Enzymatic Assay for Conjugated Bilirubin (Bc) in Serum Using Bilirubin Oxidase (BOD)

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We described an automated enzymatic assay for conjugated bilirubin (Bc) in serum using the Iatro D-Bil kit, with bilirubin oxidase (EC 1.3.3.5 BOD) from *Myrothecium* species. The specificity of the enzyme in the Iatro D-Bil kit was examined by analyzing unconjugated bilirubin (Bu), delta bilirubin (B δ), and Bc with high-performance liquid chromatography (HPLC), before and after the enzymatic reaction using BOD. The within-assay coefficients of variation (CV) of this method were 0.58 to 5.00% ($n = 20$) at 1.4 to 155.8 $\mu\text{mol/L}$. Day-to-day CVs ranged from 1.61 to 7.14% at 1.2 to 182.1 $\mu\text{mol/L}$. The analytical recovery was 96 to 101%. The presence of ascorbic acid, reduced glutathione, L-cysteine, uric acid,

urea, creatinine, glucose, lipemic material, anticoagulants, hemoglobin, or human serum albumin did not affect this assay system. The correlation coefficient between values obtained with the Iatro D-Bil kit (y) and HPLC method as reference for conjugated fractions (x) was; $r = 0.983$, $y = 0.952x + 8.851 \mu\text{mol/L}$, $Sy/x = 11.97$ ($n = 56$). We studied serum Bc levels, not including Bu and B δ , in patients with hepatic diseases or autoimmune hemolytic anemia. Levels of Bc obtained by the proposed method changed more rapidly than did those of direct bilirubin (D-Bil) obtained by diazo-dye method during the course of the diseases. *J. Clin. Lab. Anal.* 13:219–223, 1999. © 1999 Wiley-Liss, Inc.

Key words: bilirubin enzymatic assay; bilirubin oxidase; bilirubin fractions; automated analysis

INTRODUCTION

Assay of serum bilirubin is important for differential diagnosis of hepatobiliary or hemolytic diseases with jaundice. In the bilirubin assay based on the diazo reaction, serum bilirubin is determined as total bilirubin (T-Bil) and direct-reacting bilirubin (D-Bil). Indirect bilirubin is obtained by subtracting the value of D-Bil from that of T-Bil. Previously, it has been found that indirect bilirubin (I-Bil) corresponds to unconjugated bilirubin (Bu), and D-Bil, to conjugated bilirubin (Bc) (1). However, Bu and B δ react in small amounts with diazo reagent in direct assay, and some observations have been also reported with other methods such as the BOD and vanadate methods (1–4). In terms of clinical significance, values of Bc and B δ are better indicators of liver function than are the values of D-Bil, T-Bil, and hepatic enzymes such as AST and ALP (1,5–8).

As a fractional measurement of bilirubin, the HPLC method (9) and the Ektachem (BuBc) method (now Vitros) have been widely used (10,11). The Vitros method for bilirubin, which provides simple and rapid analysis, is based on the binding of bilirubin fractions to a cationic polymeric mordant, in which bilirubin fractions of B δ and neonatal bilirubin (NBIL) are calculated by use of value obtained by a “TBIL slide” and a “BuBc slide.” The Vitros method has become widely used

because Bc and B δ are better indicators of liver function than are the values of D-Bil. But this method requires special equipment; therefore, in most clinical laboratories, measurement of T-Bil, D-Bil, and I-Bil by the diazo-dye method, enzymatic method (BOD), or chemical oxidation method have been employed for routine analysis.

