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Photolysis of bilirubin in serum specimens exposed to room lighting

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Dear Editor:

For many years, the primary objective in clinical laboratory determination of serum bilirubin concentrations has been the detection of increased bilirubin concentrations [1–5]. Bilirubin was primarily of interest as a potentially toxic substance in hyperbilirubinemia of the newborn and as a marker of hepatic dysfunction or increased red cell turnover. However, increased interest in the measurement of low and normal bilirubin concentrations has been stimulated by recent findings that bilirubin is one of the major antioxidants in the circulation [6–8]. Bilirubin appears to be protective against cardiovascular disease and other pathophysiology resulting from generation of free radicals. Furthermore, bilirubin is the end product of the pathway initiated by action of heme oxygenases on heme. Heme oxygenase-1, in particular, is of great current interest as a physiological regulator and potential pharmacological target, and analysis of serum bilirubin concentrations serves as one approach for monitoring up- or down-regulation of this enzyme [6,9].

New insights into bilirubin physiology create an increased demand for accurate measurement of bilirubin within the normal range of values and may place more stringent requirements on sample handling. Bilirubin is a substance absorbing light within the visible spectrum, and it is well recognized to undergo both isomerization and oxidation in serum exposed to visible light, resulting in decreased measured bilirubin values [10–14]. Monitoring of the photodegradation is complicated somewhat by variable rates of degradation of different molecular forms of bilirubin and variable reactivity of photodegradation products with different methods for analysis of bilirubin. Although protection of specimens from light has been recognized as important for accurate bilirubin analysis [12–15], most studies have examined stability of hyperbilirubinemic specimens, and data on the stability of normobilirubinemic specimens is limited. We sought to determine the rates of bilirubin photolysis, as measured by the diazo reaction which is the most commonly-used clinical method, to establish the requirements for handling of normobilirubinemic specimens.

All serum specimens were leftover aliquots of an early morning blood draw collected from patients in serum tubes that were submitted to the laboratory for routine chemistry analysis. All specimens were used as anonymous samples according to Clinical Research Protocol approved by the Institutional Review Board. Individual serum aliquots (1 ml) were transferred into plastic tubes sealed with parafilm. Specimens were exposed at ambient temperature to fluorescent lighting with an intensity of 1,100 lumen/m² (105 footcandles) on a laboratory bench approximately 6 feet below a light fixture. To evaluate the effect of light on the kinetics

of bilirubin changes each serum specimen was transferred into two LX20 Microtubes and one tube was completely enclosed in aluminum foil. To evaluate the effect of O₂ on the kinetics of bilirubin changes one tube was flushed with N₂ gas and the serum aliquoting into the tube was also done under the flow of N₂ gas. The N₂ was reintroduced into the tube after each measurement.

All specimens were assayed, in duplicate, directly from the Microtube for direct and total bilirubin at a set time intervals with Synchron LX20 analyzer (Beckman Coulter Inc., Brea CA). The total imprecision (mean±SD) of the direct bilirubin method is 3.4±0.3, 15.4±0.8 and 88.9±2.6 µmo/l, and of the total bilirubin method is 7.4±0.8, 23.9±1.7, and 128.2±3.1 µmo/l. Analysis of changes in measured total and direct bilirubin was assessed for 20 normobilirubinemic and 5 moderately hyperbilirubinemic specimens exposed to laboratory lighting at room temperature (Table 1). For the normobilirubinemic specimens, measured values of total bilirubin decreased by an average of 59%. The decline in total bilirubin for moderately hyperbilirubinemic specimens was smaller-- 41%. Direct bilirubin decreased by slightly smaller amounts 38% and 31%, respectively, for the normobilirubinemic and moderately hyperbilirubinemic specimens.

Analysis of the kinetics of the change in bilirubin for a set of 10 specimens with more pronounced hyperbilirubinemia (total bilirubin 30–180 µmol/l) showed an approximately linear rate of decline of total bilirubin (Table 2). The rate of decline of total bilirubin averaged only about 2.3%/h which was slower than the average rate of decline for normobilirubinemic and moderately hyperbilirubinemic specimens—about 6.6%/h and 4.6%/h—in the previous experiment.

