Novel Assay for Measuring Serum Conjugated Bilirubin and Its Clinical Relevance

Yukio Morimoto,¹ Tomoaki Ishihara,² Masaharu Takayama,^{1*} Masahiko Kaito,² and Yukihiko Adachi²

¹Diagnostic Research & Development Department, R&D Division, Nesco Company, Azwell Inc., Osaka, Japan

²Third Department of Internal Medicine, Mie University School of Medicine, Mie, Japan

We have developed a new enzymatic assay for the determination of conjugated bilirubin (Bc) using stable liquid reagents. In this assay, only Bc is selectively oxidized by bilirubin oxidase at pH 5.0 in the presence of nitrilotris (methylenephosphonic acid) trisodium salt, ethylenediaminetetraacetic acid disodium manganese (II) salt, and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl. Bc is quantitatively determined from a decrease in the absorbance at 450 nm caused by Bc oxidization. The reagent solutions of the assay were developed so that they could be stably stored for one year together with bilirubin oxidase, in order to eliminate the

need to prepare working solutions every time they are required. The assay has good reactivity, differentiability, measurability, and precision. Neither ascorbic acid nor hemoglobin interfered with the measurement. Bc values determined by the assay reflected more clearly the pathophysiological condition of hepatobiliary disease patients with jaundice than the values of total bilirubin or direct bilirubin determined by conventional methods. From these observations, we concluded that this Bc assay is valuable for the evaluation of jaundice. *J. Clin. Lab. Anal.* 14:27–31, 2000. © 2000 Wiley-Liss, Inc.

Key words: serum bilirubin; conjugated bilirubin; delta bilirubin; bilirubin oxidase; high performance liquid chromatography; dry chemistry; jaundice

INTRODUCTION

Bilirubin is taken up by hepatocytes from plasma and conjugated with glucuronic acid to form conjugated bilirubin (Bc), which is then secreted into bile. Disorders that develop in the secretory pathway after conjugation (liver cell injury, intrahepatic cholestasis, biliary obstruction) lead to an elevation of the serum Bc by regurgitation. Thus, measurement of Bc may be useful in the evaluation of jaundice in various hepatobiliary diseases.

Direct bilirubin, as determined by the diazo reaction method (1), has conventionally been regarded as the Bc level. However, delta bilirubin, which is a covalently albumin-bound bilirubin, and a small portion of unconjugated bilirubin are also detected by the diazo reaction method as direct bilirubin, affecting the evaluation of jaundice. In light of this problem, we developed a novel assay that can detect only Bc, not delta bilirubin or unconjugated bilirubin.

Two methods are currently available for the determination of Bc: one applies high performance liquid chromatography (HPLC) to detect Bc fractions by the method of Lauff et al. (2) or Adachi et al. (3), while the other method uses Vitros dry chemical analysis (4) and a specially designed medical instrument (Kodak Extachem Analyzer and Vitros Chemis-

try System). However, these methods require special facilities and expensive equipment.

Various kits for the determination of bilirubin have been used in conjunction with auto-analyzers. Although a recently developed reagent kit for the direct determination of bilirubin was claimed to be able to determine only Bc, it was later found that 17% of delta bilirubin reacted with the reagent (5). Thus, the reagent's ability to differentiate Bc proved inadequate for Bc determinations. After submitting this manuscript, Doumas et al. (6) reported an enzymatic method based on rate assay for measuring conjugated bilirubin. However, this method correlated poorly with HPLC.

In this study, we have developed a novel assay that shows minimal reactivity with unconjugated bilirubin and delta bilirubin, and thus the assay can be used for the accurate measurement of Bc.

*Correspondence to: Masaharu Takayama, Diagnostic Research & Development Department, R&D Division, Nesco Company, Azwell Inc., 2-24-3, Sho, Ibaraki-city, Osaka 567-0806, Japan. E-mail: nskrd-06@mbd.sphere.ne.jp

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MATERIALS AND METHODS

Reagents

The reagents used for Bc determination were R1, a buffer solution, and R2, an enzyme solution. R1 was composed of 150 mmol/L of acetate buffer solution (pH 5.0 with lactic acid), containing 80 mmol/L of nitrilotris (methylene-phosphonic acid) trisodium salt (Dojindo Laboratories, Kumamoto, Japan) and 0.5 g/L Triton X-100. R2 was composed of 20 mmol/L of borate buffer solution (pH 10.0), 10 mmol/L of Bicine, 50 g/L of potassium sodium tartrate, 10 mmol/L of ethylenediaminetetraacetic acid disodium manganese salt, 6 mmol/L of 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), 0.5 g/L of Triton X-100, and 1,000 U/L of bilirubin oxidase (*Mirosesium berucaria* MT-1, Amano Pharmaceutical Co., Ltd., Nagoya, Japan).

