



# Effects of temperature and light on the stability of bilirubin in plasma samples

Alina G. Sofronescu, Todd Loeb, Yusheng Zhu \*

Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, United States

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## ABSTRACT

**Background:** Although it is known that bilirubin is photo-sensitive, detailed effects of both temperature and artificial light exposure on bilirubin stability in plasma have not been well investigated. We determined the impact of temperature and artificial light on bilirubin stability in plasma.

**Methods:** Plasma total and direct bilirubin were analyzed using a diazo method. The aliquots of 38 samples were stored at 3 °C and 22 °C with light protection for 2, 4, 8, and 24 h respectively before analysis. The aliquots of 20 samples with normal bilirubin and additional 20 with elevated bilirubin were exposed to artificial light for 2, 4, 8, and 24 h at 22 °C, and total and direct bilirubin were measured. The differences between the baselines and subsequent measurements were analyzed with analysis of variance.

**Results:** The baseline total bilirubin was  $9.6 \pm 8.1$  mg/dl (mean  $\pm$  SD) and the concentrations were  $9.6 \pm 8.2$ ,  $9.0 \pm 7.4$ ,  $9.0 \pm 7.5$ , and  $8.8 \pm 7.5$  mg/dl at 3 °C and  $9.5 \pm 8.1$ ,  $9.0 \pm 7.4$ ,  $9.6 \pm 8.1$ , and  $9.5 \pm 8.0$  mg/dl at 22 °C after 2, 4, 8, and 24 h ( $p > 0.05$ ,  $n = 38$ ). The baseline direct bilirubin was  $1.3 \pm 1.2$  mg/dl and the concentrations after 2, 4, 8, and 24 h were  $1.4 \pm 1.2$ ,  $1.4 \pm 1.2$ ,  $1.5 \pm 1.2$ , and  $1.3 \pm 1.1$  mg/dl at 3 °C and  $1.4 \pm 1.1$ ,  $1.3 \pm 1.1$ ,  $1.3 \pm 1.1$ , and  $1.3 \pm 1.0$  mg/dl at 22 °C ( $p > 0.05$ ,  $n = 19$ ). In samples with elevated bilirubin exposed to light at 22 °C, the baseline total and direct bilirubin concentrations were  $10.2 \pm 1.7$  mg/dl and  $5.0 \pm 1.9$  mg/dl, respectively. After 2, 4, 8, 24, and 48 h, total bilirubin concentrations were  $10.1 \pm 1.8$ ,  $10.0 \pm 1.8$ ,  $10.0 \pm 1.8$ ,  $9.3 \pm 2.0$  ( $p > 0.05$ ,  $n = 20$ ), and  $8.4 \pm 2.3$  ( $p < 0.01$ ,  $n = 20$ ) mg/dl and direct bilirubin concentrations were  $4.9 \pm 1.8$ ,  $4.9 \pm 1.9$ ,  $4.8 \pm 1.8$ ,  $4.2 \pm 1.6$  ( $p > 0.05$ ,  $n = 20$ ), and  $3.5 \pm 1.5$  ( $p < 0.01$ ,  $n = 20$ ) mg/dl. For samples with normal bilirubin levels under the same conditions, the average baseline total and direct bilirubin concentrations were  $0.7 \pm 0.1$  mg/dl and below the lower limit of quantification (LLOQ), respectively. After 2, 4, 8, 24, and 48 h, the average total bilirubin concentrations were  $0.7 \pm 0.1$ ,  $0.6 \pm 0.1$ ,  $0.6 \pm 0.1$  ( $p > 0.05$ ,  $n = 20$ ),  $0.5 \pm 0.1$ , and  $0.4 \pm 0.1$  mg/dl ( $p < 0.01$ ,  $n = 20$ ) and direct bilirubin concentrations were still below LLOQ.

**Conclusions:** Bilirubin in plasma is stable in refrigerator or at room temperature without light exposure for at least 24 h. In normal laboratory environment, a delay of up to 8 h in the measurement of bilirubin left unprotected from light at room temperature does not significantly affect the results. Under these conditions, the changes in bilirubin concentration are not clinically significant until 24 h (direct bilirubin) and after 48 h (total bilirubin).

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## 1. Introduction

Bilirubin is a metabolite of heme-containing proteins. Most of it (80–85%) results from the degradation of heme within hemoglobin molecules due to the breakdown of senescent erythrocytes, but it can also come from the destruction of erythrocyte precursors in the bone marrow, as well as from the metabolism of myoglobin and some cytochromes [1]. Heme is metabolized by heme oxygenase system to biliverdin and further processed by biliverdin reductase to bilirubin. Unconjugated bilirubin is insoluble in water, and once released in the vascular space, it will bind to plasma albumin with

high affinity. The albumin-bound bilirubin is transported to the hepatocytes, where it is mono- (15%) or di-esterified (~85%) with glucuronic acid. The resulted conjugated bilirubin is water-soluble and secreted through the biliary system.

