A new enzymatic assay for selectively measuring conjugated bilirubin concentration in serum with use of bilirubin oxidase

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Abstract

A new enzymatic assay for selectively measuring conjugated bilirubin concentration in serum with use of bilirubin oxidase (BOD) has been developed. At pH 5.5 BOD can oxidize only conjugated bilirubin in the presence of reagents such as sodium fluoride and \textit{N}-acetylcysteine which can decrease BOD reactivity to unconjugated bilirubin and bilirubin covalently bound to albumin (delta bilirubin). The resulting decrease in absorbance at 450 nm is linearly related to the concentration of conjugated bilirubin in serum. The BOD in this new assay was confirmed to oxidize conjugated bilirubin, and neither unconjugated nor delta bilirubin, based on both its reactivity to unconjugated bilirubin and HPLC results. This assay was found to give satisfactory results, such as in terms of the range of measurement, the reproducibility of the results, the lack of interference with coexisting substances in serum and the stability of the reagent solutions, in practical applications. The serum conjugated bilirubin concentrations determined using this assay correlate well with those determined by the HPLC analysis. This assay can be used for accurate monitoring of changes in the conjugated bilirubin concentration in patient sera. These findings suggest that the conjugated bilirubin assay is useful for fractional determination of bilirubin in icteric sera. © 1998 Elsevier Science B.V.

Keywords: Bilirubin measurement; Conjugated bilirubin; Unconjugated bilirubin; Bilirubin oxidase; Fractional measurement

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1. Introduction

Bilirubin is a catabolic product of haem proteins formed in the reticuloendothelial system. It exists in serum in either free form (unconjugated bilirubin), or conjugated form (monoglucuronide and diglucuronide bilirubins), or covalently bound to albumin (delta bilirubin). Serum bilirubin assays are routinely employed in the diagnosis and treatment of hepatobiliary diseases and haemolytic jaundice. Assays for total and direct-reacting bilirubin, based on bilirubin reactivity to a diazo reagent, have been widely used for differentiation of bilirubin species in serum in practical use. However, it was reported in the 1980s that the direct diazo reagent reacts with not only conjugated bilirubin, but also some unconjugated bilirubin [1–5], indicating that the measured value of the serum direct-reacting bilirubin contains the conjugated bilirubin and a part of unconjugated bilirubin concentrations. Moreover, only 76–89% of delta bilirubin was reported to be detected as direct-reacting bilirubin [6].

As alternatives, an enzymatic direct bilirubin assay and a chemically oxidative direct bilirubin assay, in which bilirubin oxidase (BOD) [5,7–9] and vanadic acid [10] are used as oxidizing agents, respectively, were developed. These assays perform well in practical use, such as in terms of an appropriate dynamic range of measurement, good reproducibility of results and good correlation of the results with diazo assay results. In these direct bilirubin assays, however, oxidation of some unconjugated bilirubin and incomplete oxidation of delta bilirubin are also reported to occur [5,8–10]. On the other hand, an enzymatic conjugated bilirubin assay performed at pH 10.0 was also reported to have a good correlation with the HPLC assay [7], but the performance was not good.

We developed a new enzymatic assay for selectively measuring the conjugated bilirubin concentration in serum with use of BOD and reaction conditions appropriate for oxidation of conjugated bilirubin and neither unconjugated nor delta bilirubin. We will describe in this report the evaluation of the fractional reactivity of reagent solutions in the assay to bilirubin species, the performance of the assay in practical use, and its usefulness in clinical applications as a patient serum bilirubin assay.

2. Principle of the bilirubin assays

In the conjugated bilirubin assay, conjugated bilirubin is selectively oxidized by BOD at pH 5.5 in the presence of reagents such as sodium fluoride (NaF) and N-acetylcysteine (NAC) which can decrease BOD reactivity to unconjugated and delta bilirubins. On the other hand, BOD can oxidize all three bilirubin species (unconjugated, conjugated, and delta bilirubins) to non-coloured corresponding substances at pH 7.8 in the presence of an anionic detergent. The
decrease in absorbance at 450 nm which occurs upon the oxidation is linearly related to the concentration of bilirubin species in serum.

