〈Original Article〉

Effects of thermostability of bilirubin subfractions on serum bilirubin measurement

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Summary Bilirubin is not only sensitive to light and oxygen, but is also easily destroyed by heat. The aim of this study was to investigate how bilirubin subfractions were affected by heat. Four icteric serum specimens (i.e., three conjugated and one unconjugated hyperbilirubinemic serum specimens) filled with nitrogen were incubated in vitro for 1.5 to 6 hours at 4, 25 and 37℃ in the dark. After incubation for six hours at all temperatures, no difference was observed in total bilirubin (TB) concentration as measured by the Syncron CX7 (Beckman Coulter). However, HPLC analysis revealed that monoconjugated bilirubin (mBc) and diconjugated bilirubin (dBc) decreased accompanied by an increase in unconjugated bilirubin (Bu), but delta bilirubin (Bd) was unchanged. Since these changes were not observed in either analysis of unconjugated hyperbilirubinemic serum, the origin of the increased Bu would be mBc and dBc. We concluded that bilirubin subfractions were stable for six hours at 4℃ under refrigeration and at least 1.5 hours at room temperature (25℃), and that the thermostability was in the order of Bd, Bu, mBc and dBc.

Key words: Thermostability, Bilirubin, Delta bilirubin, Conjugated and unconjugated bilirubin

1. Introduction

Bilirubin is sensitive to light and oxygen. The rate of photodegradation of unconjugated bilirubin (Bu) was nearly twice that of monoconjugated bilirubin (mBc) and three fold that of diconjugated bilirubin (dBc); delta bilirubin (Bd) was the most stable against photoirradiation[1]. In the presence of serum albumin, conjugated bilirubin (Bc: i.e., mBc and dBc) showed the highest rate of oxidation by peroxidase oxidation[2] and bilirubin oxidase oxidation[3] followed by Bu and Bd. The thermolability of bilirubin was reported by Morishita et al.[4]. They reported that the values of Bc and TB decreased gradually as the temperature and/or time increased, and the values of Bu increased over a 2-day period at four temperatures: -20, 4, 25 and 37℃. However, little information is available regarding the stability of bilirubin subfractions within a short time period. The aim of this study is to investigate how bilirubin subfractions are destroyed by heat during 1.5, 3 and 6 hours of storage in the dark at 4℃ (refrigerator), 25℃ (room temper-

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ature), and physiologic temperature of 37℃. This information will assist in designs of how serum specimens should be stored for bilirubin measurement.

2. Materials and methods

2.1. Serum specimens

Residual aliquots of four serum specimens were obtained from three patients with conjugated hyperbilirubinemia and one patient with unconjugated hyperbilirubinemia. They were all adult patients. Our study was in compliance with the rules for human experimentation at the Japanese Society of Laboratory Medicine. These serum specimens were deoxygenated with pure (99.9%) N_2, then incubated in vitro for 1.5 to 6 hours at 4℃ (cold room in a refrigerator), 25℃ (room temperature) and 37℃ (water bath). All serum specimens were shielded from light exposure.

2.2. Bilirubin measurements

Serum concentrations of TB and direct bilirubin (DB) were measured by the diazo-based method with a Synchron CX7 (Beckman Coulter). Indirect bilirubin (IB) concentration was calculated by subtracting the DB concentration from the TB concentration. Serum bilirubin subfractions were measured by high-performance liquid chromatography (HPLC) according to the method of Lauff[5], and each bilirubin subfraction was quantified from the relative peak area in HPLC and the TB concentration as determined by the Synchron CX7.

2.3. Statistics

Mean and standard error (SE) were calculated for each component at each time and temperature. The constancy of the serial concentration was assessed by the paired *t*-test, and *P* values were obtained. In the figures the asterisk represents $P<0.05$, which was considered statistically significant when compared with the original concentration.

3. Results

3.1. Diazo-based measurement

In both conjugated and unconjugated hyperbilirubinemic sera, no difference was observed in the TB concentrations as determined by the Syncron CX7 up to six hours at 4, 25 and 37℃ (Fig. 1). DB concentrations in the conjugated hyperbilirubinemic serum decreased according to the length of incubation time and the degree of incubation temperature, however, DB was stable up to six hours at 4℃ and 1.5 hours at 25 and 37℃. For the same time periods, IB concentrations increased remarkably. TB, DB and IB concentrations in unconjugated hyperbilirubinemic serum remained unchanged up to six ours at every temperature (Table 1).

3.2. HPLC measurement

HPLC measurement fractionated serum bilirubin into four major subfractions: Bu, mBc, dBc and Bd.

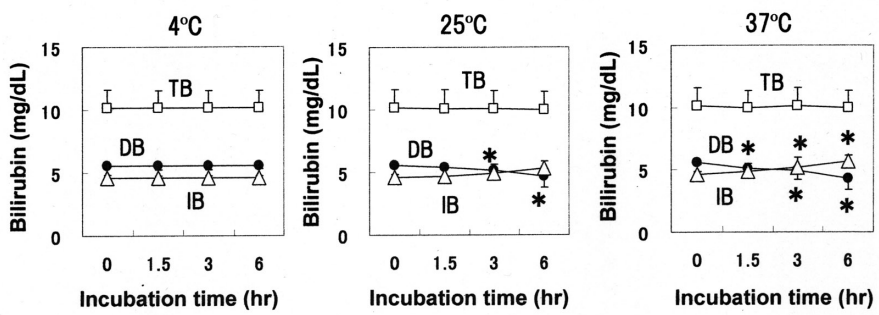

Fig. 1 Mean (and SE) concentration of TB, DB and IB versus time and three different concentrations in the dark. *Significant change from original concentration ($P<0.05$). (□) TB, total bilirubin; (●) DB, direct bilirubin; (△) IB, indirect bilirubin.