Approximately 250–350 mg bilirubin is produced daily in normal adults and the level of bilirubin is a reflection of hepatic and biliary system functions, hemolytic disease, transfusion reaction as well as inherited bilirubin metabolism disorders. Considering these clinical implications of abnormal level of bilirubin, an accurate assessment of bilirubin concentrations in blood is clinically important. Currently, most clinical laboratories measure conjugated (direct) and total bilirubin using a spectrophotometric method based on the endpoint diazo reaction. Other methods include enzymatic assay using bilirubin oxidase [2] and vanadate oxidation assay [3].

One of the factors that affect the accuracy of the test is the stability of bilirubin in blood samples. It is well known that bilirubin is a

\* Corresponding author at: Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, MSC 908, Suite 309, Charleston, SC 29425, United States. Tel.: +1 843 792 8814; fax: +1 843 792 0424.

E-mail address: [zhuyu@musc.edu](mailto:zhuyu@musc.edu) (Y. Zhu).

photosensitive substance, undergoing both photoisomerization and photooxidation and the latter is much slower than the former [4]. To prevent these reactions, clinical laboratories generally protect specimens for bilirubin tests from light exposure. However, the stability of bilirubin in serum or plasma exposed to light was only partly tested [5–10]. The influence of pre-centrifugation time and the influence of the storage temperature of whole blood, serum, and plasma on the stability of different analytes, including bilirubin were assessed [11,12]. Tanner et al. found that bilirubin was stable when stored at 15, 25 or 35 °C for up to 24 h prior to centrifugation [11]. Similarly, the study of Boyanton et al. showed that both direct and total bilirubin were stable when plasma and serum samples were maintained in the contact with blood cells at room temperature for up to 56 h [12]. However, present literature still offers limited information regarding the stability of bilirubin in plasma samples with artificial light exposure at room temperature in modern clinical laboratory environment. It is recommended that serum or plasma be physically separated from contact with cells within 2 h from the time of collection. Separated serum or plasma should not remain at room temperature longer than 8 h. If assays are not completed within 8 h, serum or plasma should be stored at +2 °C to +8 °C [13]. Blood samples collected in remote areas are sometimes assessed in central laboratories with delay and exposed to light at various temperatures for different periods of time during shipping and storage. In some laboratories, samples for bilirubin measurement may be left on the bench without light protection for some time before analysis. The aim of this study is to determine the bilirubin stability in plasma samples exposed to (i) room temperature at 22 °C and fridge temperature at 3 °C and (ii) artificial light at room temperature for different periods of time before the measurement takes place.

## 2. Materials and methods

### 2.1. Specimen collection and processing

Heparinized plasma samples obtained by venipuncture were used for the study. The samples were selected from a pool of plasma specimens that have been analyzed within 2 h after collection and then the specimens were stored in a refrigerator at 3 °C without light exposure for no more than 24 h. Thirty-eight plasma samples were used to determine the influence of temperature on the stability of bilirubin. After the measurement of the baseline, each sample was aliquoted into eight plastic tubes (250 µl each) and four of them were stored at 3 °C, while the other four at 22 °C, all protected from light exposure. Bilirubin was analyzed after 2, 4, 8, and 24 h respectively. To study the impact of light exposure on the stability of bilirubin, additional 20 samples with normal total bilirubin concentrations and 20 samples with elevated total bilirubin levels were selected from a pool of specimens already tested in the lab within 24 h. The specimens were left in their original labeled tubes (BD Vacutainer, PST Gel with lithium heparin (65 units), 3.5 ml, 13 × 100 mm, cat #367961) (Franklin Lakes, NJ, USA), placed in a tube rack on a laboratory bench, equally and continuously exposed to the artificial light normally permanently present in laboratory (mean lumens: 2660 lm; color temperature: 4100 K; cool white plus, Product #: 272484, Philips) for 2, 4, 8, 24, and 48 h at room temperature (22 °C) on the bench that is 5 ft 5 in. below a light fixture, and total and direct bilirubin were measured at specific intervals of time. They were tested in the manner similar to the study of bilirubin stability stored at different temperatures aforementioned.

### 2.2. Total and direct bilirubin measurement

Direct bilirubin in plasma was measured on a UniCel DxC 800 Chemistry Analyzer using a timed endpoint diazo method (Beckman Coulter, Inc., Fullerton, CA). Total bilirubin was measured using the

same method in the presence of caffeine, benzoate, and acetate as accelerators to form azobilirubin.