A synthetic conjugated bilirubin, ditaurobilirubin, is used in this assay as the standard bilirubin and its concentration is calibrated in terms of bilirubin equivalents by use of the Standard Reference Material 916a (National Institute of Standards & Technology).

3. Materials and methods

BOD from either *Myrothecium* sp. or *Trachyderma tsunodae* and ascorbate oxidase (AsOD) from *Acremonium* sp. were purchased from Amano Pharmaceutical Co., Ltd., Nagoya or Takara Shuzo Co., Ltd., Shiga, and Asahi Chemical Industry Co., Ltd., Tokyo, Japan, respectively, and used without further purification. Sodium salt of lauryl benzenesulphonic acid (SBS), p-toluenesulphonic acid (PTS), NAC, NaF, and potassium hydrogen phthalate were obtained from Nacalai Tesque, Inc., Kyoto, Japan. Sodium salt of flavin mononucleotide (FMN) was obtained from Yamaø Shoyu Co., Ltd., Chiba, Japan. Disodium salt of ditaurobilirubin and unconjugated bilirubin IXα were purchased from Porphyrin Products, Logan, UT and Sigma Chemical Co., St. Louis, MO, USA, respectively, and used without further purification. The other chemicals used throughout this work were of analytical grade.

Sera were obtained from patients and healthy persons. Ortho normal and abnormal control sera were purchased from Ortho Diagnostic Systems Ltd., Tokyo, Japan. Interference Check A (control material for haemoglobin, and unconjugated and conjugated bilirubins) and High Level Check BIL (control material for investigation of high bilirubin concentration) were obtained from International Reagent Corporation, Kobe, Japan.

The reagent kits for the direct bilirubin assay were purchased from Nippon Shoji Kaisha Ltd., Osaka (BOD method: Nescauto D-Bil VE) and Wako Pure Chemical Industry, Osaka, Japan (chemically oxidative (vanadic acid oxidizing) method: Direct Bilirubin E-HA Test Wako; diazo (alkaline azobilirubin) method: Direct Bilirubin II-HA Test Wako), respectively. The direct bilirubin assays performed using the three commercially available reagent kits were carried out according to the manufacturers' instruction manuals.

The BOD and AsOD assays were also carried out according to the manufacturers' instruction manuals. One unit of BOD or AsOD was defined as the amount of enzyme that catalyzes the oxidation of one μmol of substrate or the formation of one μmol of the product at 37°C in 1 min.

The total and conjugated bilirubin assays were based on mixing of two separate reagent solutions. These solutions were mixed at use so that the volumetric ratio of the first reagent (R1) to the second reagent (R2) was four to
one. For the conjugated bilirubin assay R1 comprised 120 mmol/l phthalate buffer (pH 5.5), 2.5 mmol/l NAC, 0.1 mmol/l EDTA, 50 mmol/l PTS, and 2.5 mmol/l NaF, and R2 comprised 120 mmol/l phthalate buffer (pH 5.5), 100 U/l BOD, and 4000 U/l AsOD (AsOD was added to oxidize ascorbate in serum). The pH for the total bilirubin assay was set at 7.8, at which most of the bilirubin species present in the serum are completely oxidized by BOD. The reaction was accelerated by use of an anionic detergent, SBS. For the total bilirubin assay R1 comprised 100 mmol/l HEPES buffer (pH 7.8), 32 mmol/l PTS, 0.3% SBS, and 1 mmol/l EDTA, and R2 comprised 50 mmol/l HEPES buffer (pH 7.8), 32 mmol/l PTS, 4000 U/l BOD, and 4000 U/l AsOD. The total bilirubin concentrations determined by the present total bilirubin assay correlate well with those determined by the commercially available total bilirubin assays.