Here, we report an automated enzymatic assay for serum Bc using BOD. In the assay system, Bc is oxidized to noncolored substance at pH 5.5 in the presence of sodium fluoride, *N*-acetylcysteine (NAC), and *p*-toluenesulfonate, which prevent reactivity of BOD to B δ and Bu. The decrease in absorbance at 450 nm is read and is linearly related to the concentration of Bc in serum (12). BOD can selectively oxidize the Bc fraction at pH 5.5 in the presence of reagents such as sodium fluoride and NAC because these substances inhibit the oxidation of Bu and B δ by BOD. Thus Bc fractions was measured specifically by BOD. Normal values, found using the proposed method, were in the range of 0.3 to 2.9 $\mu\text{mol/L}$ with 107 normal subjects, and were in good agreement with the Bc assay in the HPLC assay (12). In this paper,

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we evaluate the proposed method, and determine that the state of hepatic diseases correlates clinically with the Bc values measured by the Iatro D-Bil better than with D-Bil values measured by other D-Bil methods based on diazo reaction. The proposed method is rapid and reliable, and is also applicable for routine use in clinical laboratories.

MATERIALS AND METHODS

Apparatus

The proposed enzymatic bilirubin assay and conventional bilirubin assay were carried out with the Hitachi Model 7070 automatic analyzer (Hitachi Co., Ltd., Tokyo, Japan; also known as the Hitachi 911 in the U.S. and Europe). High-performance liquid chromatography (HPLC) was performed according to the procedure of Lauff et al. (9) with minor modification.

Reagents

The Iatro T-Bil and Iatro D-Bil kits for enzymatic assay of bilirubin were obtained from Iatron Laboratories, Inc. (Tokyo, Japan). The first reagent for T-Bil assay consists of *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonate (HEPES) buffer solution (100 mmol/L, pH 7.8 at 37°C) containing 32 mmol/L of *p*-toluenesulfonic acid, 3 g/L of sodium dodecylbenzenesulfonate (SBS), and 1 mmol/L of ethylenediaminetetraacetic acid (EDTA). The second reagent for T-Bil assay is HEPES buffer solution (50 mmol/L, pH 7.8 at 37°C) containing 32 mmol/L of *p*-toluenesulfonic acid, 4 kU/L of BOD, and 4 kU/L of ascorbate oxidase (ASOD).

The first reagent for Bc assay is phthalate buffer solution (120 mmol/L, pH 5.5 at 37°C) containing 2.5 mmol/L of *N*-acetylcysteine (NAC), 50 mmol/L of *p*-toluenesulfonic acid, 0.1 mmol/L of EDTA, and 2.5 mmol/L of sodium fluoride. The second reagent for Bc assay is phthalate buffer solution (120 mmol/L, pH 5.5 at 37°C) containing 100 U/L of BOD and 4 kU/L of ASOD.

Physiological saline was used as a zero calibrator. A mixture of 164.2 µmol/L of ditaurobilirubin (DTB) and 40 g/L of bovine serum albumin dissolved in 100 mmol/L Tris-HCl buffer (pH 7.3) was used as 164.2 µmol/L Bc calibrator. The calibrator value of 164.2 µmol/L DTB was determined by the use of the Standard Reference Material (SRM) 916a (unconjugated bilirubin) from the National Institute of Standards and Technology (NIST) in the U.S.

Assay Procedure

Assay was performed by the end-point assay at 37°C with the Hitachi 7070 automatic analyzer. Either the calibrator solution or serum (14 µL) is mixed with 280 µL of the first reagent and kept at 37°C for 5 min before addition of 70 µL of the second reagent. The change of absorbance is monitored from 5 to 10 min at 450 nm with reference at 546 nm.

Methods for Comparison

"Clinimate Bil" and "Clinimate D-Bil" based on the diazo method by Michaelsson (13) were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). A Hitachi Model 7070 automatic analyzer was used for conventional method. High-performance liquid chromatography (HPLC) was carried out by the procedure of Lauff et al. (9) using a 250 × 4 mm column filled with 10 µm particles, Lichrospher 100 RP-18 (Kanto Chemical Co., Ltd, Tokyo, Japan), at 41°C and a flow rate of 1.0 mL/min. The spectrophotometer was set at 450 nm. Bilirubin fractions were separated by linear gradient elution with isopropyl alcohol. Flavin mononucleotide (FMN) was used as an internal standard for the calculation of the concentration of each bilirubin fraction. The peak of α-fraction corresponded to Bu, β-fraction corresponded to monoconjugated bilirubin, γ-fraction corresponded to diconjugated bilirubin, and the peak of δ-fraction to albumin-bound bilirubin.

