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—Original Article—

Human serum bilirubin fractionation in various hepatobiliary diseases by the newly developed high performance liquid chromatography

Yukihiko ADACHI[1], Hiroko INUFUSA[1], Masaki YAMASHITA[1], Akira KAMBE[1], Kazutoshi YAMAZAKI[2], Yoshihide SAWADA[2] and Toshio YAMAMOTO[1]

[1]*Second Department of Internal Medicine, Kinki University School of Medicine, Osaka 589, Japan and* [2]*Sekisui Chemical Co., Ltd., Osaka 618, Japan*

Summary: Serum Bilirubin was fractionated by newly developed reversed phase high performance liquid chromatography (HPLC) into 5 fractions: δ (δ-Bilirubin, Bδ), γ (bilirubin diglucuronide, BDG), β (Bilirubin monoglucuronide, BMG), β′ ((Z, E,)- and/or (E, Z)-bilirubin IXα) and α ((Z, Z)-bilirubin IXα). Sera of healthy subjects and of patients with unconjugated hyperbilirubinemia showed predominantly α fraction with a small amount of β′ fraction. Trace amounts of δ fraction were detected in a few cases. The results of fractionation of serum bilirubin in 159 patients with various hepatobiliary diseases suggested that the ratios Bδ/(Bδ+BDG+BMG) and BMG/Bδ can be useful parameters to follow patients with jaundice, compared with the reported Bδ/total bilirubin which did not always reflect the jaundice stage, especially in cases with low serum bilirubin levels. *Gastroenterol Jpn 1988;23:268–272*

Key Words: *Delta bilirubin, Hyperbilirubinemia, Jaundice, Liver disease*

Introduction

In 1966, Kuenzle et al.[1] first fractionated serum bilirubin by column chromatography into four fractions: α (unconjugated bilirubin; Bu), β (bilirubin monoglucuronide; BMG), γ (bilirubin diglucuronide, BDG), and δ (tightly protein-bound bilirubin or delta bilirubin; Bδ). Weiss et al.[2] reported, using the newly developed high performance liquid chromatography (HPLC)[3], that the percentage of Bδ in total bilirubin (TB) increased in cases of jaundice with good prognosis, while it decreased with increased percentages of BMG and BDG in cases of jaundice with poor prognosis. In spite of such progress, the reported HPLC methods show low sensitivity because they need a variety of pretreatments[3] or run a small amount of serum[4,5]. Wu et al.[6] and Sundberg et al.[7] developed the multilayer film process (the Ektachem method) which fractionates serum bilirubin into 3 fractions: Bδ, conjugated bilirubin (Bc; BMG + BDG) and Bu. The Ektachem method determines TB by the diazo reaction, Bc and Bu by reflection spectroscopy, and Bδ from the difference between TB and (Bc+Bu), and the results obtained with the Ektachem method do not totally agree with those obtained by HPLC in sera both with low bilirubin concentration and unconjugated hyperbilirubinemia[8]. The present paper describes the result of fractionating serum bilirubin in various hepatobiliary diseases by using a newly developed accurate and simple HPLC.

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Address for correspondence: Yukihiko Adachi, M.D., The Second Department of Internal Medicine, Kinki University School of Medicine, Osakasayama, Osaka 589, Japan.
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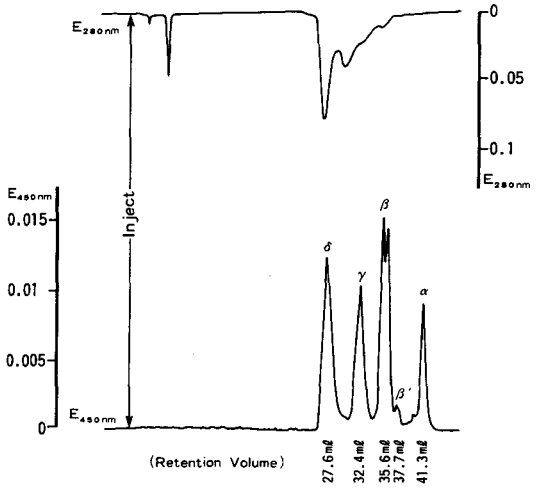

Fig. 1 *Micronex high performance liquid chromatogram*
Serum from a patient with jaundice (serum TB 170.5 μmol/L, 5 μL) was analyzed. Five peaks were shown in the elution order of δ, γ, β, β', and α. β peak was composed of two BMG isomers.

Materials and Methods

HPLC grade solvents, including acetonitrile, were obtained from Wako Junyaku (Osaka, Japan). The new HPLC (Micronex HPLC) was performed using Micronex RP-30 (φ6 mm × 150 mm; Sekisui Chemical Co., Osaka, Japan)[9]. The serum bilirubin was fractionated into 5 fractions by Micronex HPLC, i.e., δ, γ, β, β' ((Z, E)- and/or (E, Z)-bilirubin), and α fractions. A chromatogram of the serum bilirubin fractions from an icteric patient is presented in **Figure 1**.