The serum bilirubin assays were carried out at 37°C. A 14 μl of serum sample was added to 280 μl of R1, and then 70 μl of R2 was added to start the BOD reaction (these volumes can be changed according to the instrument used while maintaining the volumetric ratio). The change in absorbance at 450 nm was measured, after equilibration for 5 min, with a Hitachi 7070 or 7170 Automatic Analyzer (the secondary wavelength was set at 546 nm). The bilirubin concentration (mg/l) was calculated with reference to the absorbance change at 450 nm of a standard bilirubin solution (approximately 100 mg/l).

The HPLC analysis of serum bilirubin was performed as described by Lauff et al. [11] with minor modifications. The serum sample (20 μl) or the reaction mixture (200 μl) of serum sample with the bilirubin assay reagent solution was directly injected into the HPLC column (Lichrospher 100 RP-18, 10 mm particles, purchased from Kanto Chemical Co., Ltd., Tokyo, Japan) without treatment with sodium sulphate solution. We used a Shimadzu LC-6A pump with a Shimadzu SPD-6AV detector. Bilirubin species were separated by linear gradient elution with isopropyl alcohol and detected by measurement of absorbance at 450 nm (FMN was used as an internal standard for the calculation of the concentration of each bilirubin species).

4. Results

4.1. Optimization of conjugated bilirubin assay conditions

The pH for the conjugated bilirubin assay was set at around 5 based on reports such as of the stability of BOD [12] and the reactivity of BOD to bilirubin species [7]. At this pH, BOD oxidized completely the synthetic conjugated bilirubin, ditaurobilirubin, and oxidized unconjugated bilirubin to a considerable extent. Addition of NaF, a BOD inhibitor, and the reducing agent NAC to the assay resulted in a decrease in the level of BOD reactivity to
unconjugated bilirubin; the decrease was maximum at NaF and NAC concentrations of 2 mmol/l and 1–2 mmol/l, respectively. Because BOD oxidized approximately 6% of the unconjugated bilirubin in the assay even in the presence of 2 mmol/l NaF and 2 mmol/l NAC, we reevaluated the reaction pH. It was shown that the amount of unconjugated bilirubin oxidized by BOD in the assay was decreased to less than 1% by adjustment of the pH of R1 to around 5.5 (the pH of R2 was fixed at 5.5). The concentrations of reagent components thus determined were described in Materials and Methods.

4.2. Characterization of the conjugated bilirubin assay

Fig. 1 shows the time course of absorbance at 450 nm in the conjugated bilirubin assay. No absorbance change was observed during the initial 5 min after mixing of the serum sample with R1, and the reaction was completed within 5 min after the addition of R2. The absorbance change at 450 nm was found to be linearly related to the conjugated bilirubin concentration, up to approximately 350 mg/l. The coefficient of variation (CV) for the within-run reproducibility was less than 2.6% at a low mean conjugated bilirubin concentration (2.0 mg/l, n = 20) and less than 1% at a high mean conjugated bilirubin concentration (75.2 mg/l, n = 20). The CV at a mean conjugated bilirubin concentration of 21 mg/l was 2.3% (n = 5) in the day-to-day assays over a 5 day period. The conjugated bilirubin assay results were not influenced

![Fig. 1. Time course of absorbance at 450 nm in the conjugated bilirubin assay. The sample was mixed with R1 and the mixture was incubated for 5 min at 37°C, followed by addition of R2 to start the reaction. Samples were as follows: serum 1 (●), human serum with conjugated bilirubin at 63 mg/l (total bilirubin, 99 mg/l); serum 2 (▲), human serum with conjugated bilirubin at 9 mg/l (total bilirubin, 23 mg/l); standard (□), standard material (ditaurobilirubin, 87 mg/l); blank (◇), saline.](image_url)
by the presence of several coexistents in the serum, such as glucose (0–10 g/l), urate (0–200 mg/l), a reduced form of glutathione (0–500 mg/l), pyruvate (0–200 mg/l), lactate (0–500 mg/l), ascorbate (0–500 mg/l) and haemoglobin (0–5 g/l). Moreover, the conjugated bilirubin assay results were scarcely affected (the deviation was ± less than 0.1 mg/l at the mean conjugated bilirubin concentration of 1.5 mg/l) by the presence of anticoagulants, such as EDTA (2 g/l), sodium oxalate (2 g/l), sodium citrate (2 g/l), sodium fluoride (5 g/l) and heparin (200 mg/l), in the serum. Storage of the reagent solutions at 10°C for 30 days did not affect the above basic properties of the present conjugated bilirubin assay.