The concentration of dBc significantly decreased according to the length of the incubation time and the degree of the incubation temperature (Fig. 2). The concentration of mBc was stable up to 1.5-3 hours at every temperature, but decreased thereafter. The concentration of Bu was markedly increased according to the length of the incubation time and the degree of the incubation temperature. Bd was shown to be thermostable. In unconjugated hyperbilirubinemic serum (Table 1), the predominant bilirubin subfraction was Bu, and the minor subfractions were mBc and Bd, in which no significant changes were observed for up to six hours at all temperatures. The fraction of dBc was not observed in the unconjugated hyperbilirubinemic serum (chromatogram not shown).

4. Discussion

In this study of the stability of bilirubin subfractions, the concentrations of TB in unconjugated and conjugated hyperbilirubinemic sera remained stable for six hours at three different storage temperatures in the dark. In contrast, concentrations of DB (dBc and mBc) significantly decreased with time at 25 and 37℃. The mBc first increased after 1.5-3 hours, but declined over 3-6 hours at 25 and 37℃. The decrease in DB was accompanied by a comparable increase in IB (Bu). The concentration of Bd did not change.

Hydrolytic conversion of dBc to Bu was reported in the analysis of bile[6]. One of the mechanisms explaining this conversion is that β-glucuronidase, a member of the glycosidase family of enzymes, could catalyze the hydrolysis of glucuronic acid residues from dBc. In our study, the β-glucuronidase present in serum specimens could be responsible for the formation of Bu. Since the above changes were not observed in either analysis of unconjugated hyperbilirubinemic serum, the origin of the Bu would be dBc. That is, β-glucuronidase converted the dBc to mBc, and then the mBc was converted to Bu.

Table 1 Thermostability of bilirubin subfractions in unconjugated hyperbilirubinemic serum

Bilirubin subfractions	Incubation temperature	Incutation time (hour) 0	1.5	3	6
TB	4℃	4.1	4.0	4.2	4.0
	25℃	4.1	4.1	4.1	4.2
	37℃	4.1	4.1	4.1	4.1
DB	4℃	0.3	0.3	0.2	0.3
	25℃	0.3	0.3	0.2	0.3
	37℃	0.3	0.3	0.3	0.3
IB	4℃	3.8	3.7	4.0	3.7
	25℃	3.8	3.8	3.9	3.9
	37℃	3.8	3.8	3.8	3.8
Bd	4℃	0.6	0.6	0.6	0.6
	25℃	0.6	0.6	0.6	0.6
	37℃	0.6	0.6	0.6	0.6
mBc	4℃	0.9	0.9	1.0	1.0
	25℃	1.0	1.0	1.0	1.0
	37℃	1.0	1.0	0.9	1.0
Bu	4℃	2.5	2.5	2.6	2.4
	25℃	2.5	2.5	2.6	2.5
	37℃	2.5	2.6	2.6	2.5

TB, DB and IB were measured by diazo method, and Bd, mBc and Bu by HPLC analysis.

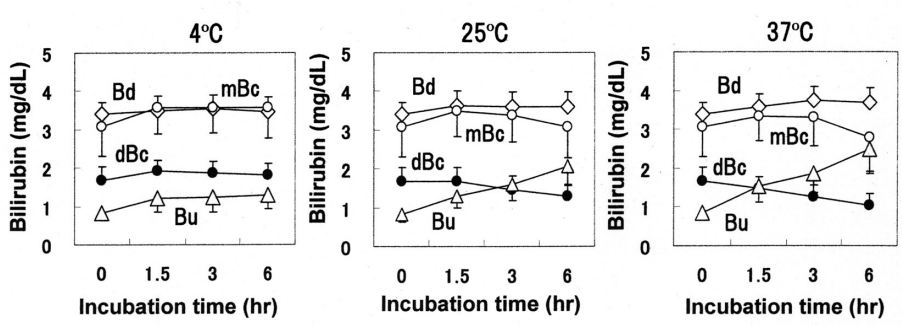

Fig. 2 HPLC measurement of serum bilirubin subfractions stored at different three temperatures in the dark. Plots are the mean (and SE) of three conjugated hyperbilirubinemic sera of Fig. 1. (◇) Bd, delta biliuribn; (●) dBc, diconjugated bilirubin; (○) mBc, monoconjugated bilirubin; (△) Bu, unconjugated bilirubin.

After six hours of incubation at 37℃, dBc and mBc decreased to 63±41% and 91±36% of the original concentrations, respectively, but Bu increased to 323±115%. The decrease in DB and increase in IB were at a much slower rate. The rates of decrease in Bc and Bu were different from those of DB (75±12%) and IB (127±25%). These slower rates in DB and IB could be explained by the presence of Bd. Bd is the most heat-stable bilirubin species. In the diazo-based measurement by the Synchron CX7, Bd gave a large direct reaction (76-89% of the total reaction) as DB and the remainder reacted as IB[7]. This result indicates a slower rate of degradation in BD compared with Bc, and also indicates a slower rate of increase in IB than Bu. In the diazo-based bilirubin measurement, serum specimens can be stable at 25℃ for 1.5 hours with stability of most subfractions, but DB will decrease thereafter accompanied by an increase of IB.

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