All steps were performed in subdued light (red light). Five to ten μL of collected sera were analyzed immediately or within a week upon storage in liquid nitrogen. Bilirubin fractions were determined by the Jendrassik-Gróf method (J-G method)[10] and Micronex HPLC using sera from 28 normal adults, from 21 cases of unconjugated hyperbilirubinemia and from 159 patients with various hepatobiliary diseases. The ratios of bilirubin fractions to TB and to each other were calculated. Student's t-test was used for statistical analyses.

Results

Micronex HPLC revealed that sera from 28 normal subjects contained 15.2 ± 5.0 μmol/L TB with neither BDG nor BMG. Bδ was found only in 2 subjects (6.5% and 3.6% of TB) and small β' fraction in 16 subjects (β'/α was 11.9 ± 9.7%). In sera from 18 patients with Gilbert's syndrome and 3 patients with type II Crigler-Najjar syndrome the serum bilirubin pattern was composed by Bu (β' fraction + α fraction) and, in 7 patients of Gilbert's syndrome, by traces of Bδ. The mean β'/α ratio was 10.3% and 7.0% in Gilbert's syndrome and in type II Crigler-Najjar syndrome, respectively. Bilirubin fractionation was performed by Micronex HPLC in sera from 159 patients with various hepatobiliary diseases.

In non-icteric patients with hepatobiliary diseases (30 cases out of 159), Bu (α fraction + β' fraction) accounted for most of the serum bilirubin (β'/α = 9.4 ± 10.1%), Bδ amounted to 5.3 ± 11.7% of TB, and neither BDG nor BMG was detected.

Bu/TB was high in compensated liver cirrhosis and chronic hepatitis (mean: 96.3 and 81.7%, respectively) and 18.9-40.2%, on the average, in other hepatobiliary diseases (**Table 1**). Mean β'/α ratio was comparable in each hepatobiliary disease (from 11.2 to 16.6%).

In sera containing Bδ, the Bδ/(Bδ+BDG+BMG) ratio was significantly lower during the increase phase of jaundice than when jaundice was remitting in acute hepatitis and obstructive jaundice. The ratio was remarkably low in fulminant hepatitis and significantly lower in decompensated than in compensated liver cirrhosis (**Table 1, Fig. 2**).

Similar changes in the Bδ/TB and Bδ/(Bδ+BDG+BMG) ratios were observed in all cases with exceptions of chronic hepatitis and compensated liver cirrhosis (**Table 1**). These exceptions may be accounted for by the high amount of Bu present in these diseases.

BMG/TB and BDG/TB ratios were high in fulminant hepatitis, in the jaundice-progression stage of acute hepatitis and of obstructive

Table 1 Serum Bilirubin Fractions in Normal Subjects, Patients with Unconjugated Hyperbilirubinemia and Patients with Various Hepatobiliary Diseases

	n	Serum bilirubin (μmol/L)	Bδ/TB (%)	BDG/TB (%)	BMG/TB (%)	Bu/TB	n#	$\frac{B\delta}{(B\delta+BDG+BMG)}$ (%)	$\frac{BMG}{B\delta}$
Normal	28	15.2± 5.0	0.4± 1.7	0	0	99.6± 1.7	2	100	0
Gilbert's syndrome	18	47.9± 14.2	2.1± 2.1	0	0	98.8± 2.1	7	100	0
Crigler-Najjar (Type U)	3	167.3± 47.9	0	0	0	100	0	—	—
Fulminant hepatitis	4	509.9± 44.9	19.8± 6.7	16.9±4.2	38.6± 7.1	24.8± 4.5	4	26.8±10.3	2.31±1.09
Acute hepatitis (progression stage)	15	169.0± 72.1	24.0± 7.9	21.2±5.2	31.2± 5.1	20.4± 9.7	15	30.6± 7.9	1.50±0.52
Acute hepatitis (recovery stage)	34	92.0± 69.2**	53.5±11.4***	6.1±6.3***	6.4± 7.7***	35.7±18.6**	34	83.0±17.0***	0.13±0.17***
Chronic hepatitis	25	22.8± 7.0	3.6± 5.8	0	0	96.3± 5.8	9	100	0
Liver cirrhosis (compensated)	14	28.9± 11.1	14.1±13.5	2.0±2.2	2.3± 2.9	81.7±17.5	10	80.0±13.7	0.092±0.083
Liver cirrhosis (decompensated)	21	238.0±157.0***	32.2± 6.8***	13.0±3.5***	19.6± 7.7***	33.7±12.0***	21	50.7± 9.3***	0.61±0.27***
Hepatocellular carcinoma	12	154.3± 87.3	32.7± 8.9	13.5±4.8	23.0±14.0	34.8±17.3	12	52.6±15.1	0.68±0.52
Intrahepatic cholestasis	10	146.7±120.5	29.2± 8.5	11.4±7.1	18.8±10.7	40.2±17.0	10	52.1±15.7	0.70±0.45
Obstructive jaundice (progression stage)	11	247.3±153.4	27.1± 6.1	20.6±3.4	33.5± 9.1	18.9±11.1	11	32.9± 6.6	1.29±0.42
Obstructive jaundice (recovery stage)	13	59.9± 38.0***	39.7± 8.4***	9.3±6.0***	8.5± 7.5***	42.5±18.2**	13	78.2±15.7***	0.21±0.17***