We examined the correlation of the conjugated bilirubin assay results with the results of the direct bilirubin assays based on the enzymatic, the diazo and the chemically oxidative methods, using samples of serum collected from 40 healthy persons and 39 patients with hepatobiliary diseases. Fig. 2 shows an example of the correlation between the results of the conjugated bilirubin assay and those of the alkaline azobilirubin assay. Although the correlation coefficient was 0.994 for a wide range of measurement results, the slope of the linear regression

![Graph showing correlation](image)
The reaction selectivity for conjugated bilirubin

The cause of the discrepancy seen in the correlation experiment results was suggested to be the difference in the reactivity of the reagent solutions to unconjugated bilirubin between the conjugated bilirubin assay and the direct bilirubin assays. The conjugated bilirubin assay was found to be specific for conjugated bilirubin in the mixed sample of unconjugated and conjugated bilirubin, as seen in Fig. 3. On the other hand, in the diazo, the enzymatic and the chemically oxidative direct bilirubin assays, the concentration of not only conjugated bilirubin, but also some unconjugated bilirubin in the mixed sample was measured. The validity of these findings was examined by HPLC analysis of the bilirubin fractions before and after the reaction. It was confirmed from Fig.
In the chromatogram for the conjugated bilirubin assay, only the peaks corresponding to the conjugated bilirubin fraction disappeared after the reaction, and unconjugated and delta bilirubins were not oxidized by the reagent solution. However, the chromatogram shown in Fig. 4b indicated that in the enzymatic direct bilirubin assay, the conjugated bilirubin was completely oxidized, and the delta and unconjugated bilirubins were oxidized to moderate extents. The same degree of reactivity of the reagent solution to unconjugated and delta bilirubins was found in the chemically oxidative direct bilirubin assay.

These findings indicate that the conjugated bilirubin assay is specific for conjugated bilirubin, being reflected in the difference in the slope of the linear regression equation in the correlation between the conjugated bilirubin assay results and the direct bilirubin assay results.

Fig. 4. Serum bilirubin fractions detected by the HPLC analysis before and after the reaction with the reagent solutions in the conjugated bilirubin assay (a) and the direct bilirubin assay (b). Sample of serum collected from a patient with hepatobiliary disease was used, with FMN as the internal standard. Four peaks were detected in the elution order of δ, γ, β, and α (δ, delta bilirubin; γ, diglucuronide bilirubin; β, monoglucuronide bilirubin; α, unconjugated bilirubin). The upper line represents the chromatogram of the mixture of the serum sample with R1 (before the reaction), and the lower that of the reaction mixture 5 min after the addition of R2 (after the reaction) (volume correction was performed before and after the reaction).
4.4. Conjugated bilirubin assay in clinical use

In Table 1 the measured total and conjugated or direct-reacting bilirubin concentrations in the sera of patients with hepatobiliary diseases and neonates are presented. It was clearly indicated that the conjugated bilirubin concentrations determined by the conjugated bilirubin assay correlate well with those determined by the HPLC analysis. In the case of the neonate sera, the measured direct-reacting bilirubin concentrations were found to agree with neither the conjugated nor the direct-reacting bilirubin (conjugated bilirubin + delta bilirubin) concentration determined by the HPLC analysis; the largest differences were seen in the total bilirubin concentration range of 30–60 mg/l. It is suggested that these deviations are due to the differences in the reactivity of the reagent solutions to unconjugated bilirubin among the assays, because of the neonate sera not having delta bilirubin so much [13–15]. It was also noted from

Table 1
Comparison of the conjugated bilirubin assay results with the direct bilirubin assay results for sera of neonates and patients with hepatobiliary diseases.