Specimens

Serum specimens were obtained from 56 patients with hepatic diseases and autoimmune hemolytic anemia (AIHA), and who underwent medical evaluation and treatment at the Osaka University Hospital.

RESULTS

Evaluation of Iatro D-Bil Assays

Bilirubin fractions were analyzed by HPLC before and after enzymatic oxidation with the Iatro D-Bil reagent (Fig. 1). After oxidation of bilirubin by BOD at pH 5.5, the peaks for δ- and γ-fractions disappeared but most of the peaks for α- and δ-fractions remained. The decreased values in ratio between after enzymatic reaction and before enzymatic reaction for each fraction by HPLC were 1% for α-fraction and 18% for δ-fraction as shown in Figure 1. We also examined another patient recovering from hepatitis, and the HPLC values before enzymatic reaction were 11.0 µmol/L for α-fraction, 4.8 µmol/L for β-fraction, 6.8 µmol/L for γ-fraction, and 20.4 µmol/L for δ-fraction, while the HPLC values after enzymatic reaction were 9.8 µmol/L for α-fraction, 20.2 µmol/L for δ-fraction, and none detectable for β- and γ-fractions. The decreased values in ratio between after enzymatic reaction and before enzymatic reaction for each fraction by HPLC were 11% for α-fraction and 1% for δ-fraction.

The dose response to Bc was linear up to 598.6 µmol/L; the regression line was $y = 95.25x + 1.16$ µmol/L ($r = 1.000$, $y = \text{Bc concentration}$, and $x = \text{dilution ratio}$).

We estimated the detection limit of the proposed method by assaying the zero calibrator (physiological saline) 10 times. The mean result (SD) was 0.171 (0.031) µmol/L. The detection limit, defined as three standard deviations above the zero standard was 0.264 µmol/L.

Fig. 1. HPLC pattern of the bilirubin fractions before and after enzymatic reaction with the Iatro D-Bil reagent. The sample before enzymatic reaction was prepared by mixing the sample and the first reagent, and the mixture was injected into HPLC. The sample after enzymatic reaction was prepared by mixing the above mixture with the second reagent (BOD), and analyzed with HPLC. The values on HPLC before enzymatic reaction were 60.2 $\mu\text{mol/L}$ for α -fraction, 46.0 $\mu\text{mol/L}$ for β -fraction, 27.9 $\mu\text{mol/L}$ for γ -fraction, and 10.6 $\mu\text{mol/L}$ for δ -fraction, while the values on HPLC after enzymatic reaction were 59.5 $\mu\text{mol/L}$ for α -fraction, 2.9 $\mu\text{mol/L}$ for β -fraction, 1.5 $\mu\text{mol/L}$ for γ -fraction, and 8.7 $\mu\text{mol/L}$ for δ -fraction. The changes in ratio between after enzymatic reaction and before enzymatic reaction were 1% for α -fraction, 94% for β -fraction, 95% for γ -fraction, and 18% for δ -fraction.

The within-assay and day-to-day reproducibilities for four different pooled human sera were tested. The within-assay CVs ranged from 0.58 to 5.00% at 1.4 to 155.8 $\mu\text{mol/L}$ and day-to-day CVs were from 1.61 to 7.14% at 1.2 to 182.2 $\mu\text{mol/L}$.

The analytical recovery of DTB (41.1, 82.1, and 164.2 $\mu\text{mol/L}$) added to four different human pooled sera ranged from 96 to 101%, and all were assayed in triplicate. The mean analytical recovery was 99%.