Comparison of all data was made between the progression and remission stages of acute hepatitis, between decompensated and compensated liver cirrhosis, and between the progression and recovery stages of obstructive jaundice.
**: P<0.01, ***: P<0.001, : the number of subjects whose serum bilirubin contained Bδ. Mean ± SD.

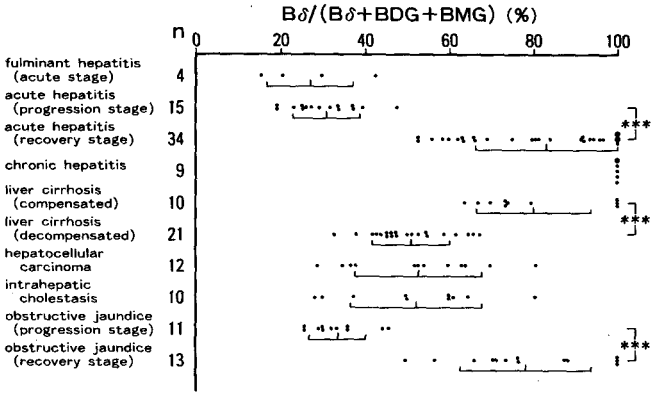

Fig. 2 *Bδ*/(Bδ+BDG+BMG) in hepatobiliary diseases
***: p<0.001. Large solid circles indicate 5 cases each.

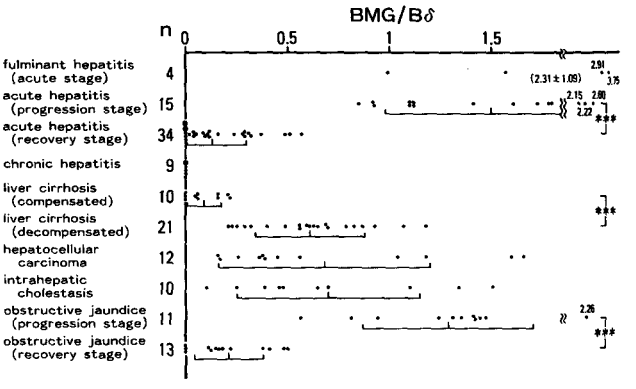

Fig. 3 *BMG/Bδ* in hepatobiliary diseases
***: p<0.001. Large solid circles indicate 5 cases each.

jaundice and significantly decreased in the recovery stage (**Table 1**). In decompensated liver cirrhosis, hepatocellular carcincoma and intrahepatic cholestasis, BMG/TB and BDG/TB ratios showed intermediate values. On the contrary, the value was very low both in chronic hepatitis and compensated liver cirrhosis. Accordingly, a significant difference was observed between the ratios in decompensated and compensated liver cirrhosis. In general, BMG/TB changes were more remarkable than a BDG/TB change (**Table 1**).

In sera containing Bδ, BMG/Bδ was also calculated (**Table 1, Fig. 3**). Opposite trends were observed in BMG/Bδ and Bδ/(Bδ+BDG+BMG), as the increase of the former was associated with a decrease of the latter and vice versa.

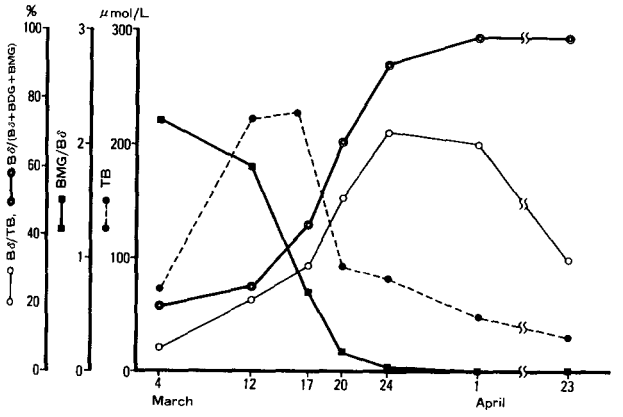

Fig. 4 *A course of a patient with acute hepatitis*
Initially low Bδ/(Bδ+BDG+BMG) increased and initially high BMG/Bδ decreased in accordance with the remission of jaundice. Bδ/TB changed similarly to Bδ/(Bδ+BDG+BMG) but to a small extent.

