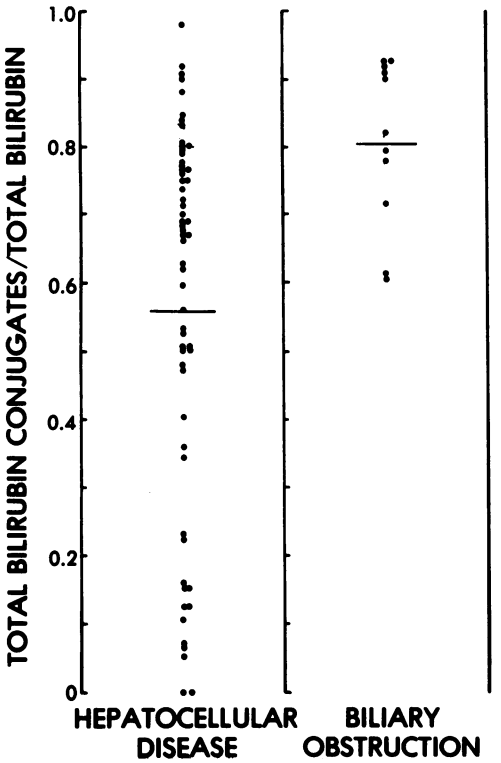

Fig. 4 *Bilirubin ester conjugates/total bilirubin by AMHPLC in patients with proven hepatocellular disease and biliary obstruction. Horizontal lines: mean values.*

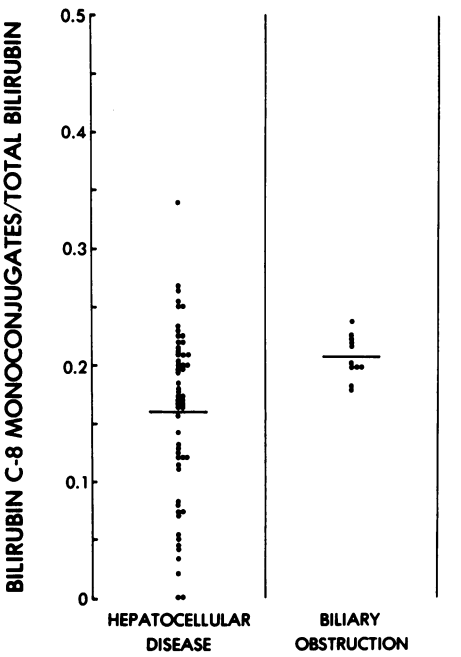

Fig. 5 *Bilirubin C-8 monoconjugates/total bilirubin in patients with proven hepatocellular disease and biliary obstruction. Horizontal lines: mean values.*

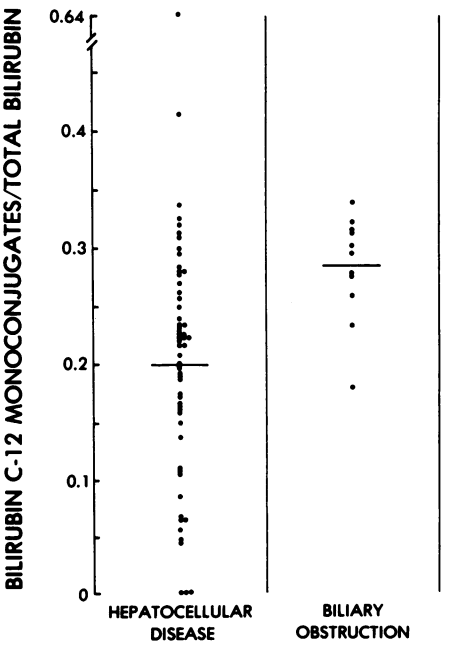

Fig. 6 *Bilirubin C-12 monoconjugates/total bilirubin in patients with proven hepatocellular disease and biliary obstruction. Horizontal lines: mean values.*

Discussion

Although it has been known since 1916[1] that more than one species of bilirubin is present in the serum of patients with hepatobiliary disease, this represents the first clinical study using a technique (AMHPLC) which individually quantifies unconjugated, monoconjugated, and diconjugated bilirubin in patient sera. Serum bilirubin was found to consist predominantly of conjugates, with monoconjugates predominating. Total ester conjugates increased in direct proportion to total bilirubin, and individual conjugated species increased directly with total conjugates (Fig. 3). Neither the proportion of total bilirubin represented by C-8 (Fig. 5) or C-12 (Fig. 6) monoconjugates or diconjugates (Fig. 7) nor the fraction of total ester conjugates (Fig. 4) permitted separation of hepatocellular disease from biliary obstruction.