For HPLC, a solution for diluting the specimen was prepared by adding Triton X-100 (final concentration: 0.5 g/L), boric acid (final concentration: 30g/L), and phosphate buffer solution (final concentration: 0.1mol/L, pH 7.2). Two eluting solutions, A and B, were prepared as follows. Eluting solution A contained 50 mmol/L of potassium dihydrogenphosphate and 50 g/L of 2-methoxyethanol (pH 2.1 with phosphoric acid), and it was used after filtration through a 0.45- μ m filter and degassing. Eluting solution B was prepared by filtering a mixture of 2-methoxyethanol, 2-propanol, and phosphoric acid (5:95:2.5) through a 0.45- μ m filter, and used after degassing.

Vitros dry bilirubin BuBc kit was purchased from Ortho Clinical Diagnostics Inc. (Tokyo, Japan). Interference Check A (control material for interference) and High Level Check BIL (control material for high bilirubin concentration) were purchased from International Reagent Corporation (Kobe, Japan). Nescauto VL T-BIL was purchased from Azwell Inc. (Osaka, Japan). The other reagents were purchased from Nakarai Tesque (Kyoto, Japan).

Preparation of Bilirubin Standards

Standard bilirubin solution was prepared just before use by dissolving ditaurobilirubin (Porphyrin Products Inc., Logan, UT) in 4% bovine serum albumin at approximate 205 μ mol/L, and measured with the total bilirubin determination reagent (Nescauto VL T-BIL). Bc solution was prepared by dissolving ditaurobilirubin in 4% bovine serum albumin at a concentration of 137 μ mol/L. Unconjugated bilirubin solution was prepared by dissolving bilirubin (Standard Reference Material No. 916a; National Bureau of Standards, Washington D.C.) in 4% bovine serum albumin at a concentration of 137 μ mol/L as described by Ihara et al. (7). Delta bilirubin was isolated from the sera of jaundiced patients by saturated ammonium sulfate precipitation and ultrafiltration as described by Ihara et al. (8). Serum samples were obtained for Bc determination from an icteric patient with cholangitis.

Bc Determination by the Novel Enzymatic Assay

Bc was determined with a Hitachi Model 7170 Auto-analyzer (Hitachi Ltd., Tokyo, Japan) using the following procedures: 240 μ L of R1 was added to 9 μ L of bilirubin specimen; after incubation at 37°C for 5 min, 80 μ L of R2 was then added, and the mixture was incubated at 37°C for 5 min. Absorbance at 450 nm was measured 5 min after the addition of R1 and 5 min after the addition of R2, and the difference in the absorbance was calculated. Then, the bilirubin level was determined by comparing the absorbance dif-

Fig. 1. Time course of the reaction of conjugated bilirubin (A), unconjugated bilirubin (B), and delta bilirubin (C). ■—■, total bilirubin assay; ▲—▲, conjugated bilirubin assay.

ference with that obtained using the standard bilirubin solution described earlier here.

HPLC Analysis

HPLC analysis was performed with a LiChrospher 100 RP-18(e) column (particle size: 10 μ m, Kanto Chemical Co., Ltd., Tokyo, Japan) according to the method of Lauff et al. with minor modification (2). An aliquot of 100 μ L of diluted sample was applied onto the column equilibrated with eluting solution A and eluted by a linear gradient from 100% A to 20% A and 80% B at a flow rate of 1.2 mL/min.

Bilirubin fractions of icteric serum samples after the enzymatic assay of Bc were analyzed by HPLC. The specimens for HPLC were prepared as follows. An aliquot of 540 μ L of R1 was added to 20 μ L of sample and incubated for 5 min at 37°C. Then 180 μ L of R2 was added, and after 5 min of incubation at 37°C, 180 μ L of sodium ascorbate (10 g/L, pH 7.2) was added to stop further oxidation of samples. Control specimens that did not undergo the oxidative reaction were prepared as described, except that bilirubin oxidase was excluded from R2.

The HPLC samples used for correlation analyses were prepared by adding 720 μ L of diluting solution and 180 μ L of sodium ascorbate (10 g/L, pH 7.2) to 20 μ L of sample.

The four fractions of bilirubin detected by HPLC analysis

were designated as δ , γ , β , and α , according to the order of retention time. Fractions α , β , γ , and δ are known to correspond to unconjugated bilirubin, bilirubin monoglucuronide, bilirubin diglucuronide, and delta bilirubin, respectively (3). The sum of the peak areas of these fractions was regarded as the total bilirubin level, and the Bc level was calculated from the sum of the peak areas of fractions β and γ .

Bc of Vitros Dry Bilirubin Chemical Analysis

Bc was determined using a Kodak Ektachem Analyzer according to the manufacturer's instructions.

RESULTS

Bc Determination

The enzymatic reaction with Bc using the novel assay progressed rapidly following the addition of R2, and was completed approximately within one min. In contrast, R2 did not react with unconjugated bilirubin and reacted only slightly with delta bilirubin (less than 10%)(Fig. 1). Thus, Bc was selectively determined by suppressing bilirubin oxidase activity toward unconjugated bilirubin and delta bilirubin.