### 2.3. Statistical analysis

The differences between the baselines and all subsequent measurements were analyzed with analysis of variance (ANOVA) with a *post hoc* Dunnett's test and  $p < 0.05$  was considered statistically significant. The allowable total error for bilirubin was <0.4 mg/dl or 20% according to Clinical Laboratory Improvement Act criteria.

## 3. Results

### 3.1. Influence of temperature

The average baseline total bilirubin concentration was  $9.6 \pm 8.1$  mg/dl (mean  $\pm$  SD) and the average concentrations after 2, 4, 8, and 24 h were  $9.6 \pm 8.2$ ,  $9.0 \pm 7.4$ ,  $9.0 \pm 7.5$ , and  $8.8 \pm 7.5$  mg/dl with recoveries of  $100 \pm 2$ ,  $100 \pm 3$ ,  $100 \pm 3$ , and  $95 \pm 5\%$  at 3 °C and  $9.5 \pm 8.1$ ,  $9.0 \pm 7.4$ ,  $9.6 \pm 8.1$ , and  $9.5 \pm 8.0$  mg/dl with recoveries of  $100 \pm 3$ ,  $100 \pm 3$ ,  $101 \pm 3$ , and  $102 \pm 6\%$  at 22 °C. There was no statistically significant difference between any of the study groups and the baseline ( $p > 0.05$ ,  $n = 38$ ) and the differences were within allowable total error of bilirubin.

To determine the stability of direct bilirubin in plasma, we selected 19 samples and stored them at 3 °C and 22 °C. The average baseline direct bilirubin concentration was  $1.3 \pm 1.2$  mg/dl (mean  $\pm$  SD) and the average concentrations after 2, 4, 8, and 24 h were  $1.4 \pm 1.2$ ,  $1.4 \pm 1.2$ ,  $1.5 \pm 1.2$ , and  $1.3 \pm 1.1$  mg/dl with recoveries of  $105 \pm 15$ ,  $101 \pm 17$ ,  $102 \pm 18$ , and  $98 \pm 17\%$  at 3 °C and  $1.4 \pm 1.1$ ,  $1.3 \pm 1.1$ ,  $1.3 \pm 1.1$ , and  $1.3 \pm 1.0$  mg/dl with recoveries of  $103 \pm 14$ ,  $98 \pm 13$ ,  $103 \pm 13$  and  $100 \pm 23\%$  at 22 °C. Similar to total bilirubin, there was no statistically significant difference in direct bilirubin concentrations between any of the study groups and the baseline ( $p > 0.05$ ,  $n = 19$ ) and the differences were within allowable total error of bilirubin.

### 3.2. Influence of the light

Effects of the light on bilirubin in samples with normal and increased bilirubin concentrations at room temperature (22 °C) were shown in Tables 1 and 2. Statistical analysis showed that in the case of samples with normal bilirubin levels exposed to the artificial light in the laboratory, there were not significant changes in the total bilirubin concentration for at least 8 h ( $p > 0.05$ ,  $n = 20$ ). However, total bilirubin levels decreased after 24 h of exposure ( $p < 0.01$ ), and there was a further decrease when exposed to light for 48 h (Table 1). Since direct bilirubin levels in majority of these samples were <0.1 mg/dl, which is the lower limit of quantification of the assay, they were not included in the statistical analysis. Samples with elevated bilirubin showed a decrease in both total and direct bilirubin levels only after 48 h exposure to light with a  $p < 0.01$  for both total bilirubin and direct bilirubin. However, the average change in total bilirubin was still within the allowable total error (20%) (Table 2).

**Table 1**  
Effect of light exposure on bilirubin in specimens with normal bilirubin.

Light exposure time	Total bilirubin	
	Mean $\pm$ SD (mg/dl)	Mean recovery $\pm$ SD (%)
Baseline	$0.7 \pm 0.1$	100
2 h	$0.7 \pm 0.1$	$95 \pm 13$
4 h	$0.6 \pm 0.1$	$90 \pm 13$
8 h	$0.6 \pm 0.1$	$86 \pm 13$
24 h	$0.5 \pm 0.1^a$	$73 \pm 11$
48 h	$0.4 \pm 0.1^a$	$61 \pm 9$

<sup>a</sup>  $p < 0.01$ .