Interference was not observed by the addition of the following substances to pooled human sera containing 7.35 to 14.9 $\mu\text{mol/L}$ of Bc: up to 5.68 mmol/L ascorbic acid, up to 1.63 mmol/L reduced glutathione, up to 2.72 mmol/L creatinine, up to 61.06 mmol/L glucose, up to 2.96 mmol/L L-cysteine, up to 3.21 mmol/L uric acid, up to 10.82 mmol/L urea, up to 95.26 mmol/L sodium fluoride, up to 15.46 mmol/L sodium citrate, up to 10.75 mmol/L EDTA, up to 200 g/L

lipemic material, up to 2460 units formazine turbidity, and up to 60 g/L human serum albumin.

The correlation between Bc values obtained by the proposed Iatro D-Bil method (y) and by the HPLC method as reference for conjugated fractions (x) was: $y = 0.952x + 8.851$ $\mu\text{mol/L}$, $r = 0.983$, $Sy/x = 11.97$ ($n = 56$) (y = Iatro D-Bil) (Fig. 2A), and the diazo-dye method (y) for HPLC method (x) was: $y = 0.918x + 31.501$ $\mu\text{mol/L}$, $r = 0.948$, $Sy/x = 20.39$ ($n = 56$) (Fig. 2B). We also compared the proposed Iatro D-Bil (y) and diazo-dye method (x): $y = 0.985x - 18.320$ $\mu\text{mol/L}$, $r = 0.983$, $Sy/x = 11.77$ ($n = 56$) (Fig. 2C).

Fig. 2. Correlation of serum bilirubin concentration: proposed method or diazo-dye method vs. conjugated bilirubin fractions by the HPLC method. (A) proposed method (y) vs. HPLC method (x); (B) diazo-dye method (y) vs. HPLC method (x); and (C) proposed method (y) vs. diazo-dye method (x), with samples from 56 hospital patients.

Typical Profiles for Bc Levels in Blood Samples From Patients

We studied the Bc levels with serial blood samples from patients during the course of hepatic diseases and autoimmune hemolytic anemia (AIHA). The bilirubin values obtained with the proposed method were compared with those from the diazo-dye method, which was shown in the percentage of Bc or D-Bil to T-Bil (Bc or D-Bil/T-Bil %) in each of the methods. Changes in Bc or D-Bil levels in patients with various hepatic diseases are shown in Fig. 3A to 3D. Figure 3A shows a patient with hepatocellular carcinoma (HCC), and whose condition deteriorated after transcatheter arterial embolization (TAE). The percentage of Bc or D-Bil/T-Bil % values with the proposed method tended to increase after discontinuation of chemotherapy on day 13, but the values by diazo-dye method did not change. In this patient, the γ -GT and ALP levels decreased rapidly on day 12 after discontinuation of chemotherapy, but melena occurred on day 21 and those levels increased because of the progressive posthemorrhagic ischemic hepatic dysfunction. Figure 3B illustrates a patient recovering from chronic hepatitis B during and following interferon (IFN) therapy. The values with the proposed method decreased from the beginning of IFN therapy—earlier than those of the diazo-dye method. A rapid decrease of

the AST and ALT activities was observed similar to that of Bc by the proposed method.

Figure 3C illustrates a patient recovering from alcoholic hepatitis. The values with the proposed method decreased earlier than did those of the diazo-dye method. The hepatic enzyme activities decreased in a patient with alcoholic hepatitis even earlier than did the values of bilirubin. In this patient, microgallstones were found by computed tomography and this may be the reason for the delayed decline of bilirubin values as opposed to those of the hepatic enzyme activities, but values of the ratio obtained by the proposed method decreased earlier than did those of the diazo-dye method. Figure 3D shows a patient with AIHA. The values with the proposed method were stable, although bilirubin levels with diazo-dye method were variable. It may be concluded that only Bc fractions are measured by the proposed method, and that values of the proposed method may be useful for diagnosis of typical hemolytic anemia.