<table>
<thead>
<tr>
<th>Present assay</th>
<th>assay 1</th>
<th>assay 2</th>
<th>HPLC</th>
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<tr>
<td></td>
<td>Total Bilirubin (mg/l)</td>
<td>Conjugated Bilirubin (mg/l)</td>
<td>Direct-reacting Bilirubin (mg/l)</td>
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<tr>
<td><strong>Neonate sera</strong></td>
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<td><strong>Hepatobiliary disease patient sera</strong></td>
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<td>63</td>
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Assay 1: alkaline azobilirubin assay.
Assay 2: enzymatic assay.
β + γ: conjugated bilirubin (monoglucuronide bilirubin + diglucuronide bilirubin).
δ: delta bilirubin.

* Serum samples collected from convalescent-stage hepatobiliary disease patients.
Table 1 that the differences between the conjugated bilirubin concentrations and the direct-reacting bilirubin concentrations in the sera of the hepatobiliary patients arise mainly from the lack of reactivity of the reagent solution in the conjugated bilirubin assay to delta bilirubin, the serum concentration of which may be very high in the convalescent stage of hepatobiliary diseases [16].

5. Discussion

A new enzymatic assay for selectively measuring the conjugated bilirubin concentration in serum by use of BOD as an oxidizing agent in the presence of inhibitors of BOD-mediated oxidation of unconjugated bilirubin was developed. The BOD in the conjugated bilirubin assay was confirmed to hardly react with unconjugated and delta bilirubins in samples of human serum.

The lack of reactivity of the reagent solution in the conjugated bilirubin assay to delta bilirubin can be attributed to maintenance of the structure of the albumin molecule in solution at pH 5.5 [17]. The reagent solution described in this report is convenient for assay of conjugated bilirubin in routine analysis, with respect to the wide dynamic range of measurement, the lack of interference of coexistent substances in serum, the excellent reproducibility of results, and the good stability of the reagent solution after constitution. The conjugated bilirubin assay is easily adapted to automated instrumentation, an advantage over the HPLC analysis.

The total and direct-reacting bilirubins in serum bilirubin measurement had been classified based on the reactivity of a diazo reagent to these species of bilirubin [18]. However, it has been reported that the direct bilirubin assay reagents react to some extent with unconjugated bilirubin [1–5]. The same phenomenon was found to occur in the enzymatic and the chemically oxidative direct bilirubin assays developed in the last decade [5,8–10]. Therefore, the direct-reacting bilirubin concentration seems to have an ambiguity in its measured value. We used the conjugated bilirubin assay to measure the concentration of conjugated bilirubin in samples of serum collected from hepatobiliary disease patients and neonates. The conjugated bilirubin concentrations determined using this assay correlate well with those determined by the HPLC analysis, and are lower than the direct-reacting bilirubin concentrations determined using the direct bilirubin assays. This may indicate that the conjugated bilirubin assay is useful for accurate measurement of serum conjugated and unconjugated bilirubin concentrations not only in cases of physiological or haemolytic jaundice, but also in case of constitutional jaundice such as in Gilbert syndrome. Delta bilirubin, which has a longer half-life than that of other bilirubin species [19], has been believed to react directly with the reagent solution in the direct bilirubin assays, but it has also been indicated to
react not completely with the reagent solution in the direct bilirubin assays [6,9,20–22]. In hepatocellular and obstructive jaundice the serum conjugated bilirubin concentration has been reported to be a more sensitive indicator than the serum direct-reacting bilirubin concentration for the monitoring of their restoration states [23]. Therefore, the conjugated bilirubin assay seems to be valuable for early judgment of the restoration state and accurate evaluation of the reflux of bile pigments in hepatobiliary diseases [23–25]. Further clinical experiments are necessary to establish the effectiveness of the conjugated bilirubin assay in various types of hyperbilirubinemia.

References