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Development of an equation to correct for hemolysis in direct bilirubin measurements

Dina N. Greene [a], Daniel T. Holmes [b], Maun-Jan Lin [a], Joy Y. Liang [a], Thomas S. Lorey [a], Robert L. Schmidt [c,*]

[a] Kaiser Permanente, TPMG Northern California Regional Laboratory, Berkeley, CA, United States
[b] University of British Columbia, Department of Pathology and Laboratory Medicine, Vancouver, BC, Canada
[c] University of Utah School of Medicine, Department of Pathology, Salt Lake City, UT, United States

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ABSTRACT

Background: Direct bilirubin is measured for the investigation of pediatric and adult jaundice. Package inserts suggest that hemolysis decreases direct bilirubin measurements, but no published studies have adequately described the extent of interference.
Methods: The influence of hemolysis on direct bilirubin quantification (Beckman AU680) was evaluated by titrating increasing amounts of hemoglobin into specimens with variable starting concentrations of direct bilirubin. An equation was derived to predict the nominal interference-free concentration of direct bilirubin as a function of measured concentration and hemolysis-index.
Results: Hemolysis decreased the direct bilirubin concentration reported by the AU680. The extent of interference is a function of both the interference-free concentration of direct bilirubin and the degree of hemolysis.
Conclusions: The concentration of direct bilirubin in hemolyzed specimens can be predicted.

1. Introduction

Direct bilirubin is quantified to assist in the evaluation of patients with suspected liver dysfunction and/or jaundice. Isolated elevation of direct bilirubin generally points to cholestasis. In adults, cholestasis can result from medication reaction, autoimmune conditions or mechanical obstruction. In neonates and pediatric patients, cholestasis can result from biliary atresia, infantile hepatitis, or total parenteral nutrition. Direct bilirubin is rarely elevated (>5% of total bilirubin) in neonates, but elevated direct bilirubin is always pathological [1]. Hospitals commonly screen all neonates for total bilirubin, but the measurement of direct bilirubin or conjugated bilirubin (herein denoted B_c) is reserved for cases where there is clinical suspicion of specific pathologies [2–4].

Direct bilirubin is not usually evaluated in isolation but is measured in conjunction with other liver markers, importantly alanine aminotransferase, aspartate aminotransferase, alkaline phophatase, and gamma-glutamyl-transpeptidase (GGT). Depending on the suspected pathology, the results will display a specific pattern. For example, recent studies have outlined that neonates with biliary atresia will have elevated direct bilirubin with concurrent elevations in GGT that are significantly higher than that of the GGT concentrations seen in other cholestatic conditions [5]. Accurate measurements are required because direct bilirubin concentration plays an important role in the diagnosis of liver pathologies. This is especially true in neonates.

Hemolysis is a common source of interference in the measurement of bilirubin. The effect of hemolysis on total bilirubin determination has been extensively investigated [6–10]. In contrast, there is relatively little information on the falsely decreased direct bilirubin results observed on hemolyzed specimens [11]. Given the importance, many institutions have strict criteria for rejecting direct bilirubin neonatal samples with elevated concentrations of hemoglobin. Strict criteria cause high rejection rates of neonatal samples, which increase laboratory expenses, delay diagnosis and necessitate repeated difficult collections. These consequences could be reduced if sample rejection criteria were relaxed. Unfortunately, limited data hampers the evidence-based relaxation of these criteria.

2. Materials and methods

2.1. Study approval

This study was considered a quality assessment project and was therefore deemed exempt by the Northern California Kaiser Permanente Institutional Review Board.

2.2. Direct bilirubin quantification

Bilirubin was measured in all samples using the Beckman AU680 (Beckman Coulter Inc.). The AU680 uses a diazo-based method (uses

* Corresponding author at: Department of Pathology, 15 N Medical Drive East, University of Utah, Salt Lake City, UT 84112, United States. Tel.: +1 801 585 2010; fax: +1 801 585 2805.
E-mail address: Robert.Schmidt@hsc.utah.edu (R.L. Schmidt).

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diazonium salt of 3,5-dichloroaniline) to quantify direct bilirubin (no accelerator) and total bilirubin (caffeine salt added as accelerator). Both the total and direct assays are calibrated against materials traceable to the National Institute of Standards and Technology (NIST) Standard Reference Material.

2.3. Hemolysis interference

Hemolysates were prepared from whole blood with a known hemoglobin concentration. Samples were centrifuging it at 3000 rpm (1610 *g*) for 10 min. The resulting plasma was aspirated and the volume removed was measured. The red cells were then washed in quadruplicate with saline. After washing, the appropriate volume of deionized water was added to make the final hemoglobin concentration 20 mg/dl. The cells were freeze-thawed three times before centrifuging to separate the cellular debris from the hemolysate. The resulting supernatant was removed and used to titrate hemoglobin into the serum samples.

Residual, non-hemolyzed adult serum samples (n = 32; starting H-index = 0) with varying concentrations of direct bilirubin concentration (range of starting concentrations 0.49–9.76 mg/dl; mean = 3.05 mg/dl, SD = 2.30) were selected from samples to be discarded and appropriately deidentified. Hemolysis was induced by titrating hemoglobin into these samples, correcting for dilution, to create a series of six–seven samples having identical direct bilirubin concentrations, but varying amounts of hemoglobin. Each sample therefore had 6–7 levels of hemolysis associated with a single, non-hemolyzed direct bilirubin quantitation. Total bilirubin, direct bilirubin, and the H-index were determined in these samples using the AU680.