**Table 2**  
Effect of light exposure on bilirubin in specimens with elevated bilirubin.

Light exposure time	Total bilirubin		Direct bilirubin	
	Mean $\pm$ SD (mg/dl)	Mean recovery $\pm$ SD (%)	Mean $\pm$ SD (mg/dl)	Mean recovery $\pm$ SD (%)
Baseline	10.2 $\pm$ 1.7	100	5.0 $\pm$ 1.9	100
2 h	10.1 $\pm$ 1.8	99 $\pm$ 2	4.9 $\pm$ 1.8	98 $\pm$ 2
4 h	10.0 $\pm$ 1.8	98 $\pm$ 3	4.9 $\pm$ 1.9	98 $\pm$ 2
8 h	10.0 $\pm$ 1.8	99 $\pm$ 2	4.8 $\pm$ 1.8	96 $\pm$ 2
24 h	9.3 $\pm$ 2.0	91 $\pm$ 7	4.2 $\pm$ 1.6	83 $\pm$ 6
48 h	8.4 $\pm$ 2.3 <sup>a</sup>	82 $\pm$ 15	3.5 $\pm$ 1.5 <sup>a</sup>	69 $\pm$ 11

<sup>a</sup>  $p < 0.01$ .

#### 4. Discussion

The present study confirms previous findings that bilirubin in blood sample without light exposure is relatively stable at various temperatures. Tanner et al. found that bilirubin in whole blood was stable at up to 35 °C for at least 24 h with a variation between  $-1.0\%$  and  $+6.8\%$  [11]. Boyanton et al. reported that total and direct bilirubin in serum and plasma were stable even over the 56-h period at room temperature (25 °C) [12].

Previous experiments have shown that bilirubin is very sensitive to phototransformation including both photoisomerization and photooxidation and the latter is much slower than the former [4]. It was recommended that bilirubin measurement should be performed immediately on drawing the specimen, or after keeping in a refrigerator for up to 2 h before examination [5]. Since then, almost all clinical laboratories have been following this recommendation. However, the conditions of O'Hagan's bilirubin light exposure experiment do not exist in modern clinical chemistry laboratories. In O'Hagan's study, a serum sample containing 9.0 mg/dl was left exposed to direct sunlight for 1 h (from 2:45 P.M. to 3:45 P.M.) on a bright spring day in Australia. It was found that the bilirubin concentration dropped to 4.8 mg/dl [5]. In clinical laboratories, samples are unlikely to be left out of the building without any protection, but it is possible that specimens may be exposed to artificial light at room temperature for various periods of time on lab benches. Our study demonstrates that in plasma samples with elevated total bilirubin concentrations, bilirubin is stable for at least 24 h without light protection at room temperature in the laboratory. O'Hagan et al. did put a serum sample on a shaded bench for 24 h and they found that the bilirubin level dropped from 0.8 mg/dl to 0.25 mg/dl. However, they tested only one sample and the concentration of bilirubin in that sample was within normal reference interval. To determine if the discrepancy between our and O'Hagan's results is due to different concentrations of bilirubin in the samples, we used 20 plasma samples with normal bilirubin concentrations to study bilirubin stability when exposed to artificial light again. Our experiment showed that both total and direct bilirubin levels did not change significantly with an average recovery of 86% for at least 8 h on the top of the bench at room temperature. In opposition to the previous observation, total bilirubin dropped only 0.2 mg/dl and 0.3 mg/dl, respectively from the baseline after 24 and 48 h light exposure (Table 1), while the allowable total error for total bilirubin is 0.4 mg/dl or 20% according to CLIA criteria. The discrepancy could be due to different sample containers used in our and O'Hagan's studies. In our study, we used labeled plastic Vacutainer tubes with plasma separator gel.

A more recent study, published by Rehak et al., recommends that exposure of specimens to light should be limited to  $<2$  h, as the losses of bilirubin are far greater than 20% [10]. In this study, however, the selected serum specimens were individually aliquoted into plastic tubes and exposed to fluorescent lighting at ambient temperature. In the case of our study, the specimens were left in the capped original plastic Vacutainer tubes with plasma separator gel and printed

identification label covering more than 2/3 area of the tube and placed in a tube rack on the laboratory bench with exposure to normal laboratory lighting. This reflects the usual laboratory practice, as in the case that the testing process is delayed and the specimens are not aliquoted, but left in their original tube on the laboratory bench. So the different bilirubin stability between our study and Rehak's may be due to different specimen tubes, the process of sample handling, and partial protection from light exposure provided by the label and the cap. In addition, as pointed out by McDonagh, different light sources with identical illuminances in footcandles but different spectral emissions might have quite different effects on the photochemistry of bilirubin. "Daylight" fluorescent light sources with high illuminance in footcandles but low spectral irradiance in the region of the bilirubin absorption band will degrade bilirubin much more slowly than fluorescent lights with high output in the region of the absorption band yet much lower illuminance in footcandles [4]. Therefore, possible difference in laboratory lighting between our and Rehak's laboratories might also contribute to the discrepancy between the present and Rehak's study. Clinical laboratories should evaluate bilirubin stability based on their own unique specimen tubes and laboratory lighting.