Examining the kinetics of the decline in total and direct bilirubin over a longer period of time also showed a nearly linear decline in measured values (Figs. 1A,B). Lines for specimens with varying initial bilirubin concentration were nearly parallel, reflecting approximately zero order kinetics for the photolysis of bilirubin. This resulted in larger proportional declines in total and direct bilirubin for specimens with low initial bilirubin values. The nearly linear decline in values argues against an internal filter effect as the primary mechanism for the lower proportional decline of specimens with high bilirubin concentrations. It is possible that some other factor besides light flux serves as a limiting factor in the photolysis of bilirubin in serum. One possible limiting component in serum is dissolved oxygen in serum. Purging of a pooled specimen with nitrogen prior to incubation did not change the rate of photolysis during a subsequent incubation, however.

Control specimens incubated at room temperature in the dark (Figs. 1C,D) did not show any change in total bilirubin (panel A), but there was a slow decline in direct bilirubin of about 15% over 24 h. The gradual decreases in direct bilirubin, with constant values for total bilirubin suggests that, at room temperature, there was a slow rate of hydrolysis of the glucuronide esters of bilirubin, which comprise the major component of direct bilirubin in most serum specimens. This slow hydrolysis should not occur for calibrator and control materials for direct bilirubin, which generally are prepared from taurobilirubin. That bilirubin conjugate is expected to have greater stability in aqueous solution than bilirubin glucuronide.

The practical consequence of our findings is that accurate measurement of bilirubin in specimens with normal or low bilirubin concentrations requires even greater care in the protection of specimens from light than for specimens with high bilirubin concentrations. Exposure of specimens to normal laboratory lighting should be limited to less than 2 hours in order to limit losses of bilirubin to <10%.

This is particularly important in providing guidance for the handling of research specimens, which may be handled as large batches with the potential for extended times of light exposure

during thawing, aliquoting and analysis. In the present study, we did not examine the photostability of bilirubin in specimens during collection and processing. Red cells may provide some limited protection of bilirubin in whole blood specimens, but, following separation of blood into a clot and serum, the serum phase is likely to have similar sensitivity to photolysis as described here. Therefore, protection of specimen tubes from light during processing and storage would be indicated, just as this is recognized as an important factor for assessment of neonatal bilirubin, where amber rather than clear samplettes are commonly used for specimen collection [15].

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Fig. 1. Kinetics of change in (A) total bilirubin and (B) direct bilirubin in individual serum specimens incubated at room temperature exposed to room lighting, (C) total bilirubin and (D) direct bilirubin incubated at room temperature in the dark.

Table 1

Photolysis of bilirubin in serum specimens exposed to 9 h laboratory lighting expressed as group means for normobilirubinemic (total bilirubin $\leq 17 \mu\text{mol/l}$) and moderately hyperbilirubinemic (total bilirubin 30–75 $\mu\text{mol/l}$) specimens.

Total Bilirubin ($\mu\text{mol/l}$) Specimens	N	0 h	9 h	Change (%)
Normobilirubinemic	20	10.7	4.4	–59%
Hyperbilirubinemic	5	43.8	25.8	–41%
Direct Bilirubin ($\mu\text{mol/l}$) Specimens	N	0 h	9 h	Change (%)
Normobilirubinemic	20	2.9	1.8	–38%
Hyperbilirubinemic	5	17.5	12.0	–31%

Table 2

Kinetics of changes in total bilirubin during light exposure of hyperbilirubinemic specimens (n=10, total bilirubin 30–180 $\mu\text{mol/l}$) expressed as mean values.

Time (h)	Total Bilirubin ($\mu\text{mol/l}$)	Change vs initial (%)	Rate of change (%/h)
0	92.0	----	----
2	87.6	–4.8	–2.4
4	85.3	–7.3	–1.8
6	79.9	–13.2	–2.2
8	75.2	–18.3	–2.3