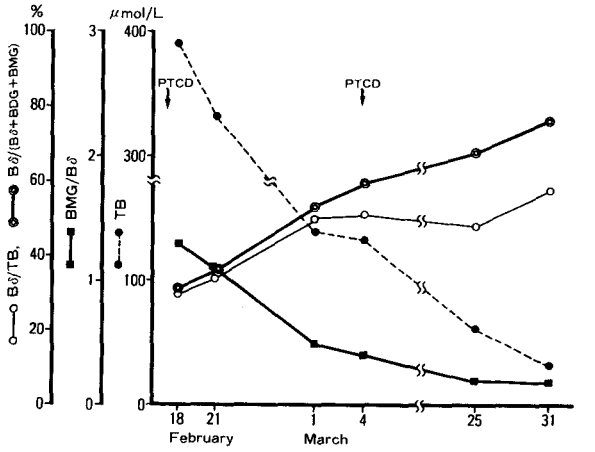

Fig. 5 *A course of a patient with obstructive jaundice (common bile duct cancer)*
Bδ/(Bδ+BDG+BMG) and BMG/Bδ changed in a warranty similarly to the patterns seen in Fig. 4. The change of Bδ/TB was not remarkable.

The courses of patients with acute hepatitis and obstructive jaundice are shown in **Figures 4 and 5**. Bδ/(Bδ+BDG+BMG) and BMG/Bδ changed remarkably in opposite directions, compared with the moderate changes of Bδ/TB.

Discussion

Micronex HPLC separated serum bilirubin into 5 fractions, δ, γ, β, β′, and α, by a single, simple pretreatment of serum in a reproducible and accurate way. The δ, γ, β, and α fractions corresponded to the corresponding fractions by Lauff's HPLC, and β′, fraction in Micronex HPLC was identified as (E, Z)- and/or (E, Z)-bilirubin IXα[9].

Recently, McDonagh[11] reported that photoderivative(s) of bilirubin appeared in sera from adults exposed for a long time to sunlight. Micronex HPLC detected the photoderivatives and revealed the presence of them in healthy adults and patients with liver diseases. Therefore, a small amount of the photoderivatives of bilirubin might be usually present in adult sera. However, β′ fraction may be increased by the exposure of serum to light because of the difficulty in collecting blood in complete darkness.

Recently Muraca et al.[12] reported the presence of trace amounts of BDG and BMG in normal serum and in cases of unconjugated hyperbilirubinemia. Since the minimal detection limits of Micronex HPLC for BDG and BMG were 5 to 10 times higher than those reported by Muraca et al.[12] when 5-10 μL serum was used in Micronex HPLC, BDG and BMG might not be detected in the serum of normal subjects and cases of unconjugated hyperbilirubinemia by Micronex HPLC. When a large amount of serum (25 μL) of normal subjects were analyzed by Micronex HPLC, the γ(BDG) and β(BMG) peaks appeared and amounted to 2.0 ± 3.3% and 4.2 ± 2.0% of TB, respectively (TB = 14.9 ± 3.3 μmol/L, n=6) (unpublished).

Bδ is reported to be produced by the covalent binding of albumin and Bu[9], BDG or BMG[13,14]. Accordingly, all of Bu and trace amounts of BDG and BMG may account for the presence of Bδ in the serum of some of the normal subjects and of Gilbert's syndrome cases.

In 159 patients with hepatobiliary diseases, we determined and compared the Bδ/(Bδ+BDG+BMG) with the Bδ/TB ratio which was previously described by Weiss et al.[2] as a good index in monitoring the jaundice. While Bδ/(Bδ+BDG+BMG) was remarkably high in chronic hepatitis and compensated liver cirrhosis, mostly at stable state, decrease in Bδ/TB was observed as previously reported by Weiss et

al.[2], suggesting poor prognosis. The ratio of Bδ/(Bδ+BDG+BMG) decreased in the jaundice-progression stage and increased in the remission stage to a larger extent than Bδ/TB. These results of the Bδ/(Bδ+BDG+BMG) study are in line with those from the corresponding Bδ/(Bc+Bδ) examined by the Ektachem method[8].

BMG/TB and BDG/TB changed in a direction opposite for Bδ/(Bδ+BDG+BMG). The change of BMG/TB was more remarkable than that of BDG/TB.

When jaundice develops, the BMG/TB ratio increased while the Bδ/TB ratio decreased, and BMG/Bδ changed more than Bδ/TB, as shown in Figs. 4 and 5. These findings suggest that Bδ/(Bδ+BDG+BMG) and BMG/Bδ at the time of serum collection can be a very useful parameter for monitoring and diagnosing the stages of jaundice without following the course in hepatobiliary diseases.

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