DISCUSSION

Serum bilirubins have been measured as D-Bil and Bu depending on their reactivity to the diazo reagent, and the Jendrassik–Grof method has been employed as a standard method for a long time (15–18). Recently, the fractional assay

Fig. 3. Typical profiles for Bc levels in blood samples from patients' serum bilirubin values expressed as the ratio of Bc or D-Bil to T-Bil in a patient with hepatocellular carcinoma (A), chronic hepatitis B (B), alcoholic hepatitis (C), and autoimmune hemolytic anemia (D). The panels show Bc (●: Iatro D-Bil) or D-Bil (■: diazo-dye method) expressed as the percent-

age of T-Bil for each method. A, On day 1, transcatheter arterial embolization was carried out and on days 6 to 13 chemotherapy was carried out. On day 21, melena occurred; B, From days 1 to day 6, interferon therapy was carried out; C, Microgallstones were found by CT on day 1.

of bilirubins has been widely used as a clinical assay test using the HPLC method or the Ektachem (now called Vitros) system, because the significance of clinical findings with β -, γ - and δ -fractions has been reported by several groups (1,5–8).

The proposed method was good in precision without interference and had reasonable analytical recovery. It gave good correlation with the HPLC method. The reagents prepared for this assay were stable for at least 1.5 months at 4°C. Required time for the assay was only 10 min and this reagent kit could be applied to other discrete multichannel automated analyzers.

Analysis of Bc fractions is important for detecting signs of recovery or exacerbation of hepatic diseases. However, currently used conventional methods for D-Bil might include the slow-reacting B δ , Bc, and small amount of Bu (2,14). To compare the value of Bc fractions among commonly used methods for D-Bil, the values obtained from each method were compared with those of the HPLC method. The D-Bil values by conventional methods correlated with the values of the Bc fractions plus the δ -fraction better than with the values of the Bc fractions measured by the HPLC method, but the proposed method gave good correlation with the HPLC method as a reference for Bc fractions assay. The proposed enzymatic assay for selectively measuring the Bc concentration in serum by the use of BOD as an oxidizing agent in the presence of inhibitors of BOD-mediated oxidation of Bu was developed by Kurosaka et al. (12). The BOD in the Bc assay was confirmed to insignificantly react with Bu and B δ in samples of human serum. Furthermore, we examined the correlation between D-Bil values obtained by another enzymatic method (3), the vanadate method (4) (y), and by the HPLC method as reference for Bc fractions (x), and found they were $y = 0.872x + 33.434 \mu\text{mol/L}$ ($r = 0.926$, $Sy/x = 23.64$, $n = 56$) for another enzymatic method, and $y = 0.799x + 40.144 \mu\text{mol/L}$ ($r = 0.919$, $Sy/x = 22.70$, $n = 56$) for the vanadate method. These results show that the proposed method seems to measure selectively the Bc fractions, and the reactivities for B δ and Bu were observed to be less those in the diazo-dye method and/or conventional methods.

B δ , which is formed from albumin and bilirubin, has a longer half-life than other bilirubins, so the assay must be performed after removing B δ in case jaundice occurs (5). B δ , expressed as a percentage for T-Bil, always fails to rise above 30 to 40% during unresolved hepatic failure (6). In such cases, Bc often remains above 50% of T-Bil until or near the death of the patient (1). The correlations between B δ /T-Bil and/or Bc/T-Bil and state of disease are more apparent when the bilirubin fractions are plotted as percentages for T-Bil than as absolute concentrations (1,5,6). In hepatobiliary jaundice, the serum Bc has been reported to be a more sensitive indicator than D-Bil, T-Bil, and ALP for the monitoring of their restoration states (7). Furthermore, in the early postoperative period after orthotopic liver transplantation, the highly reciprocal changes in the profiles of B δ and Bc fractions in serum are better markers of acute rejection than are AST and ALP (8).

Thus B δ and Bc fractions appear to be unusually sensitive and complementary markers of liver function and dysfunction. Our study indicates that the proposed method appears to reflect changes in disease conditions so that the combination of bilirubin values, the ratio of Bc to T-Bil by the proposed method, and hepatic enzyme activities seems to be useful for clinical diagnosis and monitoring of patients with hepatic diseases. From this point of view, this enzymatic assay for measuring Bc concentrations may be suitable for routine clinical use in laboratory, especially for clinical diagnosis and monitoring of patients with hepatic diseases.

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