Previous studies have shown that direct bilirubin undergoes isomerization and hydrolysis of glucuronic acid esters of bilirubin which could artifactually inflate the proportion of unconjugated bilirubin present [4]. Indeed, we found that direct bilirubin was more sensitive to light exposure at room temperature than total bilirubin with differences in an average recovery of 8% at 24 h and 13% at 48 h (Table 2). On the other hand, prolonged exposure to light causes photoisomerization, increasing direct-reacting bilirubin in bilirubin [14]. Also,  $\delta$ -bilirubin, which is covalently linked to albumin, can directly react with diazo reagent. In addition, use of wetting agents or incorrect pH buffers may increase the amount of unconjugated bilirubin measured as direct bilirubin [15].

In conclusion, the present study demonstrates that bilirubin is stable at room temperature or in refrigerator with light protection for at least 24 h, which is consistent with previous observations. Our data suggest that in normal laboratory conditions, a delay of up to 8 h in the assessment of bilirubin in plasma samples does not significantly affect the accuracy of results, even if the samples are exposed to artificial light at room temperature. Under these conditions, the changes in bilirubin concentration are not clinically significant even until 24 h (direct bilirubin) and after 48 h (total bilirubin), although they are statistically different.

#### References

- [1] Sykes E, Epstein E. Laboratory measurement of bilirubin. *Clin Perinatol* 1990;17(2):397–416.
- [2] Morimoto Y, Ishihara T, Takayama M, Kaito M, Adachi Y. Novel assay for measuring serum conjugated bilirubin and its clinical relevance. *J Clin Lab Anal* 2000;14(1):27–31.
- [3] Ameri M, Schnaars H, Sibley J, Honor D. Comparison of the vanadate oxidase method with the diazo method for serum bilirubin determination in dog, monkey, and rat. *J Vet Diagn Invest* 2011;23(1):120–3.
- [4] McDonagh AF. Photolysis and photoisomerization of bilirubin in serum specimens exposed to room lighting. *Clin Chim Acta* 2008;393(2):130.
- [5] O'hagan JE, Hamilton T, Le Breton EG, Shaw AE. Human serum bilirubin; an immediate method of determination and its application to the establishment of normal values. *Clin Chem* 1957;3(5):609–23.
- [6] Ihara H, Aoki Y, Aoki T, Yoshida M. Light has a great effect on direct bilirubin measured by the bilirubin oxidase method than by the diazo method. *Clin Chem* 1990;36(6):895–7.
- [7] Ihara H, Nakamura H, Aoki Y, Aoki T, Yoshida M. In vitro effects of light on serum bilirubin subfractions measured by high-performance liquid chromatography: comparison with four routine methods. *Clin Chem* 1992;38:2124–9.
- [8] Landt M, Hortin GL, Smith CH. Monoject amber samplette free bilirubin photolysis. *Clin Chem* 1989;35(5):889–90.
- [9] Wu TW, Dappen GM, Spayd RW, Sundberg MW, Powers DM. The Kodak Ektachem clinical chemistry slide for measurement of bilirubin in newborns: principle and performance. *Clin Chem* 1982;26(12):2366–72.
- [10] Rehak NN, Cecco SA, Hortin GL. Photolysis of bilirubin in serum specimens exposed to room lighting. *Clin Chim Acta* 2008;387(1–2):181–3.

- [11] Tanner M, Kent N, Smith B, Fletcher S, Lewer M. Stability of common biochemical analytes in serum gel tubes subjected to various storage temperatures and times pre-centrifugation. *Ann Clin Biochem* 2008;45(Pt 4):375–9.
- [12] Boyanton Jr BL, Blick KE. Stability studies of twenty-four analytes in human plasma and serum. *Clin Chem* 2002;48(12):2242–7.
- [13] CLSI document H18-A3. Procedures for the handling and processing of blood specimens; approved guideline. 3rd ed.; 2004.
- [14] Ihara H, Shino Y, Hashizume N, et al. Effect of light on total and direct bilirubin by an enzymatic bilirubin oxidase assay. *J Anal Biol Sci* 1997;20:349–54.
- [15] Dumas BT, Wu TW. The measurement of bilirubin fractions in serum. *Crit Rev Clin Lab Sci* 1991;28:415–45.