This study also confirms and extends the previously reported preliminary observation that the

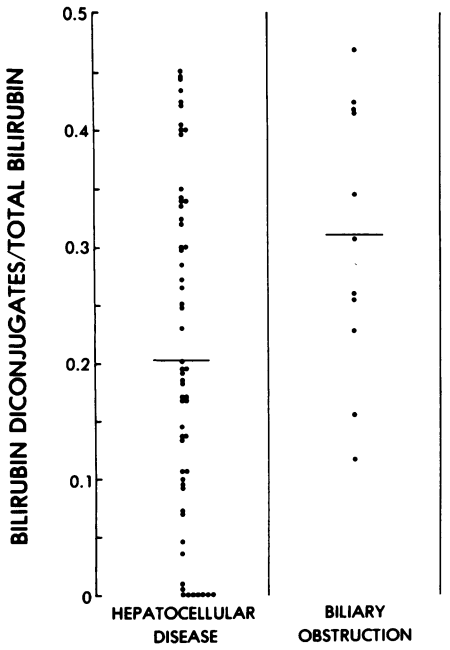

Fig. 7 *Bilirubin diconjugates/total bilirubin in patients with proven hepatocellular disease and biliary obstruction. Horizontal lines: mean values.*

AMHPLC technique yields values for unconjugated bilirubin and bilirubin ester conjugates that are considerably lower than indirect and direct-reacting bilirubin by diazo assays[11 13] (Fig. 1). This may reflect reaction of diazotised sulphanilic acid with serum substances other than bilirubin IXα and its ester conjugates.[11] Possibilities include alkali stable bilirubin conjugates,[14 15] diazo-positive non-α isomers of bilirubin IX not measured by AMHPLC,[16] or non-bilirubin substances known to interfere with the diazo reaction.[17] Alternatively, diazo-reactive bilirubin tightly linked to serum protein[18 19] might fail to be detected by the AMHPLC technique. Apparent overestimation of serum bilirubin concentration by the diazo technique has previously been noted by Bratlid and Winsnes,[9] and the relatively non-selective nature of the conventional diazo reaction may explain reports of spurious hyperbilirubinaemia in uraemic patients on propranolol therapy.[20] Moreover, the diazo reaction does not reliably quantify the fraction of total ester conjugates as measured by AMHPLC (Fig. 2). Thus, while the conventional diazo assay is empirically useful for estimating total bilirubin, its non-specificity needs to be recognised and the concept that the direct-reacting fraction specifically reflects bilirubin ester conjugates should be reevaluated.

Table *Bilirubin in urine and serum by AMHPLC**

Bilirubin (% of total)	*Serum*	*Urine*
Unconjugated	15±13†	8±6
Monoconjugates	53±3	30±9‡
Diconjugates	32±14	62±14‡

* Results of five paired serum and urine samples from four patients.
† Mean ±SD.
‡ $p<0.01$ compared with serum.

The clinical usefulness of the AMHPLC technique is likely to be its ability to accurately quantify low levels of bilirubin ester conjugates, which the diazo does not do (Figs. 1 and 2).[21 22] As ester conjugates were not detectable in serum of 22 healthy adults or six individuals with Gilbert's syndrome[11 13] but were found in 89 of the present 91 patients with hepatobiliary disease, the detection of conjugates by AMHPLC in patients with low-grade hyperbilirubinaemia strongly suggests hepatobiliary dysfunction. Conjugates would also be expected in the serum of patients with the Dubin-Johnson or Rotor's syndrome, as well as in patients with acute massive haemolysis resulting in a bilirubin production rate exceeding hepatic excretory capacity.[23 24] The sensitivity of AMHPLC in detecting hepatobiliary dysfunction remains to be assessed. The apparently high sensitivity in this study (89 of 91 patients) is probably misleading in as much as patients were selected because of hyperbilirubinaemia or other evidence of hepatobiliary disease. A more important and still unanswered question concerns the sensitivity of bilirubin ester conjugates measured by AMHPLC in the detection of hepatobiliary disease in patients with equivocal clinical findings and normal or near normal serum concentrations of total bilirubin. Indeed, both of the patients in the present series without detectable ester conjugates in serum (one with biopsy-proven cirrhosis and one with haemochromatosis) had a normal serum concentration of total bilirubin (<8 μM; <0·5 mg/dl).

The present findings also indicate that urine bilirubin consists predominantly of diester conjugates, whereas the serum contains principally bilirubin monoester conjugates (Table). This is reminiscent of previous observations in the isolated perfused rat kidney[25] and may be explained either by preferential renal clearance of diconjugates or by conversion of mono- to diconjugates in the kidney.[26]

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