The HPLC peak areas of fraction α (unconjugated bilirubin) and fraction δ (delta bilirubin) were either unchanged or little changed after the addition of R2, thus proving that bi-

Fig. 2. High-performance liquid chromatographic pattern of serum bilirubin before and after the enzymatic reaction. **A**, before the enzymatic reaction; **B**, after the enzymatic reaction. Sample of serum collected from a

patient with hyperbilirubinemia was used with flavin adenine dinucleotide disodium (FAD) as the internal standard.

Fig. 3. Linearity of the present method. High Level Check BIL was diluted and used as sample.

lirubin oxidase does not oxidize either unconjugated bilirubin or delta bilirubin under the given conditions. Almost all peaks of fractions β and γ of Bc disappeared after the addition of R2, indicating that bilirubin oxidase reacts completely with them (Fig. 2).

The measurable range and linearity determined with High Level Check BIL was maintained up to approximately 670 $\mu\text{mol/L}$ (Fig. 3). The coefficients of variation (CVs) of both within-run and day-to-day precision were 0.5–1.1% at 19.4–168.8 $\mu\text{mol/L}$ and 0.5–0.9% at 31.0–218.3 $\mu\text{mol/L}$, respectively (Table 1). Neither ascorbic acid (up to 1 g/L) nor hemoglobin (up to 2 g/L) interfered with the Bc measurement. The Bc values obtained using our novel assay correlated well with those obtained by HPLC (Fig. 4) and dry chemical analysis (Fig. 5). A good correlation in Bc values was noted between the reagent stored at 7°C for one year and the reagent prepared just prior to use ($r = 1.000$, regression formula: $y = 0.981 \times -0.31$, $S_{y \cdot x} = 0.915$).

Clinical Observation

Serum bilirubin was examined in a male jaundiced patient with cholangitis. We determined the concentrations of bilirubin, direct-reacting bilirubin, and Bc using our new enzymatic assay during disease exacerbation and recovery.

TABLE 1. Precision of the present method

	Mean ($\mu\text{mol/L}$)	SD ($\mu\text{mol/L}$)	CVs (%)
A: within-run (n = 20)			
1	19.4	0.21	1.1
2	86.5	0.57	0.7
3	168.8	0.81	0.5
B: day-to-day (n = 8)			
4	19.4	0.21	1.1
5	86.5	0.57	0.7
6	168.8	0.81	0.5

Both the serum concentrations of Bc and direct-reacting bilirubin were equally high during exacerbation of jaundice in the patient. On the other hand, the serum concentration of Bc at the beginning of recovery already indicated low levels, whereas the serum concentration of direct-reacting bilirubin remained still high (Fig. 6).

DISCUSSION

We developed a new enzymatic assay for the determination of conjugated bilirubin. HPLC data showed that unconjugated bilirubin and delta bilirubin are not oxidized, and that only conjugated bilirubin is selectively oxidized by

Fig. 4. Correlation of conjugated bilirubin measured by the present assay and HPLC method. Correlation was satisfactory with $r = 0.997$, $y = 1.005x + 1.66$, and $S_{y \cdot x} = 5.14$ in 126 specimens.

Fig. 5. Correlation of conjugated bilirubin measured by the present method and Vitros dry bilirubin Bc. Correlation was satisfactory with $r = 0.996$, $y = 1.004x + 4.85$, and $Sy \cdot x = 7.86$ in 93 specimens.

Fig. 6. Clinical course of a patient with cholangitis. Serum bilirubin analyzed at points A and B. Point A is the time of disease exacerbation, and point B is the time of recovery. Serum concentration of total bilirubin at point A is nearly equal to that of point B. STB, serum total bilirubin; SDB, serum direct-reacting bilirubin; Bc, serum conjugated bilirubin.

bilirubin oxidase at pH 5.0 in the presence of nitrilotris (methylenephosphonic acid) trisodium salt, ethylenediaminetetraacetic acid disodium manganese(II) salt and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl. It remains unclear both how these compounds inhibit the reaction of bilirubin oxidase with unconjugated bilirubin and delta bilirubin selectively, and why they do not inhibit the reaction with conjugated bilirubin. The accuracy of the assay is illustrated by the good correlation of the results with those determined by HPLC and by dry chemistry. The measurable range and reproducibility were satisfactory, with no influence of ascorbic acid (up to 1g/L) or hemoglobin. These results suggest that the assay can be used in routine laboratory work. The reagents were stable for one year at 7°C.

No evidence of delta bilirubin formation was observed by HPLC with the standard bilirubin prepared just before use in this assay.

In the clinical observation of icterus, Bc determination is superior to the conventional measurement of direct bilirubin, because the effect of covalently albumin-bound delta bilirubin was excluded. The proportion of delta bilirubin in direct bilirubin is reported to increase in the recovery stage of most icteric conditions (9). This assay is simple and useful for evaluating the prognosis of icterus associated with liver cell disorder and hepatobiliary diseases.

We concluded that the measurement of Bc is more accurate than that of total or direct-reacting bilirubin for assessing the status of hyperbilirubinemia.

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