The Vitros assay was used to confirm that the direct bilirubin was primarily composed of conjugated bilirubin (i.e. delta bilirubin was less than 10% of the direct bilirubin). The Vitros assay uses reflectance spectrophotometry to quantify glucuronide and albumin conjugates of bilirubin independently. This provided confirmation that the AU680 results were attributable to glucuronide conjugates of bilirubin.

2.4. Assessment of sample hemolysis

The extent of hemolysis was classified using a hemolysis-index (H-index) of 1+ through 5+ based upon transmission spectrophotometry on the AU680 [12,13]. Parameters were user defined as follows: normal (N), optical density (OD) < 0.0850; 1+, OD = 0.0850–0.1699 (~31 mg/dl hemoglobin); 2+, OD = 0.1700–0.349 (~62 mg/dl hemoglobin); 3+, OD = 0.350–0.6999 (~125 mg/dl hemoglobin); 4+, OD = 0.7000–0.9999 (~250 mg/dl hemoglobin); 5+, OD ≥ 1.000 (~500 mg/dl hemoglobin); abnormal (ABN), OD ≥ 3.000 (~1000 mg/dl hemoglobin).

2.5. Statistical analysis

Statistics associated with the performance characteristics (precision, linearity, method comparison) were calculated using Microsoft Excel

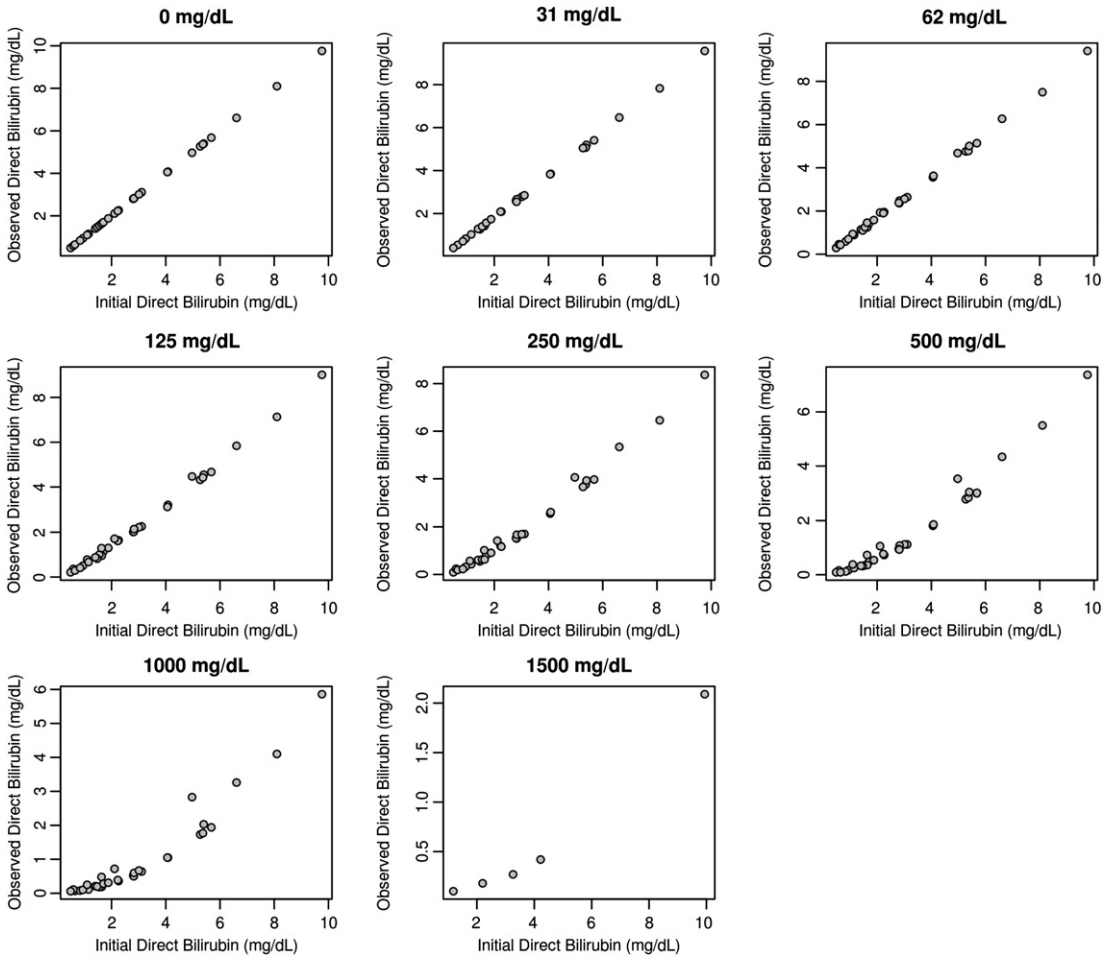

Fig. 1. The relationship between the initial direct bilirubin concentration (mg/dl) as a function of the measured direct bilirubin concentration (mg/dl) and the hemoglobin concentration. Each graph corresponds to a different concentration of hemoglobin.